Patterns of Antimicrobial Resistant *E. coli* and Genetic Interplay Between Livestock, Humans and Their Shared Environment in a High-Density Livestock-Human Population in Western Kenya

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Abstract

Patterns of Antimicrobial Resistant *E. coli* and Genetic Interplay Between Livestock, Humans and Their Shared Environment in a High-Density Livestock-Human Population in Western Kenya

Steven A Kemp

Antimicrobial resistance (AMR) is a global One Health issue. There has been a significant increase in the rates of AMR infections in both humans and veterinary medicine. The dynamics of AMR in developing countries are poorly understood, especially in community settings, due to a paucity of data on AMR prevalence and the drivers of resistance. Previous studies in sub-Saharan Africa indicated a high prevalence of multi-drug resistance (MDR) amongst *Enterobacteriaceae* such as *E. coli* in both humans and livestock. In western Kenya, there is significant overlap between humans, livestock, and their shared environments, due to the high density of smallholder farmers. This increases the risk of bacterial transmission via direct or close contact between humans and animals, or indirectly via the shared environment. The aims of this study were to determine the knowledge, attitudes and practices (KAP) amongst antimicrobial users and providers; to estimate the prevalence of carriage and identify risk factors for AMR *E. coli* including to the highest priority critically important antimicrobials (HPCIAs) amongst humans, livestock and the environment in an area of high-density smallholder farms in western Kenya and; finally, to explore AMR *E. coli* faecal carriage amongst livestock slaughterhouse workers.

Three studies were conducted; a cross-sectional KAP study of 147 antimicrobial users and prescribers understanding of AMR across Busia county; a cross-sectional study (farm) study collecting and characterising faecal *E. coli* from farmers, livestock and their shared environments, and finally examination and characterisation of faecal AMR *E. coli* amongst slaughterhouse workers. During the KAP and farm studies a structured questionnaire was used as a framework to interview all participants. During the farm study, *E. coli* isolates were isolated from farmers and livestock faeces, and from living environments and water sources on 70 farms across Busia. The slaughterhouse study was a retrospective study which collected faecal *E. coli* from workers in 142 slaughterhouses across western Kenya. All *E. coli* isolates were subjected to antimicrobial susceptibility testing using the disk-diffusion method and a subset were characterised by Whole Genome Sequencing (WGS). Non-sequenced isolates with an ESBL phenotype were subjected to PCR to determine ESBL resistance genes. Mixed effect logistic regression models were utilised to assess risk factors for carriage of AMR *E. coli* using questionnaire-derived data from the farm study.

The KAP study indicated that antimicrobials are accessed via agrovet shops, with a large proportion (~40%) being sold without a prescription. Concerning knowledge of antimicrobials; less than half of agrovets had sufficient training regarding livestock health and disease, and a registered pharmacist was often not present to dispense veterinary antimicrobials. Detailed information regarding dosage, withdrawal periods and the risks of AMR were not routinely provided by agrovets or animal healthcare assistants (AHAs) to farmers at point-of-sale. The most commonly sold/purchased antimicrobials were broad-spectrum oxytetracyclines and penicillin-streptomycin. Due to a lack of diagnostic facilities, broad-spectrum antimicrobials were often used empirically. There was good record keeping of antimicrobials sold by agrovets, but few records kept by farmers or AHAs who treated animals. Withdrawal periods were acknowledged by 80% of farmers, but only 28% had a good understanding and strictly adhered to them. A high

proportion of agrovets (69.2%), AHAs (39.7%) and farmers (29.0%) had never heard of AMR or 'resistance' before.

Faecal carriage of AMR *E. coli* on smallholder farms was high amongst sampled humans, livestock, and environment, with 95.3% of samples being resistant to at least one class of antimicrobial. Resistance to tetracycline (89.2%), trimethoprim (71.0%) and sulfonamides (69.4%) most prevalent. There was a high prevalence of MDR isolates (53.9%), with sheep and goats having the largest proportion compared to other animals. There was a moderate prevalence of ESBL-*E. coli* (14.8%) with two ESBL hotspots were identified in Nambale and Butula sub-counties. Molecular characterisation of *E. coli* isolates indicated a high diversity of *E. coli* with several similar strains found across animal, human and livestock populations between farms, confirmed by a large number of multi-locus sequence types (MLST) common to all groups. The most prevalent STs were identified in all three groups. Four distinct clonal groups with common ancestors were identified, all associated with carriage of $bla_{\text{CTX-M}}$ ESBL resistance genes. There was suggestion of clonal spread, as each member of the groups had similarities between serogroups, plasmids carried and resistance and virulence determinants, however directionality could not be determined. Two major risk factors were associated with increased risk of AMR - antimicrobial use (AMU) and animal vaccination.

There was a high proportion of faecal AMR *E. coli* amongst slaughterhouse workers (95.1%). There was a lower proportion of MDR (45.5%) and ESBL-*E. coli* (9.6%) compared to that found in farmers, livestock, and the farm environment. The largest proportion of ESBL-*E. coli* was found amongst mixed ruminant slaughterhouse workers. The most common resistance phenotypes were to tetracycline (86.2%), trimethoprim (59.3%) and sulfonamides (57.4%). A similarly wide diversity of *E. coli* was found amongst slaughterhouse workers, confirmed by numerous STs. Five isolates were identified as forming a clonal group of ST131 primarily amongst pig-only slaughterhouse workers. These were associated with serogroup O25:H4, the human pandemic clone and ESBL producer. Two other large clonal groups were associated with carriage of ESBL resistance genes, ST617 ($bla_{\text{CTX-M}}$) and ST361 (bla_{SHV}), also carrying ESBL resistance genes.

These findings indicate that there are gaps in the knowledge of antimicrobial users and prescribers and prescribing of broad-spectrum drugs. There is a high prevalence of circulating AMR *E. coli* amongst humans, livestock, and the environment as well as a moderate prevalence of ESBL-*E.coli* associated with $bla_{\text{CTX-M-15}}$ in all groups. This is important as there was little documented use of HPCIAs to provide selection pressure. There was overlap evident between humans, livestock, and the environment, as well as in slaughterhouse workers in rural settings. It is important to consider both direct and indirect transmission pathways of bacteria between humans, livestock, and the environment, using an integrated One Health approach to protect food safety and reduce the transmission of AMR bacteria. There is urgent need for surveillance systems to collect AMU and AMR data which will allow for targeted interventions and further identify reservoirs of resistance genes amongst different populations.

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Abbreviations Used

Agrovet	Agriculture & Veterinary Antimicrobial Supplier
AHAs	Animal Healthcare Assistants
AIDS	Acquired Immune Deficiency Syndrome
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
BBSRC	Biotechnology and Biological Sciences Research Council
BP	Base Pair(s)
BSAC	British Society for Antimicrobial Chemotherapy
CARD	Comprehensive Antibiotic Resistance Database
CGR	Centre for Genomic Research
CIA	Critically Important Antimicrobials
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely Adherent *E. Coli*
DDD	Defined Daily Doses
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia Coli*
EAEC	Enteroaggregative *E. Coli*
ECOFF	Epidemiological Cut-Off Value
EHEC	Enterohaemorrhagic *E. Coli*
EIEC	Enteroinvasive *E. Coli*
EMA	European Medicines Agency
EMBA	Eosin Methylene Blue Agar
EPEC	Enteropathogenic *E. Coli*
ESBL	Extended-Spectrum B-Lactamases
ETEC	Enterotoxigenic *E. Coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal *E. Coli*
GARP	Global Antibiotic Resistance Partnership
GIT	Gastrointestinal Tract
GLASS	Global Amr Surveillance System

HGT	Horizontal Gene Transfer
HICs	High Income Countries
HIV	Human Immunodeficiency Virus
HPCIA	Highest-Priority Critically Important Antimicrobials
ICE	Integrative and Conjugate Elements
ILRI	International Livestock Research Institute
IREC	Institutional Research Ethics Committee
KAP	Knowledge, Attitudes and Practices
LMICs	Low and Middle-Income Countries
MCR-1	Mobilised Colistin Reistance-1
MGEs	Mobile Genetic Elements
MHA	Müeller-Hinton Agar
MLST	Multi-Locus Sequence Typing
NA	Nutrient Agar
NACOSTI	National Commission for Science, Technology, and Innovation
NAP	National Action Plan
NCCLS	National Committee for Clinical Laboratory Standards (Now CLSI)
NDM-1	New Delhi Metallo-Beta-Lactamase-1
NGS	Next Generation Sequencing
OIE	World Organisation for Animal Health
pAmpC	Plasmid-mediated AmpC
PCR	Polymerase Chain Reaction
PMQR	Plasmid-Mediated Quinolone Resistance
rRNA	Ribosomal RNA
SCVO	Sub-Country Veterinary Officer
SNP	Single Nucleotide Polymorphism
TECOFF	Tentative Epidemiological Cut-Off Value
UTI	Urinary Tract Infection
WGS	Whole Genome Sequencing
WHO	World Health Organisation
XDR	Extensively Drug-Resistant
ZooLiNK	Zoonoses In Livestock in Kenya

Contributions to Thesis

Chapter 3 – KAP Study; Questionnaire Design

Input into and advised revisions of questions in KAP study:

Professor Nicola Williams[1]
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Chapter 1

Introduction & Literature Review

1.1 Introduction

The development of antimicrobials is one of the most important medical breakthroughs in the history of modern medicine. Their use has significantly helped to reduce the morbidity and mortality associated with bacterial infections (Andersson and Hughes, 2010). Early antimicrobials including penicillins and tetracyclines were based on naturally occurring compounds, produced by environmental microorganisms (Fleming, 1932; Martinez, 2008). Since then, several synthetic and semi-synthetic antimicrobials have been developed. In 1935, Domagk discovered and produced the first commercially available antibacterial drug, sulfonamidochrysoïdine (Prontosil, Bayer, Germany) (Domagk, 1935b). This was the first antimicrobial belonging to the sulfonamides.

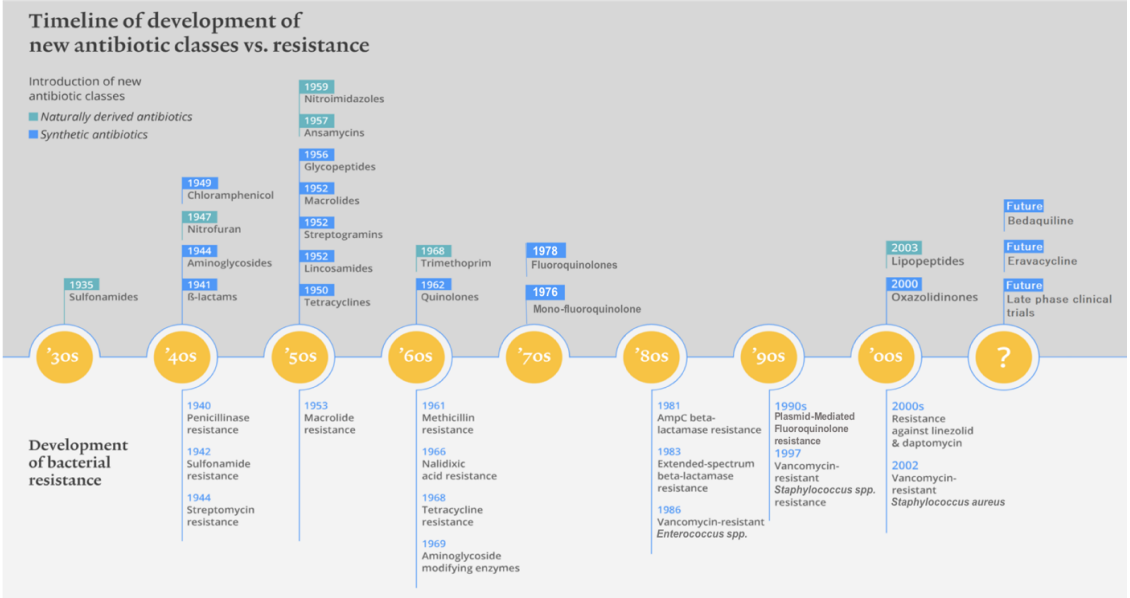
During the "Golden age of antibiotics" (1950-1960s) more than 50% of the antimicrobials commonly used today were discovered, including macrolides, cephalosporins, quinolones, and aminoglycosides (Davies, 2006). The global use of antimicrobials is constantly rising and is primarily driven by increased consumption in LMICs; latest estimates suggest that human antimicrobial consumption, expressed in defined daily doses (DDD), increased 65% from 21.1 billion DDDs in 2000, to 34.8 billion DDD in 2015 (Klein et al., 2018). In food-producing animals, in 2010, there was an estimated 63,151 tons of antimicrobials used per annum, with projections suggesting this will rise to over 105,596 tons by 2030 (Van Boeckel et al., 2015).

Antimicrobial resistance (AMR) has existed for many thousands of years, being a natural product of bacteria (D'Costa et al., 2011) but the widespread use of antimicrobials in human and animal medicine has led to more rapid development. Over the last 80 years, evidence has shown there is a direct correlation between the antimicrobial use (AMU) and AMR (Steinke and Davey, 2001; Goossens et al., 2005). With the introduction of new semi-synthetic antimicrobials, for use in human and veterinary medicine, resistance has been detected shortly afterwards (**Figure 1.1**).

Antimicrobial overuse occurs in all involved sectors – human, animal and agriculture - and this is the main driver for the spread of resistant bacteria and resistance determinants within and between these sectors and around the globe (O'Neill, 2016; Klein et al., 2018). Wherever antimicrobials are used, there is also concurrent development of reservoirs of AMR. This happens within the human population in hospitals and community settings, in animals and farm and aquaculture environments, but also in water, soil, wildlife, and many other ecological niches, as a result of pollution by sewage, pharmaceutical industry waste, and manure runoff

from farms (Marti et al., 2014; Huijbers et al., 2015). There is evidence to show that bacteria and the genes they carry can intersperse easily within and between humans, animals, and the environment (Woolhouse et al., 2015). The bacterial adaption to selection pressures within any one sector, is reflected in other sectors and actions taken to contain AMR do have an effect in other sectors (O'Neill, 2015). AMR is an inherently ecological problem which can be characterised by complex interactions involving diverse microbial populations affecting the health of humans, animals, and the environment. As such, it makes sense to address the issue of AMR by using a coordinated, multisectoral approach, such as the 'One Health' approach, discussed in Section 1.7.

Figure 1.1 Development and emergence of resistance timeline for major classes of antimicrobials. (Adapted from https://mega.online/en/articles/antibiotic-armageddon).



The emergence of AMR is a global concern for human and animal health; interaction at the human-animal-environmental interface can lead to the spread of AMR genes. In order to combat this issue effectively, intersectoral approaches which share the cost and responsibility evenly between environmental, human and veterinary health professionals is required, which laid out in the "One Health" paradigm (Fisman and Laupland, 2010).

1.2 AMR

AMR is a "global emergency" with long-reaching social, economic, and political burdens. It is the most serious public health threat of the 21st century (World Health Organization, 2012; Davies, 2013). AMR is currently estimated to cause 700,000 deaths per year, worldwide (O'Neill, 2016).

Due to the increasing amounts of antimicrobial use in human and animal medicine, AMR is developing at a rate which is faster than available control methods. By 2050, annual deaths from AMR-bacterial infections are estimated to reach 10 million, if action is not taken to tackle the issue (O'Neill, 2016). AMR is a multifactorial issue arising from the interplay between humans, their immediate environment and pathogens (Shears, 2001; Okeke et al., 2007). AMR bacteria arising in any of these sectors may spread between themselves, across species and borders. Addressing the issue will require a multisectoral, or One Health approach. To help facilitate this, the WHO has included AMR as one of the top ten threats to global health in 2019 (WHO, 2019a).

1.2.1 Current state of AMR

The state of AMR has changed rapidly over the last 15 years. In the past, major pathogens of concern included Gram-positive bacteria which circulated in hospitals - methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant pneumococcus (PRP) and vancomycin-resistant enterococci (VRE). The clinical management for both PRP and MRSA revolved around better infection control and containment in hospitals, until MRSA began to spread amongst communities (Smith et al., 2016). More recently, research has shifted focus to AMR in Gram-negative pathogens — particularly in MDR and extensively drug-resistant (XDR) Enterobacteriaceae. The emergence and rapid spread of AMR genes means that some infections are now completely resistant to all currently available antimicrobials.

A recent example of dangerous resistance genes is the New Delhi metallo-beta-lactamase 1 (NDM-1) which encodes an enzyme that confers resistance to a wide range of antimicrobials – this emerged in India and spread to the UK due to medical tourism (Yong et al., 2009). The NDM-1 gene has since been detected in (most commonly, but not exclusively) *E. coli* in surface and tap water in India and in the environment in Bangladesh (Islam et al., 2017; Khan et al., 2017), indicating the potential for NDM-1 being of environmental origin. The gene has also been identified in Arctic soil, indicating that the gene is spread in the faeces of migratory birds (McCann et al., 2019). Another example is the mobilised colistin resistance-1 (MCR-1) gene, which is a plasmid-borne gene conferring resistance to colistin – an antimicrobial of last resort; this gene was originally identified in Chinese pigs (Liu et al., 2016), but has now spread to humans and other animals in many countries (Wang et al., 2018).

The majority of antimicrobial classes are used in both human and veterinary medicine, though some are reserved exclusively for humans (e.g. isoniazid, for tuberculosis treatments) and some

are reserved exclusively for animal use (e.g. flavophospholipols and ionophores, due to toxicity to humans) (Van Boeckel et al., 2015; EMA, 2018). In human medicine, antimicrobials are predominantly used to treat clinical infections in individual patients, but there are also other prophylactic uses such as post-surgery. In Niger, an LMIC with very high risk of mortality, ciprofloxacin prophylaxis was shown to reduce the attack rate during a meningococcal group C epidemic (Coldiron et al., 2018) and twice-annually dose of azithromycin was found to reduce infant mortality (age <5) by 18% compared to control villages (Keenan et al., 2018). In veterinary medicine, there are differences between food-producing animals, and companion animals. Companion animals such as dogs, cats, horses etc. have similar antimicrobial use patterns to humans i.e. the majority of antimicrobials are to treat clinical infections (Sykes, 2013). In food animals, when a proportion of animals in a group are clinically infected and require antimicrobial treatment, medications may be administered in feed or water to an entire group. These methods of mixed treatment and prophylaxis for animals that are not infected; is defined as inappropriate use of antimicrobials in animals (Van Boeckel et al., 2017).

A particularly controversial use of antimicrobials in food-producing animals are the long-term, low-dose antimicrobials administered for growth promotion. This practice has been shown to enhance selection for AMR as the antimicrobials are administered to entire groups of animals for prolonged periods of time, often at subtherapeutic doses (FAO, 2016). This enriches the environment for AMR, thus selecting for and spreading AMR bacteria within and between groups of animals, as well as to humans through food or other environmental pathways. The imprudent use of antimicrobials is primarily for economic reasons – to allow animals to grow faster, but it is clear that use of antimicrobials for growth promotion exacerbates AMR to antimicrobials that are particularly important in both human and animal health (FAO, 2016; Carrique-Mas and Rushton, 2017; Coyne et al., 2019).

AMR is not a new phenomenon; numerous antimicrobials are based on naturally-occurring substances and so, the origin of resistance genes commonly found in clinical settings, likely originated in non-clinical settings (Alonso et al., 2001; D'Costa et al., 2011). Microorganisms which are most adapted to survival in environments that are heavily polluted with antimicrobial compounds (including heavy metal ions, and naturally-ocurring antimicrobials and antifungals) possess an excess of genes which confer protection to these stressors, including toxin transport genes (*NarK*) nitrate/nitrite antiporters and Cd^{2+}/Zn^{2+}/Co^{2+} efflux components (*CzcABC*, *CzcD*) (Hemme et al., 2010). It has been known for some time now that bacteria can acquire AMR from

other bacteria, via mobile genetic elements such as integrons, transposons and plasmids, as well as other integrative and conjugative elements (ICEs) (Boyd et al., 2009; Wozniak and Waldor, 2009). Gene exchange is a universal property of bacteria and when a bacterium is exposed to environmental stressors or antimicrobials, there is a selective pressure placed upon it (Gillings and Stokes, 2012). Bacteria faced with antimicrobial selection pressures, can enhance their fitness by acquiring and expressing resistance genes and then share those genes with other bacteria. Thus, any antimicrobial use and subsequent overuse, are important drivers of the resistance phenomenon; the other main drivers are factors such as poor sanitation and high population densities which promote the spread of resistant bacteria and their genes locally and globally (Holmes et al., 2016). This sharing enriches the environment for AMR, and will in turn, increase the opportunity for the sharing of other resistance genes between bacteria. This all contributes to the development of multi-drug resistance, posing increased risk to human health (Kümmerer, 2009; Pawlowski et al., 2016). It is not only antimicrobials which can select for AMR microorganisms; various pollutants, including agricultural, biomedical, and industrial run-off can also place selective pressures, enriching the environment for AMR. For example, the highest concentration of waste antimicrobials found in the environment, are in areas close to hospital effluent (Verlicchi et al., 2015) and soils treated with manure or used for livestock (Kay et al., 2004).

Antibiotics are explicitly designed to kill or prevent the growth of bacterial populations, but the effects depend on the antimicrobial mode of action and the bacterium. Where there is inappropriate or sub-optimal use of antimicrobials, depending on the size of the population and the antimicrobial, bacteria may undergo one of several changes: develop resistance to antimicrobials by placing a selective pressure for *de novo* resistance mutations to occur (Ashbolt et al., 2013) and increase the rate of adaptive evolution, producing genetic or phenotypic variability (Oz et al., 2014).

1.3 General AMR Mechanisms

Antimicrobial action is bacteriostatic (slow bacterial growth and reproduction) or bactericidal (kill bacteria). Their action is dependent on their mechanisms of action, and concentrations at which they are administered (Pankey and Sabath, 2004). Antimicrobials targets specifically disrupt bacterial cell machinery to prevent growth and kill them; these inhibit the synthesis of bacterial cell walls (β-lactams and bacitracin), proteins (chloramphenicol, macrolides, aminoglycosides, tetracyclines), cell membranes (polymyxins), nucleic acid (quinolones and

rifampicin) and inhibit folic acid metabolism (sulfonamides, trimethoprim) (Hooper, 2001; Samaha-Kfoury and Araj, 2003).

Antimicrobial resistance can be an intrinsic or an acquired property of a microorganism. Intrinsic resistance is the innate ability of a bacterial species to resist the mechanism of action of an antimicrobial due to inherent properties; this allows tolerance to either an antimicrobial, or an entire antimicrobial class (Munita and Arias, 2016). Acquired resistance occurs when a bacterium resists the mechanisms of action of an antimicrobial to which it was previously sensitive. This may have developed over time due to a specific selective pressure, requiring the microorganism to develop a counter-mechanism (Wright, 2005).

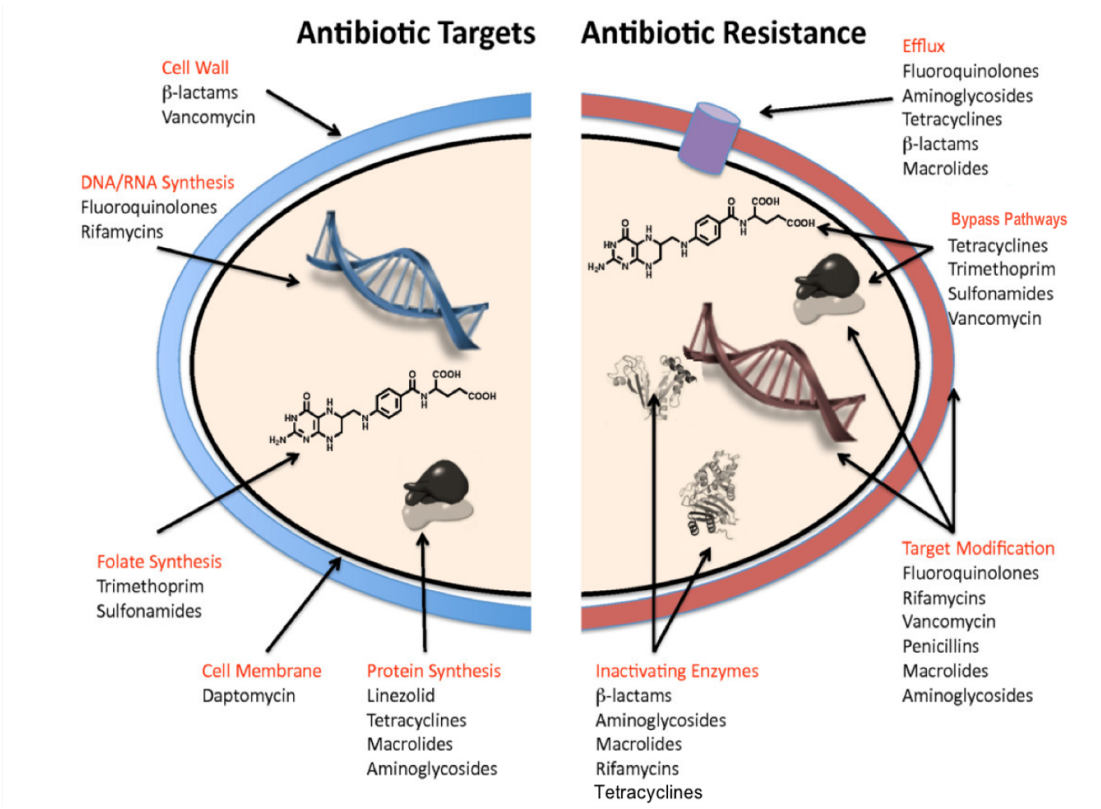
The 'antibiotic resistome', coined by Gerald Wright, refers to the collection of all the antimicrobial resistance genes and their precursors in both pathogenic and non-pathogenic bacteria. This is comprised of resistance genes not only found in pathogenic and non-pathogenic bacteria, but also resistance genes embedded in bacterial chromosomes, as well as precursor genes which can encode proteins that have some bacteriostatic or bactericidal effects (Wright, 2007). Antibiotics are indeed ancient and are thought to have been around for hundreds of millions of years (Bhullar et al., 2012), existing naturally. The sheer number of resistance genes in the resistome is a testament to the continuous evolution of molecules in both natural environments and microbial genomes. It is important to realise that whilst AMU is a key driver of AMR, both structural biology and protein biochemistry has shown that AMR initially evolved from precursors with other functions; i.e. in response to cytotoxic antimicrobial agents placing selective pressures on bacteria (Wright, 2007).

Bacterial AMR is mediated by several mechanisms. A diagram collating these methods is shown here **(Figure 1.2),** but such methods include:

1) Reducing antimicrobial molecule entry to a cell, and efflux of any accumulated antimicrobials.
2) Alteration of the antimicrobial target via mutations in binding elements.
3) Bypassing the pathway being inhibited.
4) Obtaining an alternative gene that the antimicrobial cannot bind to.
5) Chemical modifications or destruction of the antimicrobial molecule.

Many antimicrobials have intracellular targets, requiring them to gain entry through the cell membranes to have effect. To prevent entry, bacteria can alter the permeability of their membranes, preventing influx of antimicrobial molecules. This is more important for Gram-negative bacteria than for Gram-positive bacteria; the highly hydrophobic lipid bilayer is what aminoglycosides, macrolides, fusidic acid and various peptides diffuse through (Nikaido, 2003). Other antimicrobials such as β-lactams, tetracyclines and fluoroquinolones cannot pass through the outer membrane and must enter through diffusion-channels (porins) (Pages et al., 2008). In addition to limiting influx, bacterial efflux systems actively remove antimicrobial molecules from their cells. Many classes of efflux pumps have been defined; they are often substrate specific, but can also have broader substrate specificity, such as those found in MDR bacteria which can efflux fluoroquinolones, β-lactams, carbapenems and polymyxins (Delmar et al., 2014).

Figure 1.2 An overview of bacterial AMR mechanisms, indicating the mechanisms of antimicrobial actions and resistance to them. Adapted from (Wright, 2010).



Some bacteria produce proteins which block binding to the antimicrobial target; many of these are encoded on the bacterial chromosome, but clinically relevant variants appear to be carried on mobile genetic elements (MGEs). Using MGEs, AMR can be spread between related and non-related species. One of the most common method of sharing genes is via horizontal gene

transfer (HGT). HGT happens in three main ways which involve genetic recombination: transformation (naked DNA is assimilated), transduction (bacteriophages transfer genes) and conjugation (bacterial 'sexual' mating) (Manson et al., 2010). Of particular relevance are plasmids and transposons, which are important in the spread of AMR to clinically-relevant bacteria (Hayes, 2001).

Antimicrobial target site changes can be made in several ways. Target site changes primarily affect tetracyclines, fluoroquinolones, fusidic acid, macrolides and lincosamides (Connell et al., 2003; Lambert, 2005; Golkar et al., 2018). A well-defined example of the target site mechanism is described in tetracyclines; proteins belonging to the GTPase superfamily act as homologues of elongation factors – these interact with the ribosome to dislodge tetracycline from its binding site and then prevent re-binding (Donhofer et al., 2012). The target site can be modified in three different ways:

1. Mutations of the target site itself, such as those found in resistance to quinolones, prevents transcription by interfering with RNA polymerase pathways – by blocking the nascent RNA pathways, and can work synergistically with efflux-pumps (Kim et al., 2012). Mutational changes also confer oxazolidine resistance; interaction with bacterial ribosomes interferes with aminoacyl-tRNA placement, resulting in inhibition of protein synthesis (Mendes et al., 2014).
2. Production of new antimicrobial molecule targets; these are mechanistically similar to the original targets but binding of antimicrobial molecules to them has no effect. An example of this is seen in methicillin- and vancomycin-resistance *S. aureus* (M/VRSA) which has modified peptidoglycan structures (Szweda et al., 2012).
3. Over-production of antimicrobial target sites e.g. dihydrofolate reductase – this means that higher antimicrobial or inhibitor concentrations are required to bind to targets to enact the bacteriostatic activity. This mechanism works primarily on trimethoprim, but also on sulfamethoxazole, and thus, trimethoprim-sulfamethoxazole (Eliopoulos and Huovinen, 2001).

Bacterial ribosomes are one of the major targets of antimicrobials, namely the 50S and 30S subunits. Antimicrobials targeting the 30S subunit inhibit protein synthesis by preventing binding of tRNAs or by moving tRNAs via translocation (Mehta and Champney, 2002). Antimicrobials targeting the 50S subunit inhibit protein synthesis by preventing the binding of

aminoacylated-tRNAs or preventing nascent polypeptide chains travelling through the ribosomal tunnel (Lambert, 2012). Another AMR mechanism is the modification of the antimicrobial molecule, to prevent binding to these sites. (Wilson, 2014). Examples of these are aminoglycoside-modifying enzymes (AMEs) - acetyltransferases (ACC), adenyltransferases (ANT) and phosphotransferases (APH) (Ramirez and Tolmasky, 2010), and the chloramphenicol acetyltransferases (CATs), which modify chloramphenicol molecules (Schwarz et al., 2004). Another modification-type resistance mechanism is antimicrobial molecule destruction – e.g. β-lactamases hydrolyse the amide-ring in β-lactam antibiotics, which deactivates the molecule's properties (Abraham and Chain, 1940; Bush et al., 1995).

Resistance to a wide range of antimicrobial classes in human and veterinary medicine has been reported to all of our antimicrobials (Mathew et al., 1999; van den Bogaard and Stobberingh, 2000; van den Bogaard et al., 2001). A plethora of genes which encode for resistance against antimicrobials have been identified in *E. coli;* this makes it an ideal model organism for studying AMR, due to ubiquitous presence in the human microbiome. Benefits of using *E. coli* as a sentinel for this study are described in section **1.4**. A discussion of major resistance genes within *E. coli* are described here.

1.3.1 β-Lactam Action & Resistance

β-lactam antibiotics include penicillin derivatives, cephalosporins, monobactams and carbapenems. β-lactams are bactericidal, and their mode of action targets the final stage of cell wall synthesis, transpeptidation. β-lactams have a high affinity for penicillin-binding proteins (PBPs) responsible for manufacturing peptidoglycan which makes up the cell wall by forming cross-links. In the presence of β-lactams, peptidoglycan cross-links cannot form, leaving the cell wall unable to withstand changes in osmotic pressure, leading to cell lysis (Tomasz, 1979).

β-lactamase enzymes confer resistance to β-lactams by hydrolysing the β-lactam central ring – inactivating the molecule. Penicillinase was the first β-lactamase to be discovered; it was isolated from *E. coli* even before the clinical introduction of penicillin (Abraham and Chain, 1940). By the end of 2009, approximately 890 unique β-lactamase protein sequences had been identified (Bush and Jacoby, 2010). There are currently two β-lactamase classification models, and these are broadly based on either the primary structure or the functional properties of the enzyme. Molecular classifications split them into classes A, B, C and D, based on the amino acid motifs (Ambler, 1980) where each class except for B hydrolyse the β-lactam ring via an active

site serine. Class B β-lactamases are metalloenzymes e.g. New Delhi metallo-β-lactamase 1 (NDM-1), and they utilise at least one active site zinc in ring hydrolysis. The other classification model introduces functional grouping: group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-and extended-spectrum β-lactamases and serine carbapenemases; and group 3 metallo-β-lactamases (Bush et al., 1995). The most commonly occurring plasmid-mediated β-lactamase types are TEM, which confer resistance to ampicillin (Datta and Kontomichalou, 1965); SHV (which can also be found on the chromosome) confers resistance to broad-spectrum penicillins (Livermore, 1995); OXA (almost always plasmid-mediated) which confer resistance to penicillins (some newer OXA can also confer resistance to cephalosporins and carbapenems (Evans and Amyes, 2014)); and CTX-M, which preferentially hydrolyses cefotaxime (Gazouli et al., 1998). These three enzymes make up much of the β-lactam resistance encountered in *E. coli.* These β-lactamases are encoded for by *bla* genes which can be found on chromosomal cassettes and plasmids (Matthew, 1979).

In addition to bla_{TEM} *and* bla_{SHV}, *E. coli* has *AmpC* genes which encode for AmpC β-lactamases. These enzymes belong to subclass 1 and are cephalosporinases (Jacoby, 2009). *AmpC* genes are also chromosomally or plasmid-mediated; they are expressed at higher levels when on plasmids, due to promotors on the mobile elements (Schmidtke and Hanson, 2006).

CTX-M β-lactamases are commonly isolated from enterobacteriales such as *E. coli* and other enterobacteriales such as *Klebsiella* spp. (Canton et al., 2012). More than 100 variants of $bla_{CTX\text{-}M}$ have been sequenced - they are frequently reported in the literature to be increasing in frequency around the world. The rate of spread of CTX-M enzymes points to a global pandemic as they have supplanted both TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs, see **section 1.4.2.1**) and carry intrinsic resistance to 3rd and 4th generation cephalosporins (Woerther et al., 2013).

The 3rd-5th generation cephalosporins are antimicrobials which are on the list of highest priority critically important antimicrobials to human health (HPCIAs). Use of cephalosporins is known to provide selective pressures for the development of β-lactamase-producing *Salmonella* spp. and *E. coli* in animals (ur Rahman et al., 2018). Furthermore, the growing incidence of human disease due to AMR *Salmonella* and *E. coli* (World Health Organisation, 2017) requires that cephalosporins are only used as a drug of last resort, to preserve their efficacy for human health.

1.3.1.1 Extended-Spectrum-β-Lactamases

ESBLs are a group of enzymes, first discovered in 1979, which effectively hydrolyse β-lactam antibiotics with oxyimino side chains (Sanders and Sanders, 1979). They confer resistance to all aminopenicillins, variably to third-generation cephalosporins (cefotaxime, ceftriaxone ceftazidime), and the monobactam, aztreonam. ESBLs do not mediate resistance to cephamycins (e.g. cefoxitin) or carbapenems (Bonnet, 2004) and the majority remain susceptible to β-lactam inhibitors including clavulanate and tazobactam. There have been newer reports of amoxicillin-clavulanate-resistant Enterobacteriaceae however, in a new blaTEM variant (Di Conza et al., 2014). Despite much discussion, there are no official consensus on a universal definition for ESBLs; they are simply transmissible β-lactamases with expanded activity against oxyimino cephalosporins, which can be inhibited by clavulanic acid, tazobactam or sulbactam, and are encoded for by genes which can be horizontally transferred between bacteria (Paterson and Bonomo, 2005).

CTX-M enzymes originated through mobilization of chromosomal bla_{KLUA} genes from *Kluyvera* spp., which conferred resistance to 3rd generation cephalosporins (Humeniuk et al., 2002). These genes were then incorporate into mobile genetic elements (Cantón, 2008) and they have increased in prevalence, worldwide since 2000. They have now been reported around the world it is now widely considered that CTX-M enzymes have nearly displaced other ESBLs enzymes in the *Enterobacteriaceae*, including TEM, SHV and OXA ESBL variants (Coque et al., 2008a; Woerther et al., 2013; Giani et al., 2017). CTX-M enzymes are divided into clusters based on their amino acid sequences, these are currently CTX-M-1, -2, -3, -8, -14, -25, -45 and -64. As of 2018, within those clusters, there are 172 defined CTX-M enzymes, 223 TEM and 193 SHV types, though not all have an ESBL phenotype (https://externalwebapps.lahey.org/studies/). CTX-M-14 and CTX-M-15 are the most dominant and clinically relevant enzymes and they have been associated with both hospital- and community-acquired infections, as well as increasingly in animal and environmental sources (Hawkey and Jones, 2009; Bevan et al., 2017). Besides these major classes of β-lactamases, there are a number of other, less common β-lactamases: PER (Bauernfeind et al., 1996), GES (Poirel et al., 2000), VEB (Poirel et al., 1999) and SFO-1 (Matsumoto and Inoue, 1999), which all act in a similar method, but have varying activities against penicillins and extended-spectrum cephalosporins, as well as different rates of inhibition by clavulanic acid.

ESBLs are commonly associated with MDR bacteria, as resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole can also be carried on the same MGEs (Emery and Weymouth, 1997). Of important note is CTX-M producing- *E. coli*; since 2005, *E. coli* producing the CTX-M enzyme has emerged worldwide as a primary cause of community-acquired urinary tract infections and bacteraemia. As such, CTX-M is regarded as being pandemic amongst *E. coli* causing extra-intestinal infections (Canton and Coque, 2006). A single clone of MDR, CTX-M-15-producing *E. coli* (ST131) has been identified in humans and animals in many countries around the world (Rogers et al., 2010; Pitout and DeVinney, 2017). This clone is usually associated with serogroup O25, carries a large number of virulence genes, harbours MDR IncFII plasmids and are almost always resistant to fluoroquinolones (Johnson et al., 2008; Literacka et al., 2009). Isolates belonging to ST131, are also associated with other β-lactamases, but also ciprofloxacin-resistant *E. coli* that do not carry ESBL genes (Johnson et al., 2009; Oteo et al., 2009). Furthermore, ST131 isolates are pathogenic owing to the large number of infections they cause in hospital and community settings.

1.3.2 Tetracycline Action & Resistance

Tetracyclines are broad-spectrum, bacteriostatic antibiotics with efficacy against a wide range of Gram-positive and Gram-negative bacteria, as well as protozoal parasites. They have been used extensively in human and veterinary medicine for prophylaxis, growth promotion and therapeutically. The first tetracycline, chlortetracycline, was discovered in 1948 and was isolated from *Streptomyces aurofaciens* (Duggar, 1948). *S*econd-generation tetracyclines are semisynthetic analogues, developed in the 1960s e.g. doxycycline and minocycline; they have superior stability and pharmacological activity (Stephens et al., 1963). Tetracyclines remain important for both human and veterinary medicine, though increasing rates of AMR have drastically limited their effectiveness.

More than fifty tetracycline resistance genes have been described in the literature and these confer resistance in three main ways: efflux (*tetM*), ribosomal protection (*tetA)* and less commonly, enzymatic inactivation (t*etX*). The primary mechanism of resistance is via increased efflux of the tetracycline molecule from the cell, which is mediated by a family of Tet proteins which are found on the cytoplasmic surface of the cell membrane; twelve transmembrane helices force the tetracycline molecules out of the membrane by exchanging the molecule for a proton across a concentration gradient (Yamaguchi et al., 1990). There are seven different groups of efflux pump, which are sorted according to their amino acid sequences; all except one

tetracycline efflux pump belong to group 1, and they share almost 75% protein homology (Thaker et al., 2010). Group 1 efflux proteins can be found on transposons within large plasmids and are associated with other antimicrobial and heavy metal resistances (Chopra and Roberts, 2001). Another resistance mechanism involves ribosomal protection proteins - cytoplasmic proteins Tet(O) and Tet(M) – which bind to the ribosome, blocking binding of tetracycline molecules, as well as unbinding bound tetracycline from the ribosome (Connell et al., 2003). A final mechanism of tetracycline-resistance is antimicrobial inactivation. Tet(X) is a flavoprotein monooxygenase which hydroxylates tetracycline molecules, causing it to lose affinity for bacterial ribosomes, leading to non-enzymatic decomposition of molecules (Volkers et al., 2011).

In human medicine, tetracyclines are still routinely used for the prophylaxis of malaria in many places including the UK, USA and Europe (most commonly doxycycline) caused by *Plasmodium falciparum*, as well as for treatment of *Chlamydia* species *(*tetracycline*,* though this is being phased out, in favour of azithromycin) rickettsia and spirochetes (doxycycline) (Gaillard et al., 2015; Quinn and Gaydos, 2015; Rahi et al., 2015).

1.3.3 Quinolone Action & Resistance

Quinolones are synthetic, broad-spectrum, bactericidal antimicrobials – the first of which was Nalidixic acid (Lesher et al., 1962). Quinolones mechanistic action works on the cleavage of bacterial DNA-enzyme complexes associated with DNA gyrase and DNA topoisomerase IV. As double-stranded bacterial DNA is cleaved, bacterial DNA synthesis (replication and transcription) fails, which rapidly causes cell death (Hooper, 1998, 1999). After the introduction of first-generation quinolones such as nalidixic acid in 1962, subsequent second-, third- and fourth-generation fluoroquinolones have been developed by structurally modifying them e.g. adding a fluorine atom to the central quinolone ring; these fluoroquinolones have improved Gram-negative and anaerobic coverage (Ambrose et al., 1997). To date, ciprofloxacin is one of the most widely used fluoroquinolones in human medicine and to a lesser degree, veterinary husbandry (enrofloxacin is the most common).

Chromosomal resistance to quinolones is dependent on the bacteria; in Gram-negative bacteria mutations in the genes encoding DNA gyrase enzymes are more common (Zhao et al., 1997). Single amino acid substitutions in the ‘quinolone resistance determining region (QRDR)’ are enough to cause resistance to quinolones (Yoshida et al., 1990). In *E. coli,* Ser(83)Trp and

Ser(83)Leu substitutions, and mutations in Asp(87) within *gyrA* are the most commonly observed. These result in reduced binding of quinolones to gyrase-DNA complexes (Willmott et al., 1994; Aldred et al., 2014). Point mutations in *gyrB* or *parC* can also lead to enhanced levels of resistance to fluoroquinolones (Barnard and Maxwell, 2001).

Another mode of resistance to quinolones in *E. coli* pertains to membrane porins, OmpA, OmpC and OmpF; these porins control access of quinolones into cells – reduced expression of OmpF leads to reduced uptake of the antimicrobial molecule (Hirai et al., 1986). On the chromosome, the same loci which regulates porin expression also regulates efflux pumps. Any mutations at these loci (*MarRAB* and *SoxRS*) will reduce quinolone concentrations within the cell by reducing permeability of the membrane, but also by actively pumping out quinolone molecules from the cell via overexpression of pumps (Alekshun and Levy, 1997). These resistance mechanisms can only be vertically transferred to progeny; they are not transmissible to other species of bacteria, however, there are instances of plasmid-mediated quinolone resistance (PMQR) being described in the literature.

PMQR was first described in 1998 as conferring low-level, transferable resistance to quinolones and fluoroquinolones (Martinez-Martinez et al., 1998). The *qnr* (quinolone resistance) gene and subsequent QNR proteins inhibit quinolone binding to DNA gyrase- and DNA topoisomerase-complexes (Tran and Jacoby, 2002). Of relevance to *E. coli*, plasmids carrying *qnrA* (and other *qnr* genes), *qnrB* and *qnrS* have been identified globally and have been associated with ESBL genes (on the same integron) (Poirel et al., 2006). These genes have been shown to only confer low-level resistance, but they may also increase the mutation prevention concentration (Gebru et al., 2011; Hooper and Jacoby, 2015).

Another plasmid-mediated mode of resistance is a type of enzyme inactivation, mediated by the aminoglycoside acetyltransferase, *aac(6')-Ib-cr*. This can modify ciprofloxacin and reduces its activity by acetylation of the piperazinyl ring (Robicsek et al., 2006). This confers low-level resistance to some fluoroquinolones, but not to certain quinolones, which lack an amino-nitrogen on the piperazinyl ring, such as nalidixic acid. This AAC works synergistically with *qnrA* but has also been shown to confer low-level resistance in its absence too (Robicsek et al., 2006). Plasmid-mediated quinolone and aminoglycoside resistance genes are often reported to be co-associated with genes encoding for β-lactamases, most predominantly $bla_{\text{CTX-M-15}}$. These have been associated with the pandemic *E. coli* ST131 clone (Karisik et al., 2006; Jiang et al., 2008).

Finally, a PM efflux pump, *qepA,* has been identified on plasmid pHPA in *E. coli;* it preferentially targets ciprofloxacin and norfloxacin, but not hydrophobic quinolones such as nalidixic acid (Yamane et al., 2007). The prevalence of *qepA* - and its described variant, *qepA2* - resistance genes are estimated to be no more than ~1% in human clinical *E. coli* isolates, globally (Yamane et al., 2008). Other efflux pumps have been described in *E. coli*; AcrAB-TolC and OqxAB (Li et al., 2000; Hansen et al., 2007) are multi-drug efflux pumps which efflux chloramphenicol and trimethoprim molecules, as well as quinolones (Hansen et al., 2007; Sørensen et al., 2003).

Fluoroquinolones are another class of antimicrobials considered to be on HPCIA list. Use of quinolones is known to put selective pressure for the development of quinolone-resistant *Salmonella* spp. and *E. coli* in animals. Furthermore, the growing incidence of human disease due to AMR *Salmonella* and *E. coli* requires that fluoroquinolones are only used as a drug of last resort, to preserve their efficacy for human health (World Health Organisation, 2017).

1.3.4 Sulfonamide Action & Resistance

Sulfonamides or sulfa-drugs are a class of synthetic, broad-spectrum, bacteriostatic antimicrobials. They have been largely replaced by more narrow-spectrum antimicrobials but are still used in treatment of urinary tract infections, or in combination (potentiated) with other diaminopyrimidines, such as trimethoprim. The first sulfonamide, Prontosil, was created from an azo dye and was found to have activity against streptococcal infections – it was later found to be a prodrug. It was actually the metabolite, sulphanilamide, that was the active agent (Domagk, 1935a). Since then, numerous other sulfa-drugs including sulfathiazole, sulfadiazine and sulfamethoxazole have been developed and are commonly used today (Spink and Hansen, 1940; Schwartz, 1949).

Sulfonamide mode of action targets DNA metabolism by inhibiting folic acid synthesis. Sulfonamides competitively inhibit para-aminobenzoic acid (PABA), which prevents dihydrofolic acid synthesis (Woods, 1940; Swedberg et al., 1979), starving bacterial cells of thymidine and uridine which are necessary for DNA replication and transcription. By potentiating with trimethoprim, the combination also inhibits dihydrofolate reductase (Roland et al., 1979). This combination sequentially inhibits two enzymes along one biosynthetic route – allowing for the effective dosage to be reduced. Co-treatment with trimethoprim also combats the rapid emergence of resistance to sole sulfonamides, which began in the 1970s.

In *E. coli,* sulfonamide resistance is chromosomal or plasmid-mediated. The primary mechanism for sulfonamide resistance is mutations in the dihydropteroate synthase (DHPS) gene, *folP.* As sulfonamides are analogues of PABA, substitutions of Phe(28) on *folP* reduces the affinity of sulfa-drugs to bind to the active site of DHPS which prevents the production of dihydropteroic acid. However, it also reduces the affinity of PABA itself, albeit to a lesser degree (Sköld, 2000).

Plasmid-mediated resistance to sulfonamides is via dissemination of variant genes which encode for AMR DHPS enzymes (Swedberg and Skold, 1983). Originally only two genes, *sul1* and *sul2*, were thought to confer resistance to sulfonamides – they were found in equal proportions amongst sulfonamide-resistant *E. coli* and other bacterial species due to their ability to transfer horizontally across class 1 integrons (Radstrom et al., 1991). Despite *sul1* being the dominant resistance gene, more recently prevalence of *sul2* has increased amongst clinically-relevant isolates of *E. coli* in Denmark and the UK (Kerrn et al., 2002). Both *sul1* and *sul2* share 57% homology and their origins are yet unknown (Radstrom and Swedberg, 1988). In 2003, a third gene encoding sulfonamide-resistant DHPS was discovered in *E. coli* amongst pathogenic strains amongst pigs in Switzerland, *sul3* (Perreten and Boerlin, 2003). As recently as 2017, a fourth mobile sulfonamide gene, *sul4*, and is described as having potential impact on both humans and animals (Razavi et al., 2017).

1.3.5 Trimethoprim Action & Resistance

Trimethoprim is a broad-spectrum, bacteriostatic, synthetic antimicrobial which targets folic acid synthesis. It was first used in 1962 as a treatment for UTIs. It competitively inhibits dihydrofolate reductase (DHFR), which is the enzyme required to reduce dihydrofolic acid to tetrahydrofolic acid, which is a precursor of thymidine. Interference with this pathway ultimately inhibits DNA synthesis.

Resistance mechanisms against trimethoprim include provision of alternative metabolic pathways, changes in the DHFR enzymes and active efflux (Flensburg and Skold, 1987; Eliopoulos and Huovinen, 2001). There are a number of DHFR enzymes, which are categorised according to their amino acid sequences, and these are encoded for by *dfr* genes carried on plasmids and transposons (Blahna et al., 2006). Resistance can also be chromosomally encoded with two such mechanisms described in the literature, relating to the *dfr* gene. Less common is the overexpression of the DHFR enzyme which confers high-level resistance to trimethoprim,

though numerous mutational events are required to achieve this (Huovinen, 1987). More commonly, mutations which decrease the amount of available thymidylate synthetase precipitate, leading to underproduction of exogenous thymine, effectively making the DHFR enzyme redundant. This means that any binding of trimethoprim is irrelevant, as a lack of thymidine means there is a lack of dihydropteroic acid (the stage before dihydrofolic acid is produced). This mechanism only confers low-level resistance (Huovinen et al., 1995). The most prevalent trimethoprim resistance mechanism in *E. coli* is the acquisition of trimethoprim-insensitive DHFR variant resulting in high-level trimethoprim resistance (de Crecy-Lagard, 2014).

1.4 Escherichia coli

E. coli is a rod-shaped, Gram-negative member of the *Enterobacteriaceae* family. It is a gut commensal of warm-blooded mammals, including humans. Various strains of *E. coli* colonise the gut of infants shortly after birth and are mostly non-pathogenic (except in immunocompromised persons); they are one of the most highly adapted and successful facultative anaerobes of the human gut microflora (Sweeney et al., 1996). *E. coli* is a ubiquitous bacterium which has frequent exposure to a variety of antimicrobials in the gastrointestinal (GI) tract, aimed at treating other pathogens. *E. coli* is thought to act as a reservoir for AMR genes which may transfer resistance to other zoonotic or commensal organisms (Aarestrup, 2015); as such it would be a good indicator of resistance. *E. coli* is also the most comprehensively studied bacterium due to the relative ease of genetic manipulation and controlled growth in laboratory settings. They are relatively easy to isolate and investigate, and have been the subject of numerous studies which have highlighted the issue of increasing prevalence of drug-resistant commensal *E. coli* from healthy adults and children in many different countries (Osterblad et al., 2000; Nys et al., 2004; Pallecchi et al., 2007). They are also a major contaminant of carcasses during slaughtering of animals (Stopforth et al., 2006; Kabiru et al., 2015). For these reasons, *E. coli* was chosen as a sentinel organism in this study.

E. coli can be broadly split into commensal and pathogenic strains, though this is not always easy to do, and depends on numerous factors such as the occupying niche, and whether virulence genes are being expressed or not. Despite *E. coli* being the predominant non-pathogenic facultative bacterium in the GI tract, some strains have developed the ability to cause infections by acquiring virulence genes (Nataro and Kaper, 1998). The most successful virulence factors (encoding adhesins and toxins) combine to produce specific pathotypes of *E. coli* and infection with these can result in three general clinical syndromes: sepsis or meningitis,

urinary tract infections (UTIs) and enteric or diarrhoeal disease (Kaper et al., 2004). With advances in whole genome sequencing (WGS), it is apparent that these pathotypes are not as distinct as once thought and there has been some question as to whether pathotypes are still relevant. Whilst useful in tracking disease outbreaks or single-person diagnoses, pathotyping may be imprecise due to the continuously evolving nature of *E. coli*. This was the case with the German outbreak of *E. coli* O104:H4 in 2011 – it was originally assumed that the outbreak had been caused by an enterohemorrhagic strain. In fact, the outbreak was due to an enteroaggregative strain which had acquired Shiga toxin production genes (Buchholz et al., 2011).

There are several distinct diarrheagenic strains of *E. coli* which cause clinical syndromes including diarrhoeal disease, urinary tract infections and sepsis/meningitis. Intestinal pathogenic *E. coli* have been classically divided into 6 categories: enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). UTIs result from extraintestinal *E. coli* (ExPEC), known as uropathogenic *E. coli* (UPEC) (Lloyd et al., 2009) and sepsis/meningitis are caused by meningitis-associated *E. coli* (MNEC) (Russo and Johnson, 2000). Some of these classifications have further evolved, as our understanding of *E. coli* virulence factors has increased and with the increasing use of whole genome sequencing. For examples, some factors which do not neatly fit into one of these seven classifications have now been moved to subclasses e.g. DAEC which expresses the *aidA* adhesin gene is now a member of the atypical EPEC (aEPEC), and enteropathogenic EPEC has now been reclassified into typical (tEPEC) and atypical (aEPEC) (Croxen et al., 2013).

Pathotypes are identified according to the specific combination of virulence factors they carry. There are numerous different virulence determinants attributed to pathogenic *E. coli,* including adhesins, invasins, motility/chemotaxis assistance, toxins, antiphagocytic surface properties, defences against bactericidal or immune responses and genetic attributes. Adhesins are a group of proteins which are involved in the attachment of bacteria to non-biological (plastic and steel) and biological (intestinal wall) surfaces (Klemm and Schembri, 2000). Usually these proteins form distinct structures, called fimbriae which allow *E. coli* to attach to mannose receptors, resist shear force and obtain nutrients. Invasins allow invasive pathotypes of *E. coli* to invade and multiply within cells e.g. EIEC replication inside epithelial cells. Various toxins are produced by *E. coli* – these have effects on several eukaryotic processes. Common toxins include heat-

labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb) (Sears and Kaper, 1996), Shiga toxin (Stx) (Kaper and O'Brien, 1998) and cytolethal distending toxin (CDT) (De Rycke and Oswald, 2001). Siderophores are high-affinity iron-chelating compounds which allow bacteria to acquire iron from their immediate environment (even where iron concentrations are low) for various microbial systems. Enterobactin is an example of a siderophore found in Gram-Negative bacteria such as *E. coli* (Harris et al., 1979). Some pathogenic *E. coli* have K antigens, which are capsular polysaccharides that decrease binding of antibodies to the bacteria, and reduce the ability of phagocytes to recognise and engulf the bacterial cells (Van Dijk et al., 1979). Combinations of these virulence factors pertain to a specific pathotypes (**Table 1.1)**.

Table 1.1 Characteristics and associated virulence factors for the main six pathotypes of diarrhoeal-associated *E. coli*.

Pathotype	Presents as/associated with	Characteristics	Major Virulence Factors (Genes)	Virulence Functions	References
DAEC	Urinary Tract Infection Pregnancy Complications Diarrhoea in children <5 years old.	Adheres to HEp-2 cell monolayers	***Adhesins (Afa/Dr)*** *afaE-I, afaE-III, daaE, draE, draE2*	***Adhesins*** ▪ Bind to decay-accelerating factor (DAF) which protects cells from complement damage. ▪ Has cytopathic activity; develops long extensions which constrict adherent bacteria. ▪ Activates signal transduction cascades including activation of PI-3 kinase.	*(Guignot et al., 2000; Nowicki et al., 2001; Servin, 2005)*
ETEC	Rapid onset of watery diarrhoea.	Produce heat-labile (LT) and heat-stable (ST) enterotoxins. Can express both or just one or the other. Produce more than one colonising factor which help with attachment to intestinal mucosal surfaces.	***Adhesins*** *cfaB, cooA, cs3, cssA, etpA, etpB* ***Toxins*** *eltA-B, estIa*	***Adhesins*** ▪ Adhesive fimbriae – binds to oligosaccharide components of glycolipids and glycoproteins ▪ EtpA – interacts with flagellin (tips of flagella) and tethers EtpA adhesins which anchor bacteria to cell surfaces ***Toxins*** ▪ Heat-labile – activates adenylate cyclase, resulting in ion secretion ▪ Heat-stable – STa activates guanylate cyclase, resulting in ion secretion. STb increased intracellular calcium, resulting in ion secretion.	*(Nataro and Kaper, 1998; Kaper et al., 2004; Roy et al., 2009)*
EIEC	Diarrhoea Shigellosis/bacillary dysentery Haemolytic Uremic Syndrome	EIEC is an intracellular pathogen Has almost identical virulence factors to *Shigella* spp. Dysentery caused by EIEC is indistinguishable from that caused by Shigella. Contains large plasmids which are functionally interchangeable with *S. flexneri*	**Actin-based motility** *icsA/virG* **Endotoxin** *gtrA-B, gtrII* **Iron uptake** *iucaA-D, iutA* **Protease** *pic* **Toxin** *set1A-B, senB, stxA-B* **Pathogenicity islands** SHI-1-3, SRL	***Actin-based motility*** *IcsA (VirG) – intracellular movement using actin tails* ***Endotoxin*** *LPS* – resist host defences and for intracellular spread. ***Iron uptake*** *Aerobactin* – siderophore which facilitates iron uptake for intracellular growth. ***Protease*** *Pic* - protease and mucinase ***Toxins*** ▪ ShET1/2 – involved in early diarrhoeal phase ▪ Shiga Toxin – responsible for complications such as haemorrhagic colitis and haemolytic uremic syndrome. ***Pathogenicity islands***	*(Nataro and Kaper, 1998; Vargas et al., 1999; Vokes et al., 1999; Al-Hasani et al., 2001; Morona et al., 2003; Cossart and Sansonetti, 2004; Bergan et al., 2012)*

				Carries ShET1 and autotransporters SigA and Pic.	
EAEC	Traveller's diarrhoea Haemolytic Uremic Syndrome Persistent diarrhoea	No secretion of heat-labile or heat-stable toxins. Adheres to the surface of HEp-2 cells in an aggregative pattern Mainly heterogenous, but many harbour virulence plasmids	***Adherence*** ***AAFs*** *aafA-D, agg3A-D, aggA-D, R* ***Dispersin*** *aap, aatA-D, P* ***Toxins*** *astA, pet, pic, set1A*	***AAFs*** Belongs to Dr adhesin family Most EAC harbour plasmids which encode AAF/I or AAF/II and/or EAST1 and Pet toxins ***Dispersin*** Promotes dispersal of EAEC on intestinal mucosa to establish new infections and facilitate colonisation. ***Toxins*** ▪ EAST1 – Activates guanylate cyclase resulting in ion secretion ▪ Pet – Serine protease cleaves cytoskeletal proteins resulting in epithelial cell rounding ▪ Pic – protease and mucinase ▪ ShET1 – Involved in ion secretion	*(Bernier et al., 2002; Sheikh et al., 2002; Kaper et al., 2004) (Fasano et al., 1995; Henderson et al., 1999; Dutta et al., 2002; Menard and Dubreuil, 2002)*
EPEC	Diarrhoea in children <5 years old.	Creates an attaching and effacing lesion on intestinal epithelium. Does not produce Shiga toxin. Carries a large virulence plasmid which allows production of bundle-forming pili with a characteristic pattern called localised adherence.	***Adherence*** *bfpA-L,P, eae, lifA/efa1, paa* ***Protease*** *espC* ***Regulation*** *ler* **Toxin** *cdtA-C, east1* **Pathogenicity islands** EspC island LEE	**Adherence** ▪ BFP – localised adherence via pili. ▪ Intimin – as EHEC. ▪ Lymphostatin/LifA – expresses various lymphokines and inhibits lymphocyte proliferation ▪ Paa – as EHEC. **Protease** EspC – serine protease **Regulation** Ler – as EHEC **Toxin** ▪ Cytolethal Distending Toxin – disrupts chromatin which leads to growth arrest and cell death. ▪ EAST1 - Activates guanylate cyclase resulting in ion secretion. **Pathogenicity islands** ▪ EspC – contains *espC* and *orf3* virulence loci. ▪ LEE – as EHEC	(Bieber et al., 1998; Sperandio et al., 2000; Menard and Dubreuil, 2002; Badea et al., 2003; Navarro-García et al., 2004) (Schmidt, 2010)
EHEC	Watery diarrhoea, haemorrhagic colitis, HUS	Has a pathogenicity island for enterocyte effacement (LEE) Produces Shiga toxins	***Adherence*** *yagV,ecpE, yagW/ecpD, efa1, eae, paa, toxB*	***Adherence*** ▪ ECP – pilus adherence factor crucial for virulence in O157 strains. Also carried in commensal strains.	(Badea et al., 2003; Batisson et al., 2003; Rendón et al., 2007; Leo et al., 2015)

			Iron uptake *chuA, chuS, chuU, chuW* ***Protease*** *espP, stcE* ***Regulation*** *ler* ***Toxin*** *hlyA-D* *stx1A/B*, *stx2A/B* ***Pathogenicity island*** LEE	▪ Efa-1/LifA – main adhesin in non-O157 strains. Inhibits lymphocyte activation ▪ Intimin – facilitates intracellular changes – reorganises cytoskeletal proteins and polymerises actin. ▪ Paa – involved in initial bacterial adhering. ▪ ToxB – affects the production of virulence factor secretion ***Iron uptake*** Chu – excretion of cytotoxins which gain access to intracellular heme reservoir. Facilitates usage of iron from heme. ***Protease*** ▪ EspP – serine protease; cleaves coagulation factor V ▪ StcE – cleaves C1 esterase inhibitor, required for complement initiation. ***Regulation*** Ler – required for pathogenicity island activation (activates *LEE2*, *LEE3*, *tir* and *orf19* promoters) ***Toxin*** Haemolysin -broadly cytotoxic to different types of blood cell. Stimulates release of IL-1β and TNF. Stx – responsible for haemolytic uremic syndrome.	(Torres and Payne, 1997) (Lathem et al., 2004) (Sperandio et al., 2000) (Sandvig, 2001; Schindel et al., 2001) (Schmidt, 2010)

1.5 Epidemiology of AMR in Africa

1.5.1 Defining the Issue of Increasing AMR

The 2014 WHO global report on surveillance of AMR and the O'Neill report (tackling drug-resistant infections globally) have described significant gaps in country-wide surveillance systems and a lack of standardised methods for capturing AMR data and sharing it (WHO, 2014; O'Neill, 2016). In particular, the WHO report highlighted the fact that Africa and South-East Asia are two major regions in which there are no rigorous country-wide surveillance systems in place for AMR (WHO, 2014).

The lack of quantitative data on AMR, particularly in the Africa region, is problematic. As there is no readily available national (or even regional, in many cases) datasets, guidelines will have to be based on sporadic reports from all over the countries in the region, or from trend-data (which can be equally sporadic) from neighbouring countries. These data may not accurately reflect the epidemiology of disease and AMR in the area in which the guidelines are issued, and so there can be reliance on empirical therapies such as broad-spectrum antimicrobials, which can have an exacerbating effect of increasing AMR (Saleh et al., 2015). Furthermore, there is a significant gap in public health capacities, particularly in more rural areas. There are too few government-regulated physicians and veterinary staff to cover large areas, and a severe lack of diagnostic facilities in which to perform AMR testing (Mboya-Okeyo et al., 2009). Diagnostic services are needed to assess and confirm the resistance patterns of bacteria, so that appropriate antimicrobials can be used, thus reducing the selection for AMR. In the absence of systematic screening programmes, conducted by quality-assured microbiological laboratories (Exner et al., 2017), antimicrobials tend to be repeatedly used in the same patterns.

Despite limitations in diagnostic capabilities, there are other socioeconomic factors at play – access to antimicrobials, costs of those drugs and questionable quality of drugs. There is a lack of regulation regarding the sale of antimicrobials in many developing countries; antimicrobials can be purchased without a prescription and they are dispensed by either street vendors or unlicensed stockists (retail pharmacies), and may be administered by untrained persons (Barker et al., 2017; Erku et al., 2017). These drug vendors sell medications for human and animal consumption often without requesting a prescription (Zawahir et al., 2019). In addition to this, there may also be gaps in vendor's knowledge of diseases and they may resort to blind recommendation of multiple and broad-spectrum antimicrobials to ensure a positive treatment outcome. Unlicensed pharmacies can be seen as a more attractive option than (sparse)

government-owned facilities, as they can have shorter waiting times, do not charge consultation fees and may be willing to negotiate treatment options and prices, to better fit with the purchaser's financial ability. These types of pharmacies, which cater to both human and veterinary needs, especially in rural Africa, have become the new primary level of care (Kwena et al., 2008; Mukonzo et al., 2013; Zawahir et al., 2019).

There are several issues with unlicensed retail pharmacies. High ambient temperatures and humidity can quickly degrade the quality of antimicrobials during storage (Okeke and Lamikanra, 2001; Risha et al., 2002). Degraded antimicrobials may contain less than the stated dose (Lallemand et al., 2016), which means that the intended recipient (human or animal), may receive less than the optimal dose of the drug. Sub-therapeutic dosing can select for AMR bacteria. There is also a problem of counterfeit drugs, in which the drug may contain little or no active substance of the antimicrobial or the wrong substance. The influx of counterfeit and sub-standard antimicrobials into the pharmaceutical markets in some regions is a major problem (Ozawa et al., 2018) as these preparations of reduced potency also result in pathogens being exposed to sub-therapeutic concentrations of the drug. Studies have observed that the highest prevalence of poor-quality medicines was observed in African countries, where 18.7% of antimicrobials were substandard or falsified (there were more substandard antimalarials (19.1%) than antimicrobials (12.4%)) (Ozawa et al., 2018). These findings were in agreement with the report produced by the WHO regarding falsified medical products (Tabernero et al., 2014; WHO, 2017b).

Antimicrobial misuse is another large factor contributing to the increase in AMR. Particularly relevant for African settings, is the diagnosis and treatment of malaria; as there is improved diagnosis for malaria, global transmission is slowly declining, however it has also highlighted the lack of testing available for other infectious diseases. In these cases, patients who tested negative for malaria, were still given a course of antimicrobials (Sandlund et al., 2013; Hopkins et al., 2017). There are clinical treatment algorithms in place, as well as guidelines published by the WHO, which have sought to optimise antimicrobial prescriptions in resource-limited settings, but there is still an unmeasured overuse of antimicrobials (Vasan et al., 2014; Gera et al., 2016; Chem et al., 2018). Healthcare providers should play an essential role in the treatment and prevention of diseases, but in informal settings, prescription of antimicrobials (or lack thereof) are frequently inappropriate; they can be for an inappropriate antimicrobial, the wrong dose, or an antimicrobial may not be necessary (Ayukekbong et al., 2017). Healthcare

professionals, and those people working in retail pharmacies, may be restricted by poor dissemination of research information. As access to current information on AMR patterns within the country is challenging (and not always available) the same antimicrobials are routinely recommended to purchasers. As there is little capacity to perform AMR testing, and patients do not necessarily have the money, or wish to pay for these tests, there is an over-reliance on broad-spectrum antibiotics such as tetracyclines and penicillins. Long-term, inappropriate use of broad-spectrum antimicrobials can contribute to the development of resistance; a selective pressure is placed not only on the disease-causing bacterium, but also on commensal bacteria (such as *E. coli*) (Hansen et al., 2017).

Finally, non-human use of antimicrobials can contribute to the development of AMR. As discussed, in African settings, a high proportion of people engage in mixed crop-animal farming. Antimicrobials have historically been used for prophylaxis and treatment of animals, as well as for growth promotion. Antimicrobials are also used directly and indirectly in crop farming – directly sprayed onto crops to prevent disease and treat disease, or indirectly, through antimicrobial residues found in animal manure in a host of low and middle-income countries (LMICs), which are used to fertilise fields (Tasho and Cho, 2016; Collignon and McEwen, 2019). In Kenya, particularly high levels of antimicrobial drug residues were detected in meat intended for consumption (Mitema et al., 2001a), though this could be due to AMU or lack of information surrounding withdrawal periods.

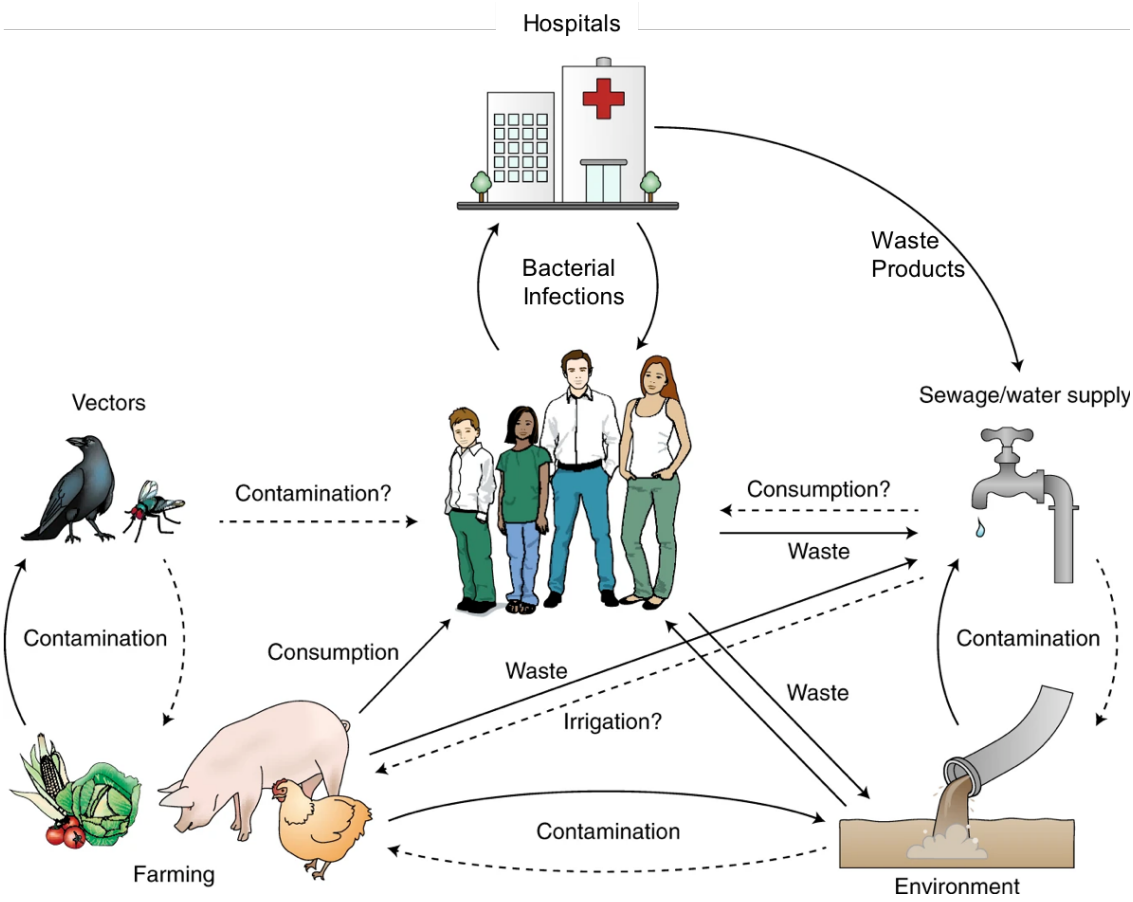
Overuse of antimicrobials can be attributed to their ease of access. In the absence of government-controlled veterinary services, veterinary paraprofessionals (animal healthcare assistants, AHAs) may travel to farms and treat animals. There are strict laws regarding the delivery of antimicrobials and these are regulated by three different laws in Kenya – the Animal diseases Act, the Veterinary Surgeons and Veterinary Para-professionals Act and the Pharmacy Poisons act (Global Antibiotic Resistance Partnership, 2011; National Council for Law Reporting, 2012b). Theoretically, AHAs must be registered with the Kenya Veterinary Board to practice as a paraprofessional. However, in rural areas, there is extremely limited access to veterinary services from registered veterinary surgeons. This means that there is reliance on private veterinary paraprofessionals who may dispense antimicrobials inappropriately. Animal owners may freely access drugs without prescription from retail pharmacies (or Agrovet shops, which stock agricultural products and antimicrobials for animals) and treat their animals, or get para-professionals to do it (Global Antibiotic Resistance Partnership, 2011). Kenyan law also states

that “A person shall be qualified for registration as a veterinary paraprofessional if the person is a citizen of Kenya and has successfully completed a post-secondary school training course in animal health science lasting two years or more at an institution approved by the Board and has a certificate, diploma or degree and has served an internship of not less than twelve months under supervision of a registered veterinary surgeon” (National Council for Law Reporting, 2011). As there is insufficient capacity to fully regulate private veterinary paraprofessionals, it is unclear if their training is up to date or not.

As previously discussed, there is some link between AMR bacteria and their spread between animals, humans, and the environment. In rural African areas, three major dissemination pathways of AMR bacteria are possible. Firstly, close contact with animals is frequent, as animals are usually free to graze around farms, and humans’ domiciles are often near chicken coops, cattle huts, and sheep pens. Human sewage is perhaps also an issue – many farms do not have toilets inside the main house, rather dug latrine pits are more common. These are not treated with chemicals, and are simply filled in when they are full, and a new one dug nearby (WSP, 2004). Antimicrobial residues in faeces can leach into the surrounding soil and into water sources; contaminated water may then be drunk or crops may take up residues through the soil (Tasho and Cho, 2016). Animals treated with veterinary antimicrobials may also defecate and urinate in the vicinity of human dwellings, further allowing the spread of residues (**Figure 1.3**). There may also be some issues with use of expired antimicrobials – socioeconomic reasons may prevent an antimicrobial user to continue using antimicrobials from a previous treatment, instead of disposing and purchasing a new batch. Furthermore, there can be contamination of the environment with the disposal of unused antimicrobials.

Whilst it is currently unknown what the magnitude of AMU in food-producing animals is to human AMR, the extremely close contact between humans and their animals warrants further investigation into dissemination and transmission pathways between these groups.

Figure 1.3 Possible routes of transmission of AMR bacteria between humans, animals and their shared environments. Dashed lines are presumed routes. Adapted from (Walsh, 2018).



1.5.2 General Trends of Prevalence of AMR in Sub-Saharan African Countries

A number of studies conducted over the last 30 years have investigated the prevalence of AMR in the *Enterobacteriaceae* in African countries. These studies have all highlighted the increasing prevalence of AMR Gram-negative bacteria, including *E. coli,* in a host of community and clinical settings, as well as in food-producing animals in Africa. These studies show that there is an increasing prevalence of AMR bacteria, and high-level resistance to commonly used antimicrobials such as oxytetracycline, aminopenicillins, sulfonamides and phenicols (Laxminarayan et al., 2013; Leopold et al., 2014; Van Boeckel et al., 2014; Tadesse et al., 2017; Founou et al., 2018). In human populations, the need for these antimicrobials is driven by the endemicity of diarrhoeal disease, respiratory tract infections, malaria and HIV/AIDs in sub-Saharan African countries (WHO, 2018), as well as the increase burden of zoonotic diseases such as brucellosis, leptospirosis and Q fever (Cleaveland et al., 2017). As the incidence of these diseases increases, there is increased demand for antimicrobials for prophylaxis and therapeutic treatment of humans, but also to treat the animals which spread these diseases.

There is a paucity of AMR data in many African countries, at the time of writing, 23 out of 54 countries did not have any robust, published, AMR studies. There have been several systematic reviews and meta-analyses of AMR in the African continent during the last 10 years – each of these has attempted to collate data from published studies which detail AMR in various bacteria such as *E. coli*, *S. aureus*, *S. typhi* etc. Most of these studies focus on human, clinical infections, though there are also sporadic studies conducted in community settings and in food-producing animals too. The most recent systematic review of human-derived AMR in Africa assessed 144 studies across the continent to form an insight into AMR in the African context. Tadesse et al were able to calculate the prevalence of AMR, median resistance and inter-quartile ranges for a number of bacterial species, and they used these to calculate a standardised measure of overall resistance in human populations from the data (Tadesse et al., 2017). Three other systematic reviews were also examined, regarding AMR *E. coli* in humans, however these were limited to smaller parts of Africa i.e. Eastern Africa only, Western Africa only and these only encompassed a far smaller number of studies (14).

A limitation in this collation of AMR across African countries is that their comparison was made from different patient groups, and across different countries. This approach gives an overview of resistance across Africa, but this may also have normalised peaks of resistance to specific antimicrobials, in different countries reducing the ability to detect country specific outbreaks. It is arguable that due to the large number of *E. coli* isolates tested, there is sufficient resolution to deduce general trends. Similarly, not all studies included in this analysis utilised the same laboratory methodologies, however, the majority of studies used the disk diffusion method (81.9%) and CLSI guidelines (72.9%). As such, the variation in AMR methodology on the validity of the final results was suggested to be minimal. This data is shown here, however, individual studies are examined in more detail in Sections **1.5.2.1 – 1.5.2.3**.

Data pertaining to human AMR *E. coli* has been extracted from Tadesse et al (2007) systematic review of AMR in human participants, in clinical and community settings, and is presented here (**Table 1.2**). The highest prevalence of AMR in human-derived *E. coli* was to macrolides, penicillins, tetracyclines, β-lactams and potentiated sulfonamides. Alongside this data, the review highlighted that a third of African countries had no publicly published data and that there is a need for standardisation of microbiological identification of bacteria and testing methodologies of AMR, in order to allow for national and international surveillance of AMR.

Table 1.2 Collated prevalence of AMR *E. coli* from a systematic review of AMR in human participants, in clinical and community settings. Antimicrobials are sorted in descending order of prevalence, according to their class. Extracted from (Tadesse et al., 2017).

Antimicrobials	Antimicrobial class	*# of Isolates*	Median Prevalence (%)	IQR
Tobramycin	Aminoglycoside	677	32	(12.3–43.2)
Gentamicin	Aminoglycoside	8282	29.8	(8.4–45.3)
Amikacin	Aminoglycoside	5422	0.2	(0–24.5)
Meropenem	Carbapenem	3402	0	(0–5.7)
Cefalotin	Cephalosporin	515	56.9	(23.5–63.5)
Ceftriaxone	Cephalosporin	2800	31.5	(6.9–47.7)
Cefuroxime	Cephalosporin	3925	30	(19.7–51.2)
Cefotaxime	Cephalosporin	5173	26.8	(8.3–64.5)
Cefepime	Cephalosporin	957	21.8	(5.8–42.5)
Ceftazidime	Cephalosporin	2773	19.5	(10.0–55.8)
Cefoxitin	Cephalosporin	535	8.3	(2.9–44.1)
Nalidixic Acid	Quinolone	2960	36	(12.7–53.8)
Ofloxacin	Fluoroquinolone	1294	30.4	(9.8–47.9)
Norfloxacin	Fluoroquinolone	876	25.6	(15.0–46.3)
Ciprofloxacin	Fluoroquinolone	7877	23.2	(7.7–35.6)
Levofloxacin	Fluoroquinolone	751	19.2	(8.7–47.6)
Erythromycin	Macrolide	675	81.6	(29.9–86.5)
Nitrofurantoin	Nitrofuran	5087	14	(4.5–25.1)
Amoxicillin	Penicillin	5500	88.1	(81.4–92.6)
Ampicillin	Penicillin	2951	86.7	(69.2–96.7)
Penicillin	Penicillin	43	62	(52.9–90.6)
Amoxicillin/Clavulanic Acid	Penicillin + β-lactam	6764	43.5	(30.8–61.9)
Chloramphenicol	Phenicol	2963	40.9	(11.3–58.0)
Trimethoprim/Sulphamethoxazole	Potentiated Sulphonamide	7493	80.7	(69.8–85.6)
Tetracycline	Tetracycline	2896	76.2	(72.6–87.9)
Doxycycline	Tetracycline	302	54.5	(12.8–72.3)
Oxacillin	β-lactam	411	91.5	(22.2–98.5)
Piperacillin	β-lactam	132	58.4	(35.1–95.0)
Imipenem	β-lactam	1613	0.2	(0–5.5)
Piperacillin/Tazobactam	β-lactam + β-lactamase inhibitor	235	21	(11.1–30.6)

A similar analysis was performed to gauge the general trends of AMR *E.coli* associated with food-producing animals, across Africa. Another systematic review of AMR in food animals in Africa was conducted by Founou et al; 6 studies specifically examining AMR from a mixture of healthy, sick, or dead food-producing animals and animal products. Data pertaining to *E. coli* in food-producing animals was extracted here (**Table 1.3**). A high prevalence of AMR was found in Animal-origin *E. coli*, especially to ampicillin and tetracyclines. A more variable prevalence of resistance to potentiated sulfonamides and phenicols was also noted.

Table 1.3 Collated prevalence of AMR *E. coli* from 6 studies of AMR *E. coli* in food-producing animals (cattle, pigs and poultry) from a mixture of farms and slaughterhouses. Colour-coding indicates high prevalence (red) to low prevalence (green). Adapted from (Founou et al., 2018). Blank space indicates no data present.

				Antimicrobial Class													
				Penicillin	Cephalosporin		Aminoglycoside					Quinolone	Fluoroquinolone		Tetracycline	Phenicol	Nitrofuran	Potentiated Sulfonamide
Guidelines	# of Isolates	Resistant isolates (%)	MDR isolates (%)	Ampicillin	Cefuroxime	Cefotaxime	Gentamicin	Streptomycin	Kanamycin	Amikacin	Neomycin	Nalidixic acid	Ciprofloxacin	Enrofloxacin	Tetracycline	Chloramphenicol	Nitrofurantoin	Trimethoprim-sulfamethoxazole
EUCAST	371	16.4		6.5			2.0						1.5		11.2			4.0
EUCAST	162	100		100								55	55					
EUCAST	67	100	100				6.0			6.0		89.5			94			73.1
NCCLS	187		91.6	90.4	49.5	0	84.5			56.0					91	82.5		62.0
NCCLS	116	100	100	72.9	65.7		11.4					35.7			91.4	72.9	72.9	44.3
CLSI	235	65.5	37.9	40.6			1.0	36.0	11.6						28.6	4.0		22.0
CLSI	154	96.2	69.5	82.5				50.3			24.0	37.7	22.1	25.3	75.3	42.9		
Calculated Overall Resistance*		54.8	42.6	51.2	13.1	0	14.3	12.5	2.1	8.4	2.9	19.2	10.0	3.0	43.6	24.3	6.5	21.9

Each row represents a different study, data is for AMR in food-producing animals only. Missing data was not reported. ***Calculated Overall Resistance**: proportion of isolates resistant to each antimicrobial, divided by the total number of isolates collected across all studies. **MDR isolates:** resistant to 3 or more classes of antimicrobial. All resistance figures are percentages.

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As with the human derived *E. coli* dataset (**Table 1.2**), this collation of food-producing animal *E. coli* has its own limitations. There was no data on antimicrobial consumption included in the studies due to lack of availability. There was no correlation between resistance to specific antimicrobials, particularly those regarded as HPCIAs, and resistance or virulence genes, as these were not published. This is reflective of the limited laboratory capacity in Africa. The included studies were similar in the origin of animals (similar sized farms/slaughterhouses), farming and slaughterhouse practices and study design. Despite this, there were 3 different AMR guidelines used (CLSI/EUCAST/NCCLS), so studies should not necessarily be deemed as fully comparable. However, this gives a broad overview of AMR trends in food-producing animals across Africa.

1.5.2.1 Detailed Prevalence of AMR *E. coli* in hospital and community settings

Most studies relating to AMR in Africa are either clinical or community based. The incidence of both hospital-acquired and community-acquired infections are hard to gauge as there are inconsistencies in reporting methods and the panels of antimicrobials tested. Furthermore, studies are often limited to small geographic areas. Various reports suggest a decrease in the incidence of hospital-acquired infection due to the improvement being made in infection and prevention controls, though AMR still remains prevalent (Wangai et al., 2017). However, numerous other studies show that many healthcare facilities have ineffective plans in place (Mugomeri, 2018; Oji et al., 2018). In community-acquired infection literature, it is common to see an increasing number of AMR infections. This is mainly due to use of broad-spectrum antimicrobials as empirical therapies, unregulated access to antimicrobials, self-medication etc. (Al-Kubaisi et al., 2018).

In sub-Saharan Africa, diarrhoeal diseases are one of the most common causes of hospitalisation and death, particularly in children (GBD 2016 Diarrhoeal Disease Collaborators, 2018). ETEC is the predominant cause of travel-associated diarrhoea and in cases of children under the age of five, in developing countries. ETEC is typically associated with acute and chronic diarrhoea. Persistent diarrhoea results in a high proportion of deaths by nutritional deficit (Levine and Edelman, 1984; Okeke et al., 2000; Okeke, 2009). In 2013, the Global Enteric Multicentre Study published a contrasting report indicating that EPEC, not ETEC, was one of the most commonly isolated *E. coli*, associated with moderate-to-severe, infant diarrheal disease in sub-Saharan Africa and South Asia (Kotloff et al., 2013). Diarrhoeagenic *E. coli* has a disproportionately larger impact in LMICs due to high population densities, lack of infrastructure and slower responses to

disease outbreaks, a high proportion of people with compromised immunity due to comorbidities such as HIV/AIDS or parasitic diseases, and lifestyles in which daily life depends on animals. Diarrhoea persisting in patients is routinely treated with azithromycin, doxycycline, trimethoprim-sulfamethoxazole and fluoroquinolones (Paredes-Paredes et al., 2011; Taylor et al., 2017). Whilst there is a case for treating moderate-to severe diarrhoea with antimicrobials, in most cases antimicrobial therapy is not suggested, because the illness is usually self-limiting. Antimicrobial therapy may lead to adverse events, and unnecessary treatments exacerbate AMR development (Zollner-Schwetz and Krause, 2015).

A study from Guinea-Bissau screened 408 children under the age of 5 years presenting with fever or tachycardia to paediatric emergency wards, for faecal carriage of ESBL-producing *E. coli*. Susceptibility to cefotaxime, ceftazidime, gentamicin, tobramycin, tigecycline and amoxicillin-clavulanic acid was tested with the VITEK2 system and susceptibility to trimethoprim-sulfamethoxazole, piperacillin-tazobactam, ciprofloxacin and meropenem was tested with the antibiotic disc diffusion method, using EUCAST guidelines. In the study, 32.6% of participants carried at least one ESBL-producing *E. coli* (47.7%) or *K. pneumoniae* (52.3%) (Isendahl et al., 2012). The predominant β-lactamase gene family was $bla_{CTX\text{-}M}$ (96.4% in *E. coli*). Three isolates were determined to belong to the pandemic clone ST131. Quinolone resistance was reported in 81.9% *E. coli* isolates, as well as 43.4% resistance to aminoglycosides, and 94.0% resistance to trimethoprim-sulfamethoxazole, however no carbapenem resistance was found. (Isendahl et al., 2012). A similar study in Cameroon examined ESBL-producing *Enterobacteriaceae* from 358 faecal samples of outpatients and healthy volunteers, *E. coli* represented the most common species associated with ESBL production (78.4%). The majority of ESBL-producers carried $bla_{CTX\text{-}M\text{-}15}$ (98.0%) and resistance was common to gentamicin (61.0%), ciprofloxacin (75.6%), nitrofurantoin (34.1%) and trimethoprim/sulfamethoxazole (97.5%) (Lonchel et al., 2012). In Benin, the prevalence of ESBL-*E. coli* in nosocomial infections was determined and found in 35.5% isolates. There was a high prevalence of resistance to amoxicillin (92.8%), ampicillin (94%), trimethoprim-sulfamethoxazole (85.7%), ceftriaxone (58.3%) and gentamicin (54.8%) (Anago et al., 2015). In Tanzania, a study examining the carriage of *E. coli* in stool samples (and milk from animals) from three different cultural groups indicated a high prevalence of ampicillin (68.4%), tetracycline (52.5%), trimethoprim (54.2%), sulfamethoxazole (57.5%) and streptomycin (40.6%) resistance (Caudell et al., 2018) This study did not look for ESBL-*E. coli* however.

In Ethiopia, a study examining UTIs showed that of 228 cultured urine samples, only a small percentage were significant bacteriuria cases (9.2%). Of these, *E. coli* was isolated in a third of cases; resistance to a number of antimicrobials was described, including ampicillin (100.0%), amoxicillin (100.0%), clindamycin (28.6%), ciprofloxacin (14.3%), tetracycline (28.6%) and trimethoprim/sulfamethoxazole (28.6%) (Beyene and Tsegaye, 2011). In another UTI study in Rwanda, examining resistance in *E. coli* in community- and hospital-acquired UTIs, showed high prevalence of resistance to various antimicrobials including: amoxicillin (93.0%), nitrofurantoin (28.1%), nalidixic acid (61.3%), amoxicillin/clavulanic acid (63.1%), gentamicin (41.5%) and trimethoprim/sulfamethoxazole (80.0%) (Muvunyi et al., 2011).

Few studies have investigated the socioeconomic costs of AMR. In Tanzania, a cohort study examining the risk of fatality after admission to hospital (of children between the ages of 0-7) with systemic infections, revealed that AMR was a major risk factor in patients with septicaemia (Blomberg et al., 2007). In Uganda, patients with ESBL-producing *Enterobacteriaceae* and MRSA were more at risk of death than those without, in cases of surgical site infections (Seni et al., 2013). Numerous studies in high-income countries (HICs) have highlighted that patients with AMR-associated infections have higher rates of mortality, require longer hospital stays and have higher overall healthcare costs when compared to patients with antimicrobial-susceptible infections (Mauldin et al., 2010; Neidell et al., 2012); it is likely to be the same in LMICs.

A Kenyan survey of more than 900 *E. coli* isolates, isolated from urine, blood and diarrheal clinical cases over an 18-year period showed high rates of resistance to β-lactam antimicrobials; 27.0% of isolates were ESBL-producing, and 57.5% of those ESBLs showed resistance to aztreonam, ceftazidime and other cephalosporins (Kiiru et al., 2012). The report suggests that AMU correlates to the AMR resistance phenotypes being reported such as ampicillin, trimethoprim/sulfamethoxazole, streptomycin, and amoxicillin/clavulanic acid. This report also indicates moderate resistance to third-generation cephalosporins and fluoroquinolones (Kiiru et al., 2012).

The overall prevalence of AMR *E. coli* appears to fluctuate between different parts of Africa. It is particularly important that policy makers have accurate data on hospital-acquired infections, in order to better implement infection control policies, reducing the need for antimicrobial therapies. In many LMICs there are few auditing systems, and so rates of AMR are never truly known, even by healthcare staff. For community-acquired infections, most studies suggest a

general increase in AMR infections. The highest proportion of resistance is to oral antimicrobials, which can be acquired without prescription from retail pharmacies or community hospitals (Kalungia et al., 2016; Mboya et al., 2018).

1.5.2.2 Detailed Prevalence of AMR *E. coli* in animal settings

Smallholder farms are the most common type of farming in sub-Saharan Africa. The World Bank estimates that approximately 70% of people engage in some degree of farming in Kenya (and in other sub-Saharan African countries), and the majority of them live around or below the poverty line (Mondiale, 2008; Wiggins et al., 2010). Traditionally, farming encompasses keeping animals including indigenous east African Zebu cattle, sheep and goats in the low-rainfall, semi-arid and arid parts of the country, though in more recent years there has been a major shift towards smaller holdings and marketed milk production using imported (or exotic) cattle in mixed, crop-livestock systems. The production system in these areas includes mainly dairy cattle, other livestock (mostly poultry, sheep, and goats), cash crops (e.g., coffee, tea, horticulture) and subsistence crops (e.g., maize, vegetables). Various studies have investigated the prevalence of AMR *E. coli* in food-producing animals, though there are far fewer studies than in human cases, in Africa. Studies which analyse *E. coli* also appear to be far fewer than in other enteric bacteria such as *Salmonella* spp. and *Campylobacter* spp. in animals.

In a stratified random sample of Zambian dairy cattle on 104 different small, medium, and commercial-sized dairy farms, faecal *E. coli* was analysed for AMR. Diarrhoea in the cattle was the most common reason for treating with antimicrobials (mean number of cases was 54.1%); the estimated prevalence of resistance across the different farming systems (376 isolates) was found to be surprisingly low in almost all of the antimicrobials tested - tetracycline (10.6%), ampicillin (6.0%), sulfamethoxazole/trimethoprim (4.5%), cefpodoxime (1.9%), gentamicin (0.9%) and ciprofloxacin (0%) (Mainda et al., 2015). Mainda et al suggest that there was a higher prevalence of AMR *E. coli* from dairy cattle in commercial-sized farms, compared with medium- and small-scale farms, however there was no statistically significant difference. The differences they reported in prevalence of AMR *E. coli* from different sized farms could be attributed to differences in testing methodology, however the study does not explain those differences. Of note was a significant association between exotic breeds (imported Friesian and Jersey breeds) and a higher rate of isolation of AMR *E. coli* compared to local breeds (Zebu). Future work could be undertaken in order to assess if local breeds are more inherently resistant to specific diseases, and therefore require fewer antimicrobials to maintain their health.

In a Ghanaian study, 210 stool samples were collected from farm animals, including goats, sheep, pigs and poultry and *E. coli* isolated. There was a high prevalence of resistance of AMR *E. coli* in 8 of the 9 tested antimicrobials: cefuroxime (97.7%), ampicillin (95.7%), tetracycline (91.6%), chloramphenicol (80.9%), gentamicin (75.0%), co-trimoxazole (68.3%) and amikacin (60.8%). Of these isolates, 97.7% were MDR (resistant to 3 or more classes of antimicrobial, according to disc-diffusion test). There was no reported resistance to cefotaxime in *E. coli* from any of the animals in the study. There was correlation between the high prevalence of resistance of all animal isolates, and the high rates of antimicrobial usage in animal husbandry in Ghana (Donkor et al., 2012). The antimicrobials commonly used in Ghanaian animal husbandry practices correlated with AMR in animal *E. coli* isolates, especially in tetracycline and penicillins. These antimicrobials have been described in other studies as being widely available on the market, which suggests that they have been used extensively in veterinary and human medicine (Newman et al., 2011).

AMR is also an issue for aquaculture – several classes of antimicrobials are commonly used in large quantities in the fish industry, particularly in LMICs, where they are not regulated. This has resulted in a number of African aquaculture products containing high antimicrobial residues - this is not only a public health issue, but also an economic one, as exports may decrease or products may become devalued due to AMR (Okocha et al., 2018). An example of this is oxytetracycline residues detected in African catfish, in Nigeria. The study showed that the majority (95%) of questioned fish farmers frequently administered antimicrobials (in feed and water) to their fish without veterinary prescription, and did not observe withdrawal periods (Olatoye and Basiru, 2013)

There are many studies which have looked specifically for ESBL, plasmid-mediated AmpC (pAmpC) and carbapenemase-producing *E. coli* in farm animals in various African countries; detection is often associated with MDR and infections caused by pAmpC-producing Enterobacteriaceae have high therapy failure and mortality rates (Rensing et al., 2019). Whilst surveillance of ESBL-*E. coli* is important, very few of these studies report the resistance profiles to individual antimicrobials (Alonso et al., 2017). The potential for inter-host spread of AMR and MDR clones through close contact with animals or ingestion of contaminated products is a public health risk. Over the last 10 years, there has been a large increase in the prevalence of resistance to HPCIAs, including to fluoroquinolones and third/fourth generation cephalosporins

amongst commensal *E. coli* in healthy livestock species. In most cases, resistance to both antimicrobial families are co-selected and disseminated not only by clonal spread but possibly also to via HGT, or via plasmids carrying *qnr* or *aac(6)Ib-cr* and *bla* genes (especially, of the CTX-M group). Furthermore, carbapenem- and colistin-resistant *E. coli* strains are also emerging among husbandry animals in Africa, which demonstrates the urgent need of a better control of the usage of veterinary drugs and the implementation of effective surveillance programmes to stop the dissemination of MDR and ESBL- *E. coli* strains (Mitgang et al., 2018).

One such example of a study incorporating examination of ESBLs, alongside resistance phenotypes is a Tunisian study of 136 faecal samples collected from healthy poultry across 36 different farms; these were tested for AMR using the disc and double-discs diffusion methods according to CLSI guidelines. There was a moderately high prevalence of ESBL-*E. coli* according to double-disc diffusion test approximately 30%. Sixty-seven CTX-resistant *E. coli* isolates (including duplicates from 57 of the faecal samples) had MDR phenotypes, including to tetracycline (94%), nalidixic acid (89.5%), norfloxacin (71.6%), trimethoprim–sulfamethoxazole (73.1%), gentamicin (6.0%) and amikacin (6.0%). All the isolates were susceptible to imipenem. Only one isolate carried $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$ genes; $bla_{\text{TEM-1}}$ was detected in 26 isolates (38.8%). *QnrS1* was detected in 2 $bla_{\text{CTX-M-1}}$ producing *E. coli* and *QnrB5* in one $bla_{\text{CMY-2}}$ isolate and the *aac(6')-Ib-cr* gene in 2 $bla_{\text{CTX-M-15}}$ and one $bla_{\text{CTX-M-1}}$ producing isolates (Mnif et al., 2012). This study highlights the importance of investigating not only resistance phenotypes, but also to dig deeper in the genomes of isolated *E. coli* in order to assess patterns of spread between animals, humans and their environments, by understanding the resistance and virulence genes carried on plasmids.

There is some evidence suggesting the transmission of AMR bacteria and genes between animals, humans, and the environment, though there is difficulty in determining precisely if there has been transmission or not. A recent systematic review examining the evidence of transmission of bacteria between humans and animals was conducted by Muloi et al; showed that 8 studies (18%) suggested evidence of transmission of AMR from food animals to humans, 25 studies (56%) suggested transmission between animals and humans with no specific direction, and 12 studies (26%) indicated there was no proof of transmission. As many of these studies have relied on MLST and the presence of similar bacteria in hosts, it is becoming more prudent to make use of high-resolution whole genome data analysis with systematic

longitudinal, epidemiological evidence to fully demonstrate AMR transmission between food animals and humans (Muloi et al., 2018).

1.5.2.3 Detailed Prevalence of AMR in African environments

There have been extremely few African studies examining the presence of antimicrobial residues and AMR in the environment. As a large proportion of the African population live in rural areas with restricted access to clean drinking water and sub-optimal sanitation, there is a significantly increased risk of transmission of diseases. It is a good idea to investigate environmental isolates, alongside human and animals in order to assess factors which facilitate the exchange of AMR genes between environmental microbiota and human and animals' commensals and pathogens.

A recent study in South Africa examined the prevalence of AMR and potentially pathogenic *E. coli* from treated wastewater (Adefisoye and Okoh, 2016). In this study, 223 *E. coli* isolates were tested using disc diffusion tests, according to CLSI guidelines - there was high prevalence of AMR *E. coli*, with resistance to ampicillin (55.6%), cephalexin (51.1%), nalidixic acid (31.4%), tetracycline (60.1%) and chloramphenicol (22.9%). There was also a moderately high prevalence of MDR isolates (32.7%). Additionally, associated resistance genes were detected using PCR - *strA* (88.2%), *aadA* (52.9%), *cat I* (15%), *cmlA1* (4.6%), *bla* $_{TEM}$ (56.4%), *tet(A)* (30.4%), *tet(B)* (28.4%), *tet(C*) (42.2%), *tet(D)* (50%), *tet(K)* (11.8%), and *tet(M*) (68.6%).

Existing studies have highlighted hotspots for environmental contamination (such as effluent from hospitals) (Harris et al., 2014; Huijbers et al., 2015); however, very few studies have attempted to integrate all three components of the One Health spectrum to understand the dynamics of transmission.

1.6 Kenya's Current Policy on AMR

Since this study began in 2015, Kenya has implemented a national action plan (NAP) to tackle the growing crisis of AMR. In 2009, the Global Antibiotic Resistance Partnership (GARP) Kenya was established – this group was tasked with undertaking a situational analysis of both antimicrobial use and resistance in Kenya in August 2011 (Global Antibiotic Resistance Partnership, 2011). Findings of the situational analysis led to Kenya hosting its first AMR awareness week in November 2013. Events were held to highlight issues surrounding antimicrobial use and resistance. The following month, a regional antimicrobial stewardship

workshop was held, in December 2013. The outcome of the workshop provided recommendations to the National Infection Prevention and Control Committee at the Ministry of Health in Kenya. All findings of the situational analysis completed in 2011 were disseminated to health managers in 45 counties in Kenya. In March 2014 it was decided that the threat of AMR should be a focal point for discussion and mitigation. The Infection Prevention and Control Unit formed the National strategic plan, with two strategic objectives specific for AMR: a) establish a national AMR surveillance system, and b) appointment of a National Antimicrobial Stewardship Advisory Committee (Wesangula et al., 2016). In June 2017, the National Policy on Prevention and Containment of Antimicrobial Resistance was published (Government of Kenya, 2017).

The plan sets out a number of objectives which it hopes to achieve, these are:

1) Improve the awareness and understanding of AMR by implementing effective surveillance systems and commit to research, communication, education, and training.
2) Improve the knowledge base and gaps in evidence on AMR via surveillance and research.
3) Reduce infections associated with AMR-bacteria by improving sanitation, hygiene and infection prevention and control measures.
4) Optimise the use of antimicrobials in animal, human and plant health.
5) Support sustainable investment, targeted towards Kenyan needs, and increase investment in medicines, diagnostic tools, vaccines, and other necessary interventions.

The National Policy on Prevention and Containment of Antimicrobial Resistance should be a good start to unifying AMR issues and numerous challenges and action points are discussed (Government of Kenya, 2017); these action points are summarised in (**Table 1.4**)

Table 1.4 Summary of issues and action points in the Kenya National Policy on Prevention and Containment of Antimicrobial Resistance (Government of Kenya, 2017).

Objective	Issue	Action
Increase understanding of AMR by implementing effective surveillance systems	General population has a high risk of exposure to AMR bacteria, particularly at human-animal-environmental interface.	Raise awareness across all sectors by targeting human, animal, and agricultural practices as well as consumers and school children. To be done via public communication campaigns.
Strengthen knowledge base and evidence of AMR	Lack of integrated surveillance systems as well as poor diagnostic and laboratory capacity.	Develop and support a national, integrated action plan to combat AMR. Enforce compulsory reporting of AMU and AMR so that a national database can be maintained. Also increase the capacity of laboratories to assist with reporting.
Reduce AMR by adopting preventative measures	A broad range of antimicrobials used in livestock production systems, leading to high prevalence of AMR.	Core infectious disease control practices to be implemented, including better biosecurity, hand hygiene, food and water safety and promotion of vaccination programmes instead of prophylactic AMU.
Optimising the use of antimicrobials in human, animal, and human health	Antimicrobials are used in all aspects of human, veterinary, and environmental life, including for aquaculture and crop-production systems, for non-therapeutic uses. There is frequent over-prescription in hospitals and unregulated access to antimicrobials in non-hospital settings.	Set up a clear antimicrobial supply chain to reduce the number of illegal outlets providing antimicrobials. Also enforce legislation on the prudent use of antimicrobials and ensure that county governments guarantee access to essential antimicrobials at all levels.
Regulation of monitoring antimicrobials	Various laws exist to govern antimicrobial use in Kenya, including the Kenya National Drugs Policy and the National Livestock Policy. There is weak enforcement due to lack of funding and capacity to monitor compliance.	Restructure laws governing AMU and better enforce importing and manufacture of human and veterinary antimicrobials to reduce counterfeits and illegally imported antimicrobials.

1.7 The One Health Paradigm as a Solution

One Health has been defined by the One Health commission as "the collaborative effort of multiple health science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment" (One Health, 2018). The One Health approach (**Figure 1.4**) is designed to aid in designing and implementing various programmes, policies, legislations, and ongoing research where multiple sectors work together to achieve better public health outcomes.

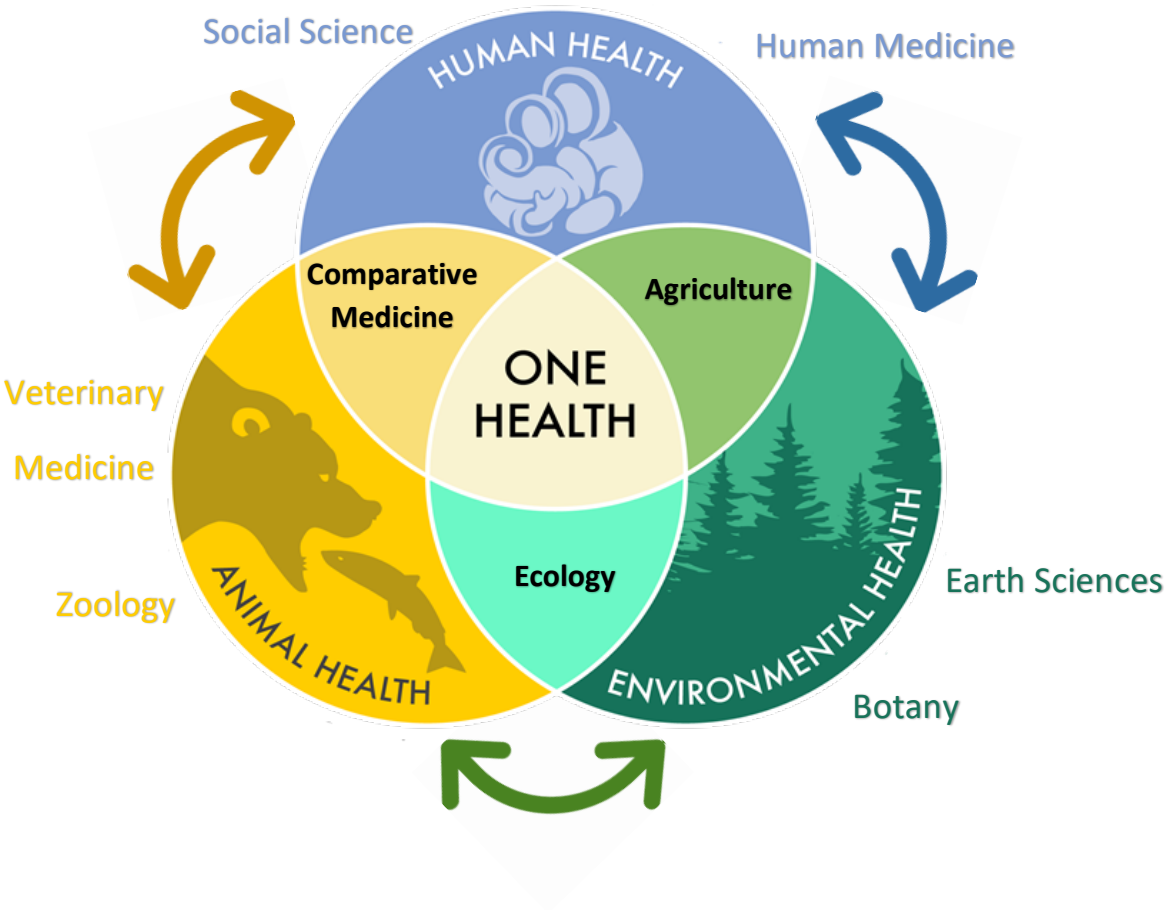
Specific areas in which a One Health approach is particularly relevant are in food safety, control of zoonoses and combatting AMR. The One Health concept recognises that humans and animals are mutually dependent on one another, and they have a shared environment, as well significant overlap in the microbes which infect both (Zinsstag et al., 2012); indeed as many as 75% of human infectious diseases that have emerged or re-emerged in recent decades are zoonotic in origin (Cutler et al., 2010). Efforts directed by individual sectors cannot prevent or eliminate issues such as AMR and spread of zoonoses when there is crossover with other sectors. A good example of this is prevention of rabies in humans; only by targeting the animal source of the rabies virus (i.e. vaccinating dogs) can humans become protected. Similarly, information on circulating influenza strains in animals are important for the selection of human vaccines for potential influenza pandemics. Finally, AMR bacteria can be transmitted between humans and animals via direct contact or through contaminated food. In order to prevent ongoing transmission of AMR bacteria, a well-coordinated multifactorial approach is therefore required.

AMR has highlighted the importance of a 'One Health' approach and has shifted the responsibility of tackling it cross multiple sectors, namely across human health, animal health, agriculture, and environmental platforms. To effectively detected and respond to AMR, zoonotic outbreaks and food safety issues, epidemiological and laboratory data needs to be shared across sectors.

Epidemiological, microbiological and social science research is needed at population levels across the One Health spectrum in order to fill the large gaps in knowledge of AMR in low-resource settings like Africa (Rousham et al., 2018). This calls for a robust surveillance system for monitoring environmental contamination with antimicrobial residues and emergence of

AMR. For any surveillance system to be effective, especially in the context of AMR, it needs to be truly 'One Health' in its approach. Until now, a full country-wide level of integration has not occurred in any part of Africa; all data from multiple sectors are generally considered separately; Africa is well-positioned to benefit from an integrated approach (Kamani et al., 2015). The Global AMR Surveillance System (GLASS) was launched by the WHO and is a good first step at having an international surveillance system for reporting AMR and identifying global health security threats. This calls for a certain level of capacity building and standardisation of the coordinating labs; it is expected that smaller countries can benefit greatly from the improvement in lab capacity. It is hoped that this surveillance platform will grow and move slowly into the agriculture and environment sectors too (Seale et al., 2017; World Health Organization, 2017).

Figure 1.4 Venn diagram the relationship between human, animal and environmental sectors in the One Health paradigm. (Adapted from University of Alaska Fairbanks https://www.uaf.edu/onehealth/).



Surveillance studies such as the Zoonoses in Livestock in Africa study (ZooLiNK) (Falzon et al., 2019), from which this thesis stems are a good start in filling in gaps in AMR data in western Kenya. Studies such as these can aid policymakers by educating them about the prevalence and

risks of AMR. These policies can then be filtered down to antimicrobial users and providers, through communication campaigns (Pehrsson et al., 2016). In LMICs, adopting a One Health approach may be a better solution for tackling AMR, by drawing together different bodies and standardising reporting across all sectors. In this way, a targeted approach to tackling AMR, in the vein of the Kenyan NAP (Fleming Fund, 2019) can be achieved.

1.8 Aims of this study

This study is part of a larger surveillance study being conducted in three western counties of Kenya called ZooLiNK. The study aims to help Kenya develop an effective surveillance system, dedicated to zoonotic infectious diseases, and fill in the many gaps in the carriage of AMR *E. coli* in humans, animals, and their environments. This will be achieved using a One Health approach, by facilitating cross-disciplinary partnerships between veterinarians, physicians, ecologists, economists, and public health professionals.

The work in this thesis will first determine the general understanding of antimicrobials by users (farmers and animal healthcare workers) and providers (animal healthcare workers and retail pharmacies) using questionnaires-guided interviews, giving a basis of the most prudent audience to target in surveillance plans. Following that, *E. coli* isolates from farmers, farm animals and their environment will be characterised to investigate the potential for sharing of *E. coli* strains, mobile genetic elements and resistance and virulence genes between those groups. To achieve these aims, antimicrobials users and providers were recruited across Busia county (Chapter 3) and then cross-sectional sampling of farms was undertaken (Chapter 4). Finally, a retrospectively collected cohort of *E. coli* from slaughterhouse workers was collected, to examine the carriage of AMR *E. coli* in the faeces of slaughterhouse workers (Chapter 5).

Chapter 2

General Materials & Methods

2.1 Literature Review Strategy

A systematic literature search was carried out using multiple electronic databases (PubMed, Web of Science and Scopus), for research articles published in peer-reviewed journals. No geographical, language or date restrictions were used. A combination of keywords was used to find search results, and these included antibiotic resistan* (encompassing resistant and resistance), drug resistan*, antimicrobial resistan*, *Escherichia coli*, *E. coli,* Enterobacteriaceae, human, livestock, food-producing animal*, food animal*, pig, poultry, cattle, sheep, goats. Grey literature including reports (GLASS report (WHO), Kenyan Government (annual reports) and the Centre for Disease Dynamics, Economics & Policy Global Antibiotic Resistance Partnership (CCDEP-GARP) and conference proceedings (poster or oral presentations) was also searched using the same keywords. Articles and grey literature were excluded if they were written in a non-English language or if there was no reference to *E. coli* (the bacterium of interest) within the abstract or text body. Article searches were performed by considering both the article titles and abstracts. Data extraction from articles and grey literature was performed by one author (SK) only.

2.2 Ethics Statement

Before data and sample collections, ethical approval was sought from ILRI-IREC (International Livestock Research Institute - Institutional Ethical Research Committee), which is accredited by the National Commission for Science, Technology, and Innovations (NACOSTI) in Kenya. Approval was also sought concurrently, from the University of Liverpool Veterinary Research Ethics Committee (VREC). Permission to engage with farmers and antimicrobial providers was given by the District Veterinary Officer of Busia county, and then also by each of the seven sub-county veterinary officers. Informed consent was obtained from all participants, both verbally and written (or with a thumbprint in cases of potential illiteracy). The aims of the study as well as participants rights to withdraw were fully explained in both English and Kiswahili; each participant was given enough time to ask questions regarding the study, before giving consent. To ensure good ongoing links with the district veterinary services, copies of questionnaires were provided, and regular contact was made with the sub-county veterinary officers for the duration of the studies.

2.3 Study Site

The study population was a mixed crop-livestock farming community in Busia County, western Kenya (0.434° N, 34.242° E) which supports one of the highest human and animal population

densities in Eastern Africa (**Figure 2.1**). The area is broadly representative of other communities spanning the Victoria Lake Basin in Kenya, Uganda, and Tanzania. The study area covered approximately 1630km^2, in a 45km radius from Busia town, where the ILRI/BUSIA lab is based. There is a high density of human (1.4 million people) (OpenData, http://www.opendata.go.ke), cattle (340,000) and domestic pig populations (55,000) in this region (Divisional Livestock Production Office data). Busia county also has a high proportion of smallholder farms; up to 90% of people are thought to engage in crop-livestock farming.

2.3.1 KAP Study

Taking into account the anticipated time it would take to recruit and interview participants, it was advised by Jane Poole at the International Livestock Research Institute (informed by previous KAP studies that had been conducted in Kenya), that approximately 70 farms were an appropriate target for the proposed timeframe of 8 weeks of fieldwork. Busia County is divided into seven sub-counties, and so random sampling of farmers was stratified by sub-county to to adhere to logtical consideration of sharing vehicular access. Maps of Busia were generated in QGIS v3.2 (QGIS Dev Team, www.qgis.org/en/site) and overlaid with 1km^2 grids. Farms were randomly selected with a random number generator, which corresponded to each grid number. The centre of each grid was input into Google Maps (Google 2017, California, USA) and using the satellite navigation feature, we travelled to the physical location indicated. The nearest farm to the co-ordinates was then selected for recruitment. Where there was no farm at, or close to the co-ordinates, a repeat randomly generated point (constrained to the grid), with new co-ordinates was generated. Where these points were closer than 500m to a previously recruited farm, a new random point was generated at grid level. This constraint was designed to capture farms from different urban and peri-urban locations within each sub-county, as opposed to presumably highly similar farming neighbours. Once we arrived at the farm, GPS of the actual farm or agrovet were captured using a mobile phone and stored in a secure database for direct follow-up in future studies.

Figure 2.1 Location of field site in relation to Kenya. The field laboratory (green star) is located in Busia township, within Busia county. (0.434° N, 34.242° E)

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Agrovet shops and AHAs was recruited systematically, with assistance from the sub-county veterinary officer from each sub-county. These officers accompanied all visits and were able to assist in directing us to all agrovet shops in each sub-county. The most senior person (or only person present) in each agrovet shop was interviewed, except for shops which were closed on more than two occasions during repeat visits. A comprehensive list of all known AHAs was collected from sub-county district officers; all AHAs were recruited by phone and all agreed to participate – once recruited, we travelled to a convenient location for the AHA and gave the interview at the roadside.

2.3.2 Farm Study

A second cross-sectional study was designed, to collect faecal *E. coli* from livestock, farmers, and the environment. The GPS coordinates captured from farms during the KAP study were used to locate, approach and attempted to recruit the same 70 farms to the cross-sectional study, however, 27 farmers declined to participate. Therefore, 27 new farms were recruited using the same random method as for the KAP study.

2.4 Data Collection

2.4.1 KAP Study

A detailed questionnaire was designed, and all participants were interviewed orally using that framework. Different groups of respondents were given a relevant mixture of open and closed questions, and these were used to ascertain participants' education, access to veterinary antimicrobials, prescribing patterns and knowledge of ABR and withdrawal periods. Questionnaires were designed in Adobe® Acrobat® Pro DC (Adobe, San Jose, United States) and coded electronically using AppSheet® (AppSheet c/o Solvebot Inc., Seattle, Washington). Participants were interviewed in English or Kiswahili by bilingual Kenyan research members, being asked each of the questions in the questionnaire. All answers were given verbally by the participant and these were recorded as verbatim transcribed text into the coded questionnaire on a mobile phone or tablet, by the interviewer.

2.4.2 Farm Study

Prior to bacterial sampling, the farm study collected additional questionnaire data. Some of the questions had crossover from the KAP study; such questions included knowledge of AMR and withdrawal periods, the incidence of disease during the prior 3 and 12 months (to the questionnaire) amongst animals, and purchase of antimicrobials. An additional subset of questions was asked to gather data regarding slaughter practices, use of personal protection equipment, human illness, and hygiene practices. This was conducted in the same way as in the KAP study i.e. participants were interviewed in English or Kiswahili by research members using the questionnaire as a framework.

2.5 Microbiological Methods

Various samples were collected during the farm study, including livestock and human faeces, water from the main water sources on farms and environmental boot swabs of living areas on farms. A retrospective study examining slaughterhouse workers also collected faecal samples,

but in a different way. All samples were immediately processed at the Busia/ILRI lab and then shipped to Liverpool University where they were purified and subjected to further characterisation. Collection of these samples and isolation and purification of *E. coli* are detailed in the respective chapters. Once purified, isolates were subjected to antimicrobial susceptibility testing and further characterisation.

2.5.1 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on all isolates using the disc diffusion method detailed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). Following overnight incubation, several colonies were selected from the NA plate and homogenised by vortex in 3ml of sterile distilled water to achieve a 0.5 McFarland standard. The suspension was inoculated onto a Müeller Hinton Agar plate (MHA) using a sterile cotton swab and rotary plate, to obtain a confluent lawn. For the standard antimicrobial sensitivity panel, seven antimicrobial discs were applied to each plate using a disc dispenser: ampicillin 10µg, chloramphenicol 30µg, ciprofloxacin 5µg, gentamicin 10µg, sulfathiazole 1000µg, tetracycline 30µg and trimethoprim 5µg. For ESBL confirmation, the combination double-disc diffusion method was used (M'Zali et al., 2000). Three pairs of antibiotic discs containing ceftazidime (30µg), cefotaxime (30µg) and cefpodoxime (30µg) with and without clavulanic acid (10µg) were applied to inoculated MHA plates. All plates were then incubated aerobically overnight at 37°C. Following incubation all plates were photographed using a laboratory camera mounted to a pedestal (to ensure photos were taken at fixed height) and downloaded to a computer. Zones of inhibition were measured using the Fiji distro of ImageJ2 (Schindelin et al., 2012) and transferred to a spreadsheet. Isolates of the standard antimicrobial panel were considered to be resistant if their size in mm was less the published breakpoint; suspected ESBL isolates were confirmed as being an ESBL if the zone of inhibition for the cephalosporin with the clavulanic acid disc was at least 5mm greater than the zone of its counterpart without clavulanic acid.

Zones of inhibition for all antimicrobials except for sulfathiazole and tetracycline were categorised as resistant or sensitive according to EUCAST human clinical breakpoints (EUCAST, 2017). Tetracycline was interpreted according to BSAC human clinical breakpoints (BSAC, 2015). There are no published breakpoints for sulfathiazole and so a tentative epidemiological cut-off value (TECOFF) was estimated using the distribution of zones of inhibition. After all susceptibility testing has been completed and zones of inhibition measured, a histogram was constructed indicating their frequency (mm). A bimodal distribution was observed (**Figure 2.2**) – the left cluster of peaks indicate antimicrobial-resistant bacteria and the right cluster of peaks indicate susceptible bacteria. The TECOFF was set between the peaks representing the largest proportion of isolates within the two populations (Morrissey et al., 2014); for susceptible isolates this value is 32mm and for the resistant isolates, this was 6mm. As such, isolates with zones of inhibition of <19mm were considered to be resistant, and those ≥19mm were 'susceptible'.

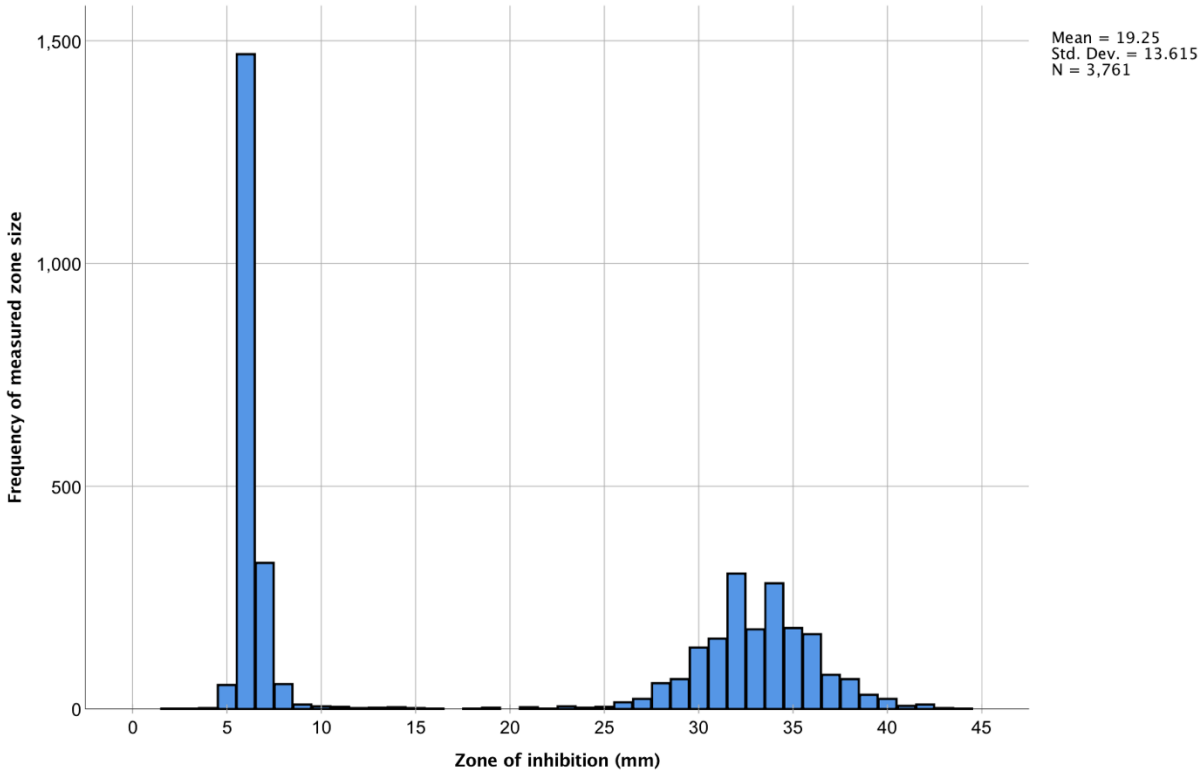


Figure 2.2 Bimodal distribution of zones of inhibition for the antimicrobial sulfathiazole, measured using the PC method. A total of 3763 *E. coli* isolates from pigs, poultry, sheep, goats, cattle, humans and the environment were used to determine the bimodal distribution.

All culture media was sourced from Lab M Ltd, Bury, UK; antimicrobial discs were obtained from MAST Group Ltd., Bootle, UK; and cefotaxime and ceftazidime powder were sourced from Sigma-Aldrich Company Ltd., Gillingham, UK.

2.5.1.1 Use of software to measure zones of inhibition

Typically zones of inhibition are measured with a ruler. An alternative method using a camera and a computer to measure these zones was trialled, alongside the ruler method. 2078 zones of inhibition were measured traditionally with a ruler and simultaneously photographed, using a pedestal mounted camera to ensure consistency in height from each plate. Concordance between the ruler measurement and the Fiji measurement was determined using a Bland-Altman test (Bland and Altman, 1986). Measurements of both ruler and computer were input into a spreadsheet, named RULER and PC. The difference between the two was calculated by using "PC" – "RULER". The mean of the two measurements was calculated using ("PC" + "RULER") / 2. A one-sample T-Test was run to determine how close to 0, the mean is. In this case, the mean (difference) was found to be -0.2862mm. i.e. the pc method underestimated the average zone of inhibition size by 0.29mm. The PC method was found to be 98.6% sensitive and specific (**Table 2.1**). In total, the PC method showed only two discrepancies, one false-positive and one false-negative.

Table 2.1 Concordance between the use of a ruler and computer software to measure zones of inhibition following a disk-diffusion test. Results indicate a subset of resistant isolates (n=608) detected using both methods.

		Antimicrobials Tested							
		CIP	CHLOR	GENT	TET	SULFA	AMP	TRIM	Total
Method Used	PC	1	12	0	149*	164	116	166**	608
	RULER	1	12	0	150	164	116	165	608
	Concordance	100.0%	100.0%	N/A	99.3%	100.0%	100.0%	98.8%	99.8%

*Isolate was resistant but measured as sensitive. **Isolate was sensitive but measured as resistant.

2.5.2 Isolate Storage

Isolates destined for long-term storage at -80^{o}C, were inoculated into Microbank™ cryovials (Pro-Lab Diagnostics U.K, Cheshire UK) as previously detailed. Briefly, colonies from a pure isolate culture were inoculated into the cryopreservative fluid to achieve a turbidity of approximately 4 McFarland standard and inverted numerous times to ensure binding of the organisms to Microbank™ beads. Excess cryopreservative was then removed using a sterile pipette. Isolates were recovered by removing a single bead from the cryovial using sterile forceps and inoculation of the bead onto nutrient agar for aerobic incubation at 37^{o}C for 18-24 hours.

2.5.3 Polymerase Chain Reaction (PCR)

All isolates that were morphologically consistent with *E. coli* (shiny, metallic-green colonies on eosin-methylene blue agar) were confirmed by detection of the *uidA* gene (McDaniels et al., 1996). PCR assays were also used to assess the carriage of β-lactamase resistance genes (bla_{SHV}, bla_{TEM}, bla_{OXA} and bla_{CTX-M}), to the grouping of isolates carrying a bla_{CTX-M} resistance gene, and also to identify isolates belonging to the O25b-ST131 clade (Clermont et al., 2009). The procedure for each of these assays follows a similar pattern, beginning with DNA extraction.

2.5.3.1 DNA Extraction

Cell lysates were prepared by inoculating several purified *E. coli* colonies from a NA plate into 1ml of sterile distilled water (to an opaque turbidity) in 1.5ml Eppendorf tubes. Suspensions were thoroughly homogenised by vortex and boiled at 100°C for 10-15 minutes in a heat block. DNA lysates were stored at 4°C for short-term working and then transferred to a -20°C freezer for long-term storage.

2.5.3.2 PCR Substrates and Primers

All PCR assays were made up in 25µl volumes and utilised 5x FIREPol® 12.5mMCl2 Master Mix Ready to Load (Solis Biodyne, Tartu, Estonia), comprising 1U FIREPol® DNA polymerase, 80mM Tris-HCl, 20mM $(NH4)^2SO_4$, 0.02% w/v Tween-20, 2.5mM $MgCl_2$ and 200µM of each dNTP per reaction plus blue and yellow dye. Reactions testing for the presence of *uidA* and extended-spectrum beta-lactamase (ESBL) resistance genes were undertaken in reaction volumes of 25µl constituting 4µl of master mix, 5pmol of each primer and 1µl of DNA lysate with the addition of sterile molecular grade water (Sigma-Aldrich, Dorset, UK) to make up the reaction required reaction volume. PCR assays for the detection of *E. coli* O25b-ST131 were undertaken in reactions containing 4µl of master mix, 10pmol of each primer, 3µl of DNA lysate and sterile molecular grade water making up a reaction volume of 25µl. A lysate of a bacterial isolate known to carry the gene of interest and sterile molecular grade water were included as a positive and negative control in each PCR run.

All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and PCR reactions were undertaken using ABI 2720 Thermal Cyclers (Applied Biosystems, California, USA). Target genes, amplicon sizes and various conditions for all PCR assays carried out can be seen in **(Table 2.2).**

Table 2.2 Gene targets, primers and conditions for each PCR assay used to confirm and characterise *E. coli*.

Gene Target	Primer	DNA Sequence 5'→3'	Amplicon size (bp)	Annealing (°C)	Reference
uidA	uidAF	CCAAAAGCCAGACAGAGT	623	58	(McDaniels et al., 1996)
	uidAR	GCACAGCACATCAAAGAG			
bla_{TEM}	TEMF	CATTTCCGTGTCGCCCTTATTC	800	60 Multiplex	(Dallenne et al., 2010)
	TEMR	CGTTCATCCATAGTTGCCTGAC			
bla_{SHV}	SHVF	AGCCGCTTGAGCAAATTAAAC	713		
	SHVR	ATCCCGCAGATAAATCACCAC			
bla_{OXA}	OXAF	GGCACCAGATTCAACTTICAAG	564		
	OXAR	GCACCCAAGTTICCTGTAAGTG			
bla_{CTX-M}	CTX-MU1	ATGTGCAGYACCAGTAARGTKATGGC	593	58	(Boyd et al., 2004)
	CT-MU2	TGGGTRAARTARGTSACCAGAAYCAGCGG			
bla_{CTX-M} Group 1	CTX-MGrp1F	CCCATGGTTAAAAAATCACTGC	876	55	(Carattoli et al., 2008)
	CTX-MGrp1R	CAGCGCCTTTTGCCGTCTAAG			
bla_{CTX-M} Group 2	CTX-MGrp2F	ATGATGACTCAGAGCATTCGC	876	55	(Hopkins et al., 2006)
	CTX-MGrp2R	TGAGAAACCGTGGGTTACGAT			
bla_{CTX-M} Group 9	CTX-MGrp9F	ATGGTGACAAAGAGAGTGCAAC	893	55	(Batchelor et al., 2005)
	CTX-MGrp9R	TTACAGCCCTTCGGCGATG			
ST131 *trpa*	trpAF	GCTACGAATCTCTGTTTGCC	427	60	(Clermont et al., 2009)
	trpAR	GCAACGCGGCCTGGCGGAAG			
ST131 *pabB*	pabBF	TCCAGCAGGTGCTGGATCGT	347	65	
	pabR	GCGAAATTTTTCGCCGTACTGT			

F = Forward primer, R = Reverse Primer

2.5.3.3 Visualisation of PCR Products

All amplified PCR products were visualised by gel electrophoresis, on peq-GREEN (Peqlab, Fareham, UK) stained 2.0% agarose medium (150ml) or large (250ml) gels, produced using Hi-pure EEO agarose (Biogene, Cambridge, UK) in 1 x Tris-acetate-EDTA (TAE) buffer (Sigma-Aldrich). 10µl of 100bp DNA ladder (Solis Biodyne, Tartu, Estonia) was added to the first well of each gel and for all reactions, 8µl of products was loaded into each well following the DNA ladder. All gels were run in electrophoresis tanks containing 1xTAE Buffer. To obtain maximum differentiation of DNA bands, all gels were run at 100V for 60 mins (medium gel, 150ml) and 90 minutes (large gel, 250ml). PCR products were visualised under an ultraviolet transilluminator using the UVItec gel documentation system (UVItec, Cambridge, UK). Gel images were printed for analysis and saved using UVIProMV (UVItec, Cambridge, UK).

2.6 Whole Genome Sequencing

A subset of *E. coli* isolates from the farm study (**Chapter 4**) and the slaughterhouse study (**Chapter 5**) were selected for whole genome sequencing. Selection of isolates and the method by which isolates were sequenced were different for both studies and are detailed in the respective chapters.

WGS services were provided by two different groups. For the farm study, all isolates were sequenced by the Liverpool Centre for Genomic Research (CGR). For the slaughterhouse study, all isolates were sequenced by MicrobesNG, a BBSRC-funded collaboration between the University of Birmingham and the University of Sheffield. The exact methods used for DNA extraction, library preparation and sequencing are detailed in the respective chapters.

2.6.1 WGS Workflow

A standard workflow was designed to analyse both sets of WGS results. Illumina pair-end reads (forwards and backwards) were first downloaded each from the CGR and MicrobesNG servers. FastQ files were assessed for quality using FastQC v0.11.7 (Andrews, 2010). Poor quality reads (score <20) and any detected primers or adapters were removed using Trimmomatic v0.36 (Bolger et al., 2014). Forward and reverse reads were then mapped to a reference *E. coli* genome (*E. coli* K12 MG1655; NCBI Reference Sequence: NC_000913.3 (Blattner et al., 1997), using the Burrow-Wheeler Alignment (bwa mem v0.7.17) (Li, 2013). Once BAM files had been made, a further QC step to check the mean mapping quality scores and coverage in relation to the reference genome was performed with QualiMap2 (Okonechnikov et al., 2015). In any instance where there was poor coverage across the genome (<10x) after mapping, genomes would be excluded from further analysis. Any reads which did not map to the reference genome were assembled *de novo* into contigs, using SPADES v3.12.0 (Bankevich et al., 2012). Speciation analysis of those contigs was then performed using Kraken v2.0.7 (Wood and Salzberg, 2014) and an in-house database downloaded from NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/sra/). Non-*E. coli* contigs were excluded from further analysis.

2.6.2 Analysis of Sequencing results

A brief comparison of methods was made regarding resistance and virulence gene data. Up to date resistance and virulence sequences from several different databases were downloaded, and the frequency of detection of common resistance and virulence genes was assessed. Databases were manually compiled on 18th October 2018 and used to screen mapped reads.

Databases screened included ResFinder (Zankari et al., 2012), CARD (McArthur et al., 2013), NCBI Antibacterial Resistance Reference gene database (NCBI A Accession: PRJNA313047, ID: 313047), EcOH (Ingle et al., 2015), PlasmidFinder (Carattoli et al., 2014) and VDFB (Chen et al., 2016).

Initial analysis was performed with the most up to date version of the ResFinder database (generated on 9th October 2018). To date, there have been several updates to the ResFinder programme and database; resistance genes such as *bla*$_{CARB}$ and *bla*$_{FRI}$ genes, updates to the fusidic acid database, the addition of mcr-9.1 to the colistin database and various qnr mutations have been added to the quinolone database. These changes have not largely altered the results presentd in this thesis.

All reads were blasted against the up to date ResFinder (Zankari et al., 2012) and VirulenceFinder (Joensen et al., 2014) databases (18th October 2018) to assess carriage of acquired resistance and virulence genes. In each case, a 90% threshold for identification was set and a minimum query length of 60% was utilised. Where no resistance or virulence genes were detected, this was re-checked using a lower threshold for identity of 75% for identification.

To identify plasmids carried by isolates, the PlasmidFinder tool for Enterobacteriaceae was used (Carattoli et al., 2014) using an identity threshold of 95% and minimum coverage of 60%. Where no plasmids were detected, this was re-checked using a lower threshold for identity of 75% for identification.

In silico serotyping of isolates was undertaken using SerotypeFinder v2.0 (Joensen et al., 2015) in order to assess the O and H serogroups of each isolate using a 85% identity threshold and query length of 60%.

Multilocus sequence typing (MLST) was determined using a custom batch script (Seemann T, mlst GitHub https://github.com/tseemann/mlst) which blasts assemblies or consensus fasta files against the *E. coli* #1 schema (https://pubmlst.org/escherichia/) (Jolley and Maiden, 2014). From this a sequence type (ST) was assigned according to the *adk, fumC, gyrB, icd, mdh, purA* and *recA* loci. For any detected ST131 isolates, *fim* typing was undertaken using a custom script to blast genomes for *fimA* and *fimH* genes (Totsika et al., 2011). Literature was then searched to match the fimtype to known ST131 isolates.

In silico phylogenetic typing (phylotyping) of all sequences was performed using the tool Clermont Typing (Beghain et al., 2018), which assigns a Clermont phylogenetic type (A, B1, B2, C, D, E, F and Cryptic Clade I) to each sequence. As of 2019, the Clement phylotyping tool also identifies the new phylogroup, G (Clermont et al., 2019).

Finally, maximum-likelihood phylogenetic trees were constructed by using concatenated single nucleotide polymorphisms (SNPs) of all sequences. SNPs were called against the *E. coli* K12 Reference genome (U00096.3) using Samtools mpileup (Li et al., 2009) (minimum coverage, 1x; minimum number of reads, 2; minimum variant frequency 0.2; minimum SNP quality, 30; minimum read mapping quality, 25) and VarScan (Koboldt et al., 2009) to produce VCF files. Non-variable positions were extracted from the VCFs using a custom Perl script to produce pseudosequences of concatenated SNPs of the same length. Phylogenetic trees were constructed using IQ-Tree (Nguyen et al., 2015), using 1000 bootstrap replicates and the GTR model. Maximum-likelihood trees were rooted using the *E. coli* K12 Reference genome (U00096.3). Tree topology was checked using Figtree v1.4.4 (Rambaut, 2012) and then annotated using the interactive tree of life (iTOL) v5.0 (Letunic and Bork, 2016).

Chapter 3

Knowledge, Attitudes & Practices of Antimicrobial Users and Providers: A cross-sectional survey in an area of high-density livestock-human population in Western Kenya

3.1 Introduction

AMR is a growing, global problem. AMR is a multifactorial issue, exacerbated by inappropriate dispensing and use of antimicrobials, varying quality of antimicrobials (Padget et al., 2016), disproportionately higher mortality impact (in LMICs) than in HICs, lacking access to healthcare facilities (Bebell and Muiru, 2014), few available resources for enacting and enforcing national action plans, and a lack of targeted infrastructure to combat AMR bacterial transmission (Jasovsky et al., 2016). There are complex interactions between factors, but it is likely that unregulated access to a wide variety of human and veterinary antimicrobials plays a key role in the exacerbation of developing AMR in sub-Saharan Africa. Whilst there has been evidence to suggest that animals can act as reservoirs of AMR bacteria, the complex epidemiology of AMR and the degree of the contribution of veterinary antimicrobials use on human AMR are still being investigated (Muloi et al., 2018).

Agriculture is one of the key contributors to Kenya's economy, representing 27.3% of its GDP and employing more than 70% of Kenya's rural population (Food and Agriculture Organization, 2014). More than three-quarters of Kenyans own and maintain small-holder, mixed crop-livestock farms with traditional livestock, such as pigs, poultry, sheep, goats and cattle. These traditional, low-input systems often have challenges with biosecurity, which can result in a higher incidence of disease amongst animals – this can lead to the increased need to treat animals (Grace, 2015). One of the challenges of rapidly growing populations is the increased demand for animal products, and this requires a shift in agricultural practices. In many sub-Saharan African countries, there is an ongoing shift to commercialisation and intensification of farming, from subsistence farming (Chen et al., 2018). Similarly, intensification of livestock production can also be associated with the increased use of antimicrobials to maintain animal health; there is evidence to suggest that AMR and MDR bacteria is more prevalent in larger, intensified farms, though it is still unclear if this is due to more antimicrobial use (AMU) or differences in management practices (Ström et al., 2017). Also, with the increased demand for meat, there has been a rise in diarrhoeal and food-related illnesses, linked to contamination in the value chain (Alarcon et al., 2017a; Carron et al., 2018) providing a vehicle for the transmission of foodborne enteric pathogens such as *E. coli* and *Salmonella* spp. (Okeke, 2009; Kotloff et al., 2013).

The sale of veterinary antimicrobials is covered by the Pharmacy and Poisons Act in Kenya (National Council for Law Reporting, 2012a) and is now the responsibility of the Kenyan

Veterinary Medicines Directive. To legally sell antimicrobials (for human and animal use), the law requires the presence of a registered pharmacist. This is infrequently adhered to as farmers can purchase any veterinary antimicrobial at informal, or unlicensed agrovet shops or retail pharmacies without a prescription, even though these are required by law (Esimone et al., 2007; National Council for Law Reporting, 2012a; Luseba and Rwambo, 2015; Kalungia et al., 2016). Furthermore, 78% of veterinary medicine outlets were found to be operated by unqualified persons (Kenya Veterinary Association, 2016). To operate legally, the premises owner must possess a valid license from the local veterinary office and have a qualified pharmacist present, though many do not – due to finite resources, ensuring that correct licensing is in place can be challenging (Luseba and Rwambo, 2015). As such, unregulated access to antimicrobials, and AMU in farming settings risks contributing to the development of AMR.

There is a paucity of data on the true prevalence of AMR in Kenya, as there are limited laboratory capacity and no rigorous, systematic, national surveillance systems for zoonotic-related diseases in place. To successfully manage the issue of ABR, the Global Antimicrobial Resistance Partnership was tasked, in 2011, to conduct a situational analysis to identify gaps in available data, by identifying groups working on resistance issues and support their research (Global Antibiotic Resistance Partnership, 2011). As of June 2017, a National Action Plan on Prevention and Containment of Antimicrobial Resistance in Kenya was designed (Government of Kenya, 2017). This aims to improve awareness and understanding of AMR by committing to a national surveillance programme and improve community knowledge of AMU and the consequences of AMR. This will be implemented by the Fleming Fund from 2019 onwards (Fleming Fund, 2019). A particularly important gap to fill would be the current understanding of the consequences of using HPCIAs in animal therapies. The WHO and European Medicine Agency (EMA) list 3rd-5th generation cephalosporins, fluoroquinolones, and polymyxins as HPCIAs for human health. This list (updated most recently in 2018) is intended for public health and animal health authorities, practising physicians and veterinarians and stakeholders involved in managing antimicrobial resistance, to ensure that all antimicrobials, especially these HPCIAs, are used prudently both in human and veterinary medicine (OIE, 2017; WHO, 2019b).

Relatively few studies have examined the attitudes, knowledge and practices relating to antimicrobial use or considered the disease burden and elevated need for antimicrobial therapies when investigating ABR in Kenya. The present study is the first to determine the access to, and understanding of AMU and AMR, as well as the practices surrounding AMU in western

Kenya. The focus was on both antimicrobial providers and users in an area of extremely high density of both humans and animals (Fèvre et al., 2017), chosen as to examine the relationship between human and animal AMU and AMR.

3.2 Materials & Methods

3.2.1 Study Area and Population

The study population was a mixed crop-livestock farming community in Busia County, western Kenya (0.434° N, 34.242° E) which supports one of the highest human and animal population densities in Eastern Africa. This area is broadly representative of other communities spanning the Victoria Lake Basin in Kenya, Uganda, and Tanzania. The study area has a cattle population of around 340,000 and a domestic pig population of around 55,000 (Divisional Livestock Production Office data). Busia county has a high proportion of smallholder farms, with an estimated 90% of people engaging in some degree of crop-livestock farming.

Three main groups were identified as being antimicrobial users or prescribers: animal healthcare workers (AHAs) and veterinary surgeons, smallholder farmers, and agrovet shop workers and owners. The inclusion criteria were defined as “drug stockist shops (agrovets), smallholder farmers who keep livestock (including pigs, poultry, cattle, sheep and goats) for the intent of sale or consumption of the animals or any animal products, veterinary practitioners and private and public/government-employed AHAs”. Exclusion criteria included “Children under 16 years of age, those that do not speak either English or Kiswahili and farmers who no longer own any animals.

3.2.2 Study Design

It was anticipated (according to advice from Jane Poole at ILRI) that approximately 70 farmers could be recruited in the proposed 8-week fieldwork timeframe. As Busia county is divided into seven sub-counties, random sampling of farmers was stratified by sub-county to adhere to logistical consideration of vehicular access, and time required by veterinary officers to accompany us during the study. To randomly select each farm, each sub-county was divided into 1km^2 grids using QGIS v.3.2 (QGIS Dev Team, www.qgis.org/en/site) and numbered sequentially. A random number generator was used to indicate a grid number – when a grid was selected, a random point within the grid was generated within QGIS v3.2 and the co-ordinates of this point input into Google Maps (Google 2017, California, USA). In the field, using Google Maps satellite navigation feature, we travelled to the physical location indicated. The nearest farm to the co-ordinates was selected for recruitment. In instances where there was no farm at the co-ordinates, a repeat randomly generated point, with new co-ordinates was generated. Where these points were closer than 500m, a new random point was generated.

Systematic interviewing of agrovet shops and AHAs was conducted with assistance from the sub-county veterinary officer, from each sub-county. They accompanied all visits and they were able to direct us to all agrovet shops in each sub-county. The most senior person (or only person) in each agrovet shop was interviewed in each sub-county, except for shops that were closed on more than two occasions during repeat visits. A comprehensive list of all known AHAs was collected from sub-county district officers; all AHAs were recruited by phone and all agreed to participate – once recruited, we travelled to a convenient location for the AHA and gave the interview at the roadside.

3.2.3 Questionnaire Design and Implementation

A detailed questionnaire was designed, and all participants were interviewed orally using that framework. Different groups of respondents were given a different mixture of open and closed questions, and these were used to ascertain participants' education, access to veterinary antimicrobials, prescribing patterns and knowledge of ABR and withdrawal periods. Questionnaires were designed in Adobe® Acrobat® Pro DC (Adobe, San Jose, United States) and coded electronically using AppSheet® (AppSheet c/o Solvebot Inc., Seattle, Washington). Participants were interviewed in English or Kiswahili by bilingual Kenyan research members, being asked each of the questions in the questionnaire. All answers were given verbally by the participant and these were recorded as verbatim transcribed text into the coded questionnaire on a mobile phone or tablet, by the interviewer.

The questionnaire comprised of four sections each relevant to the aforementioned groups of people (**Appendix I, Figure I-iii**). Questions were designed to determine participant's education, access to veterinary antimicrobials, prescribing patterns of antimicrobials and knowledge of antimicrobial resistance and withdrawal periods. Questions specifically asked of farmers focused around access to veterinary antimicrobials, basic information on animals kept (date of acquisition, vaccination status etc.), which diseases they frequently treat and their understanding of antimicrobial resistance and withdrawal periods for animal products including meat, milk, and eggs. Questions asked to AHAs/agrovets/veterinarians focused more on access to antimicrobials, sales/prescription patterns and responsible use of antimicrobials. Furthermore, data on the use of some of HPCIAs according to the EMA list (EMA, 2015), including fluoroquinolones, 3rd-5th generation cephalosporins and polymyxins were collected.

The exact geolocations of each farm and agrovet shop were recorded using the AppSheet Geocapture feature and corroborated with Google Maps. Location data of all farms and agrovet shops was then transferred to QGIS v3.2 (QGIS Dev Team, www.qgis.org/en/site) to produce additional maps.

3.2.4 Pilot Testing of Questionnaire

Pilot testing of the questionnaire with different respondents allowed the specific terminology to be better targeted towards Kenyan customs/culture e.g. in questions including the word 'vaccination', distinguishing information had to be included, as some Kenyan people confused vaccinations with injectable antimicrobials. Further alterations were made to the wording of some questions according to revisions suggested by Dr Salome Bukachi (University of Nairobi, co-PI of ZooLiNK project), to ensure that Kenyans clearly understood all questions.

The initial version of the questionnaire was piloted on field team staff at IRLI, Nairobi (two clinical officers, and two veterinarians). Minor modifications in wording were made according to their comments e.g. to 'stay in a place' means 'to live in a place' to Kenyans. Following minor modifications, the questionnaire was again piloted on a sub-country veterinary officer visiting ILRI, prior to being taken to the field. The first time the questionnaire was used on a farmer and a member of agrovet staff was also considered to be pilots to ensure the smooth operation of the coded questionnaire. After this testing period, the questionnaire was considered to be fit for purpose and was used unadjusted, for the remainder of the study.

3.2.5 Ethical Approval and Permissions

Ethical approval was sought locally from ILRI, Nairobi and the University of Liverpool Veterinary Science Research Ethics Committee before dissemination of the questionnaire. Before beginning any work in Busia county, state permission was sought from the Acting County Director of Veterinary Services and it was also necessary to approach and gain the permission of each sub-county Veterinary Officer (SCVO). Whilst travelling through each sub-county, it was requested that we travel alongside the relevant SCVO to farms and agrovet shops, so that participants understood that we had the appropriate permission to conduct our work. In cases where the SCVO was unable to travel with us, a representative of the office was brought along. In cases where this was not possible, we contacted the village elder and sought permission to conduct our study.

3.2.6 Data Analysis

Answers were stored within the AppSheet application until an internet connection was available. Data were then uploaded to a secure server in XLS format for later analysis. Data were imported from the XLS file into Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and some descriptive analysis (e.g. frequency tables of participant demographics), was conducted using SPSS Statistics 25.0 (IBM SPSS Statistics for Windows Version 25.0, (New York: IBM Corp). Multiple-choice questions with responses on 5-point Likert scales were condensed down to a 3-point Likert; “Never” and “Rarely” were condensed into “Never or Rarely” and “Sometimes” and “Often” were condensed into “Sometimes and Often” following piloting of the questionnaire, to ensure each category had enough data to compare.

Open-ended questions were analysed on a question-per-question basis using a thematic approach (Nowell et al., 2017). Briefly, text responses were read twice to ascertain a general comprehension of the responses. A large number of themes were identified and input into an Excel spreadsheet. Once all responses had been categorised, similar themes were condensed together. To ensure reproducibility, these themes were cross-checked by Dr Gina Pinchbeck (University of Liverpool). All data was then imported into SPSS Statistics 25.0 (IBM SPSS Statistics Version 25.0, New York: IBM Corp).

3.3 Results

3.3.1 Participant Demographics

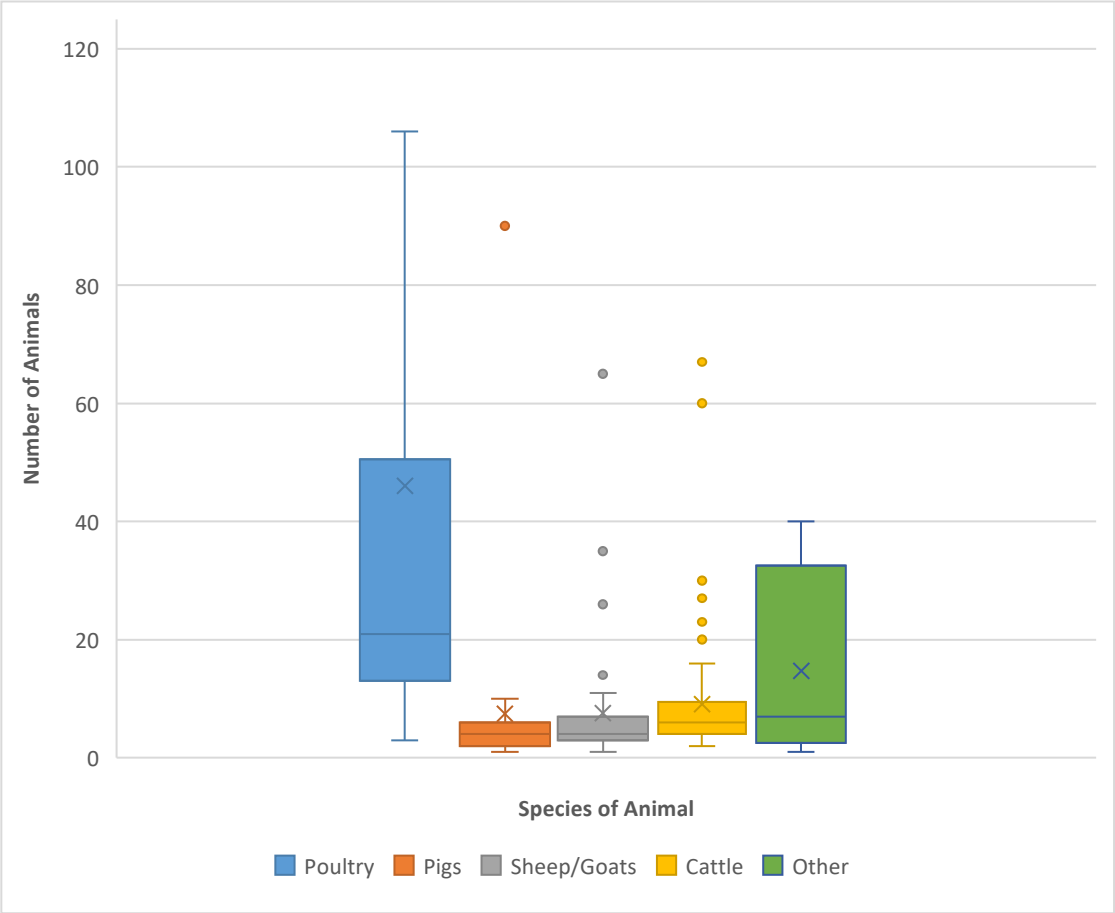
In total, 70 farmers, staff at 49 agrovet shops, 27 AHAs and 1 veterinarian surgeon were recruited. For the purpose of analysis, the (only) veterinarian surgeon located in this study was included in the AHA dataset. The majority of farmers and AHAs were male, though agrovet staff (agrovets) were as likely to be male or female (**Table 3.1**). The primary age bracket for all groups was 25-44. The main/most senior person in each agrovet shop was interviewed and the majority of these identified themselves as an agrovet assistant or the owner of the shop. AHAs had several titles and other jobs, including livestock officers, animal health technicians or laboratory staff. Most participants had been working in their current position for longer than 3 years.

Table 3.1 Demographics of interviewed agrovet staff, AHAs and farmers.

Demographic Factors		Agrovet Staff (n=49)		AHAs (n=28)		Farmers (n=70)	
		n	%	n	%	n	%
Gender	Male	25	51.0	27	96.4	48	68.6
	Female	24	49.0	1	3.6	22	31.4
Age	18-24	8	16.3	-	-	2	2.9
	25-44	35	71.4	20	71.4	40	57.1
	45-64	5	10.2	6	21.4	17	24.3
	65+	1	2.0	2	7.1	11	15.7
Position	Animal Healthcare Worker	1	2.0	14	50.0	-	-
	Artificial Insemination Technician	-	-	1	3.6	-	-
	Sub-country Veterinary Officer	-	-	3	10.7	-	-
	Agrovet Assistant	39	79.6	1	3.6	-	-
	Laboratory Staff / Vet Technician	1	2.0	3	10.7	-	-
	Livestock Officer	-	-	5	17.6	-	-
	Veterinarian	-	-	1	3.6	-	-
	Manager	1	2.0	-	-	-	-
	Owner	9	18.4	-	-	-	-
Length of time at job	<1 Year	14	28.6	1	3.6	-	-
	1-2 Years	4	8.2	-	-	-	-
	>3 Years	31	63.3	27	96.4	-	-

Most farmers kept a similar group of core animals including cattle (dairy and beef production systems), poultry, goats, pigs, and sheep on their farms. A small minority of farmers also kept one or more 'other' animals including ducks, turkeys, rabbits, and donkeys (**Figure 3.1**). The average number of cattle kept by farmers was 6 animals, compared to 21 poultry. Only four farms reported keeping 'other' animals, including doves (n=5), donkeys (n=3) and rabbits (n=4).

Figure 3.1 The average number of each species of animal, kept by farmers on 70 smallholder farms across Busia County, Western Kenya. Tails indicate range, dots indicate outliers, crosses indicate median and lines within the boxes indicates the mean.



3.3.2 Qualifications & Training of antimicrobial providers

All agrovets and AHAs, and 40% of farmers had completed at least secondary education (**Table 3.2**). Of the AHAs, almost all had attained a college diploma and received specific training in livestock health and disease, and the majority had also received specific training to dispense antimicrobials for animal use. Conversely, less than half of agrovets had a college diploma or formal training in livestock health and disease, and more than half did not have training to dispense veterinary antimicrobials. Whereas AHAs obtained most of their training from formal courses (college diploma/certificate or university), agrovets were more likely to receive either no training or informal (on-the-job) training. Almost a third of agrovets participated in short courses provided by pharmaceutical companies when they delivered antimicrobials to the agrovet shop, though they were only educated regarding the specific antimicrobial being supplied (**Table 3.3**).

Table 3.2 The highest level of education achieved by agrovet workers, AHAs and farmers.

Education	Agrovets (n=49)		AHAs (n=28)		Farmers (n=70)	
	n	%	n	%	n	%
No Formal Education	-	-	-	-	4	5.7
Primary Education	-	-	-	-	24	34.3
Secondary Education	27	55.1	3	10.7	33	47.1
College (certificate/diploma)	20	40.8	23	82.1	7	10
University	2	4.1	2	7.1	2	2.9

Table 3.3 Specific training undertaken or received, by antimicrobial providers.

Question	Responses	Agrovets (n=49)		AHAs (n=28)	
		Number	%	Number	%
Have you had specific training in Livestock Health and/or Diseases?	Yes	26	53.1	28	100
	No	23	46.9	-	-
Have you ever received training or are you still training to dispense antimicrobials for animal use?	Yes	21	42.9	23	82.1
	No	28	57.1	5	17.9
Nature of Training	Professional Qualification	8	16.3	26	92.9
	Pharmaceutical company	15	30.6	-	-
	None/Informal Training	22	44.9	2	7.1
	Cannot Remember	3	6.1	-	-

3.3.3 Access to Antimicrobials

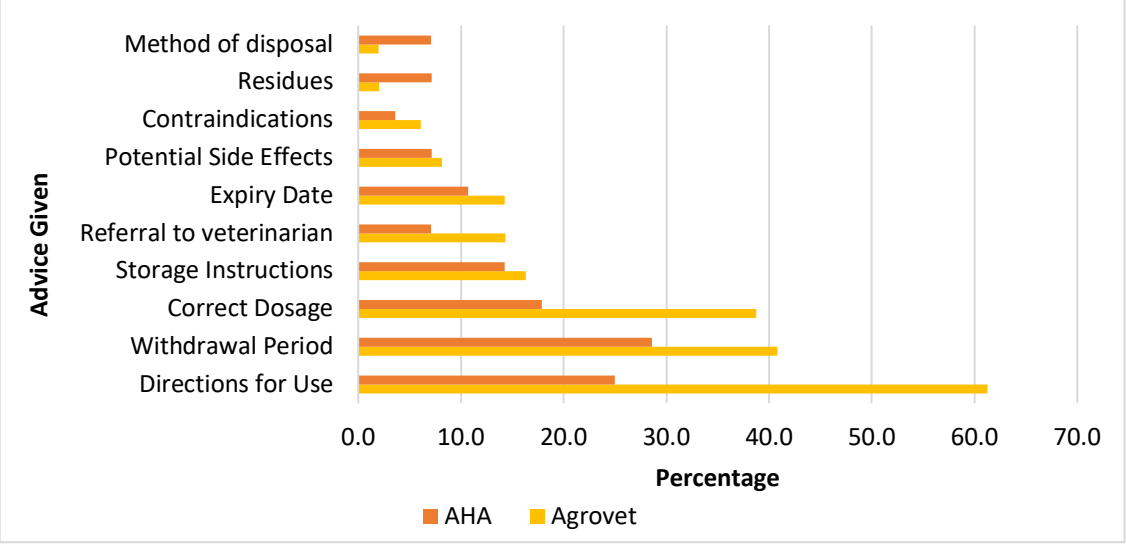
All antimicrobials were purchased directly by farmers or by AHAs (who then used their own supply to treat animals) at agrovet shops, which highlights the key role these facilities play in supplying antimicrobials. Antimicrobial stock and vaccines were always purchased by local agrovets shops from two larger wholesale agrovet shops within Busia county, who in turn obtained antimicrobials directly from manufacturers/distributors. Specific agrovet shops were chosen by farmers for several reasons including the “close distance to [their] farms”, ability to “get drugs on credit” and for “wide selection” and “good stock availability”. Most AHAs (92.9%) and agrovet staff (85.7%) reported that there were no restrictions on the quantity or classes of antimicrobials which could be purchased. Farmers also did not report any restrictions when purchasing antimicrobials from agrovet shops, even without a prescription. The majority of farmers first sought the advice of a veterinarian or AHA before deciding to purchase antimicrobials (78.6%) though. More than half of farmers (54.3%) never requested specific

antimicrobials without first discussing with an agrovet or AHA. A small minority of farmers (12.9%) purchased antimicrobials without first obtaining advice from an agrovet or a prescription from an AHA. Farmers who purchased antimicrobials without consultation did so "using [their] own knowledge" or "already had a prescription from a veterinary officer from a previous consultation". A small proportion of farmers also used antimicrobials previously prescribed or purchased, "[having antimicrobials leftover] from previous use".

3.3.4 Information provided at point-of-sale

Agrovets and AHAs indicated that they provided various information to farmers when they purchased antimicrobials. When presenting at an agrovet shop or when deciding which antimicrobial to prescribe, the most commonly offered information offered by both providers was directions for use, withdrawal periods and correct dosing for animals (**Figure 3.2**). AHAs and agrovets usually gave this information by consulting antimicrobial packaging or recalling from their knowledge and experience. Overall, agrovets were more likely to provide information regarding use to farmers, than AHAs; half of AHAs stated that they gave no information to farmers when they purchased antimicrobials.

Figure 3.2 Information given to farmers at point-of-sale of antimicrobials by agrovets (n=49) and during recommendation or prescription by AHAs (n=28).



Both AHAs and agrovets considered several factors when deciding which antimicrobial treatment to prescribe or sell. Both groups indicated that the cost of the antimicrobial (affordability to the farmer), customer preference and treatment effectiveness were major considerations. In agrovet shops, customer preference (65.3%) was the primary factor for sales. AHAs primary consideration was the effectiveness of the antimicrobial (57.9%) and then the

affordability to the farmer (39.3%). Farmers were primarily concerned with the cost of antimicrobials (44.3%), followed by their effectiveness (40.0%). A small minority of farmers also considered antimicrobial availability and the distance they needed to travel to purchase specific types of antimicrobials as their main point of consideration (5.7%).

3.3.5 Antimicrobial Prescription Patterns

More than half (57.1%) of AHAs stated that they provided a prescription for farmers to fulfil at an agrovet shop, the rest routinely carried a selection of their own antimicrobials, and they treated animals at the time of consultation. Due to logistical issues (such as distance to travel to a farm), half of AHAs (50.0%) stated that they provided a farmer with a prescription over the phone, according to the described clinical signs. In these cases, once the prescription had been fulfilled by an agrovet, most AHAs (89.3%) would travel to the farm to treat the animal(s) themselves; the rest would give some instructions on how to treat the animal. Most AHAs followed up with the farmer to ensure that the designated treatment regime had been completed and was successful (85.7%). Approximately 60% of agrovets dispensed antimicrobials against a prescription from an AHA, leaving 40% who sold antimicrobials without a prescription.

3.3.6 Antimicrobial Usage/Sale Patterns

The most commonly purchased/prescribed/used antimicrobials were oxytetracycline, penicillin-streptomycin and sulfa-drugs (**Table 3.4**). The question asked was "What are the five most common antimicrobials you sell/prescribe/use" – as between 1-5 antimicrobials were volunteered as answers, each time the antimicrobial was mentioned, it was counted. Therefore, percentages do not equal 100%. Oxytetracycline was indicated as the most common antimicrobial to be used by farmers and AHAs, followed by penicillin-streptomycin. Antimicrobials were used therapeutically by farmers (85.7%) and AHAs (100.0%) and sold for therapeutic purposes by agrovets (98.0%). However, some farmers (37.1%) and AHAs (28.6%) also used antimicrobials prophylactically; numerous agrovets (38.8%) said that they sold antimicrobials to be used prophylactically. Antimicrobials were also used relatively frequently as growth promoters by farmers (37.1%), but only one agrovet shop stated that they explicitly sold antimicrobials to be used as a growth promoter.

Reported use of HPCIAs was extremely limited. There was no reported use or sale of 3rd-5th generation cephalosporins or fluoroquinolones, and there was only a single occasion volunteered by a farmer (1.4%) who reportedly treated animals using polymyxins (colistin). No

agrovets and only a single AHA (3.6%) were aware of what HPCIAs are, but this information was provided to all participants after the end of the questionnaire, to reinforce their importance. The majority of AHAs and agrovets were not aware of any specific guidelines which should be consulted regarding the prescription or sale of antimicrobials. Some AHAs cited guidelines from the Kenya Veterinary Board (n=6) or instructions from the County Veterinary Officer (n=3). Agrovets cited pharmaceutical guidelines (n=3) or Kenya Veterinary Board guidelines (n=7). One agrovet knew of the existence of some guidelines but was unable to recall which body issued them.

Table 3.4 List of the most commonly used/purchased/prescribed antimicrobials according to farmers, agrovets and AHAs, to treat livestock. Up to 5 answers most common antimicrobials were volunteered as answers, therefore each antimicrobial was counted as the percentage of time it was volunteered as a most common antimicrobial.

Antimicrobial	AHAs (n=28)		Agrovets (n=49)		Farmers (n=70)	
	n	%	n	%	n	%
Oxytetracycline	26	92.9	46	93.9	55	78.6
Penicillin-streptomycin	27	96.4	39	79.6	33	47.1
Sulfachloropyrazine	9	32.1	27	55.1	-	-
Sulfadimidine	9	32.1	13	26.5	2	2.9
Trimethoprim & Sulfadiazine	9	32.1	8	16.3	4	5.7
Tylosin & Doxycycline	-	-	18	36.7	2	2.9
Sulfamethoxazole	3	10.7	8	16.3	-	-
Gentamicin	6	21.4	-	-	1	1.4
Tylosin	4	14.3	-	-	-	-
Tetracycline	1	3.6	3	6.1	-	-
Fosfomycin & Tylosin	-	-	4	8.2	-	-
Sulfamethoxazole & Trimethoprim	-	-	4	8.2	-	-
Erythromycin	2	7.1	-	-	1	1.4
Gentamicin & Doxycycline	-	-	3	6.1	-	-
Neomycin	-	-	3	6.1	-	-
Cefalexin	1	3.6	-	-	1	1.4
Metronidazole	1	3.6	-	-	1	1.4
Ampicillin	1	3.6	-	-	-	-
Streptomycin	1	3.6	-	-	-	-
Amoxicillin	-	-	1	2.0	-	-
Dexamethasone**	-	-	1	2.0	-	-
Erythromycin & Oxytetracycline	-	-	1	2.0	-	-
Colistin*	-	-	-	-	1	1.4

*Highest Priority Critically Important Antimicrobials **not an antimicrobial but described by the respondent as one.

All farmers stated that one or more of their animals had required some antimicrobial treatment during the previous 12 months. More than half of farmers treated their animals at regular

intervals: every 3-months (28.6%), every 4-months (24.3%) and every 6-months (20.0%). To assess the correct antimicrobial dose for animals, farmers determined the weight of animals by eye (88.6%), or by using a weighing band (8.6%). Others did not consider weight but gave a dose as instructed by agrovet staff or AHAs (24.3%). The majority of farmers administered antimicrobials by intramuscular injection (66.9%) except for poultry, where the preferred method of dispensing antimicrobials was in water (43.5%). Farmers stated that they gave the same dose (mg/kg) of antimicrobial to each of their animals, irrespective of their weight or species e.g. imported dairy cows were given the same dosage as indigenous Zebu cattle.

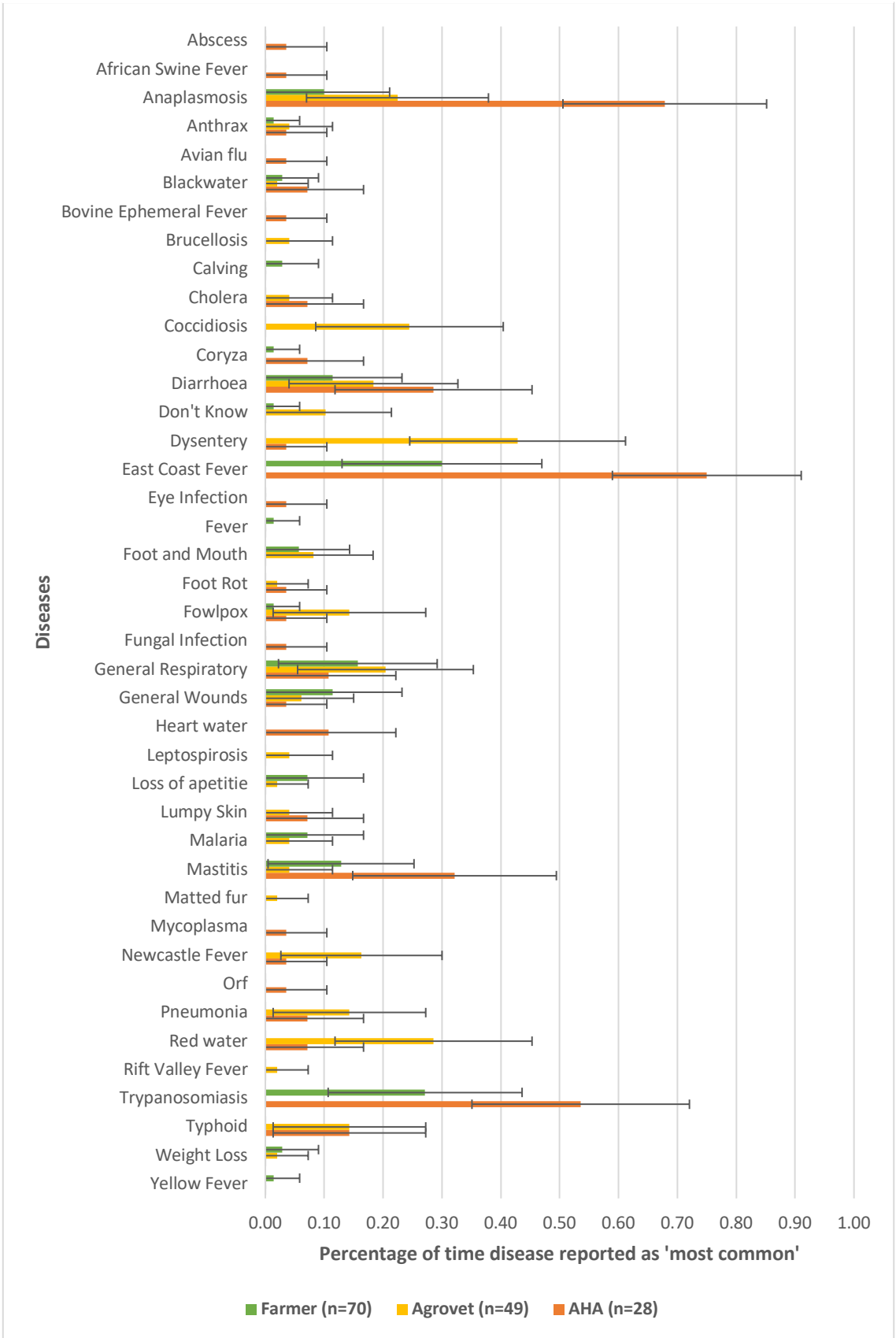
3.3.7 Most common diseases treated with antimicrobials

A large variety of diseases were reported during the questionnaire as affecting all farm animals. Treatment was almost always syndromic due to lack of available diagnostic facilities; diseases were usually diagnosed by AHAs or by agrovets according to visual examination of an animal or from the described clinical signs. More than half of AHAs said that they regularly perform blood smears (53.6%) to look for parasitic infections, but there was no report of culture and AMR testing. All participants were asked to describe the five main diseases affecting animals, for which antimicrobial treatment was given – usually, three or four diseases were given and each of these was counted as the number of times they were mentioned, thus percentages do not add up to 100%. Across all interviews, agrovets reported 39 different diseases, AHAs reported 29 different diseases and farmers reported 19 different diseases. There was some overlap between the reported diseases, though the most common diseases did not differ significantly by group (**Figure 3.3**), except for the reporting of anaplasmosis, east coast fever and dysentery. The most common diseases according to AHAs and farmers was East Coast fever, and according to agrovets was dysentery. Combined, the most common diseases were East Coast fever, anaplasmosis, trypanosomiasis, diarrhoea, and general respiratory diseases.

3.3.8 Record Keeping

A large majority of participants kept some form of records regarding antimicrobial purchase or use - AHAs (64.3%), agrovet shops (71.4%) and farmers (48.6%). Agrovet shops usually maintained a sales book to track sales and purchases. All sales books (where present) were inspected, and the agrovet-volunteered ‘top five most commonly sold antimicrobials’ were cross-referenced against these books. There was good concordance with those antimicrobials recorded in the record books and the volunteered responses. Half of farmers (50.0%) had some records of antimicrobials they administered to their animals; however, the records did not often

Figure 3.3 The most common conditions or infections cited requiring antimicrobial treatment by farmers, agrovets, and AHAs. Bars are 95% CI.

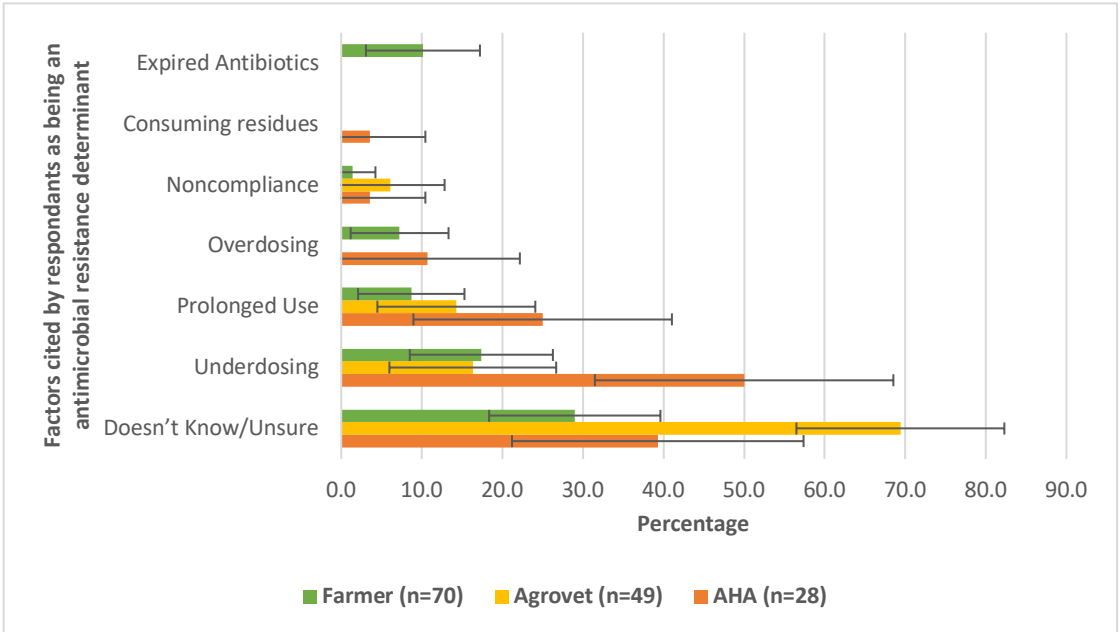


contain specific antimicrobial names or dosages. When questioned, farmers were often unable to name the antimicrobials used as an AHA had provided and administered the treatment, and not recorded it for them (corroborating the previous point that AHAs do not provide detailed information regarding antimicrobials to farmers). Of those farmers that self-treated animals, some kept the discarded packaging, which were examined. Almost all the interviewed AHAs stated that they recorded treatments they gave in books owned by the farmer, however, almost all the farmers reported that the AHA maintained and kept the record book for their farm.

3.3.9 Understanding and Management of AMR

A large proportion of agrovets, AHAs and farmers were unable to give an accurate definition of 'AMR' or 'antibiotic resistance'. A large proportion of agrovet staff (69.2%), AHAs (39.7%) and farmers (29.0%) stated that they had never heard the terms before. Of those farmers, agrovets and AHAs who had heard of AMR, volunteered explanations for the cause of AMR included "underdosing", "prolonged use" and "not following agrovet or AHA instructions" (**Figure 3.4**). Farmers also attributed resistance to "bacterial mutation" (2.9%), misdiagnosis by an agrovet/AHA (15.9%) and using "counterfeit antimicrobials" (1.4%). Some participants who were unsure about AMR still guessed at what it is, and what causes it. Some answers included: "when you treat an animal and it doesn't respond", "when the animal is tired, the antibiotic will not work" and "cheap drugs no longer work, but the more expensive ones do". An accurate definition of AMR was given to all participants after they had answered, to ensure correct understanding in the future.

Figure 3.4 Most common responses given by participants indicating what they thought were the main causes of antimicrobial resistance. Bars are 95% CI.



Farmers and AHAs were asked if there had been cases of AMR on farms. Agrovets were also asked if AMR had been reported to them when farmers purchase antimicrobials. The majority of agrovets indicated that they "[did] not know" or there was "no reported" resistance to antimicrobials (61.2%). Some stated that there was AMR, but they did not know to which antimicrobial (16.3%). A small minority of agrovets reported resistance to oxytetracyclines (10.2%), penicillin-streptomycin (4.1%) and sulfa-drugs (8.1%). AHAs reported higher rates of resistance to oxytetracycline (41.4%) and penicillin-streptomycin (27.6%), but no resistance to sulfa-drugs. Farmers suggested that they had not encountered resistance in more than half of treatments given to their animals (58.7%) (though this could mean that treatment was simply successful in these cases). Oxytetracycline resistance was the most commonly reported (20.6%), and a small minority also reported penicillin-streptomycin (7.9%) and sulfa-drug (1.6%) resistance. A small subgroup of farmers suggested that there had been resistance but were unsure to which antimicrobial (11.1%).

AHAs and agrovets were asked, "If you recommend or prescribe an antimicrobial and the farmer returns to you and complains that it hasn't treated the disease, what do you do?". AHAs stated that they would undertake a blood smear (53.6%) or perform a bacterial culture (7.1%), or PCR (3.6%), the rest would give an alternative antimicrobial. A quarter of agrovets involved a more experienced agrovet, an AHA or the owner of an agrovet shop (28.6%) where they suspected or had reported AMR. A similar proportion of agrovets would suggest an alternative antimicrobial without gaining more information regarding the animal (26.5%) and a small number of agrovets said that they had not encountered the issue before (22.4%). The rest of agrovets would first try to obtain more information i.e. ask about more clinical signs, and then recommend an alternative antimicrobial.

Withdrawal periods are important to observe, to avoid consumption of antimicrobial residues through meat and milk products. Farmers had different levels of knowledge regarding these: "no understanding" (12.9%), whereby the farmer had no knowledge of withdrawal periods, "minor understanding" (34.3%) whereby the farmer had heard of withdrawal periods, but quoted incorrect withdrawal periods for milk, meat and eggs, and "good understanding" (27.1%), whereby the farmer had good knowledge of withdrawal periods and was easily able to quote the correct lengths of time for each antimicrobial they regularly treated animals with. These responses were cross-checked with antimicrobial packaging to ensure accuracy. The remainder of farmers were aware that withdrawal period information is printed on packaging,

and they sometimes referred to this (18.6%) to ascertain the correct length of time for withdrawal prior to consuming products or selling animals for slaughter.

There were also some misconceptions regarding withdrawal periods - some farmers thought that they only applied to milk (12.9%), or meat (1.4%). The majority of farmers did not sell or consume animals or products during withdrawal periods (75.7%). However, some farmers said that they ignored withdrawal period recommendations (17.1%) as it wouldn't harm them; commonly farmers would give antimicrobial residue-containing animal products (usually milk) to their dogs (14.3%), or still allowed a calf to suckle (44.3%). One farmer stated that he regularly gave contaminated milk to his animals, despite understanding residues: "[I] give to the calves and the dog. [I] understand that resistance may develop in these animals, but [I] choose to ignore it".

Finally, a number of farmers (20.0%, n=14) stated that they either had themselves given or had heard of other farmers dispensing human-intended antimicrobials to various animals (pigs and poultry, mainly). These included treating poultry with and pigs with metronidazole for diarrhoea and tetracycline and amoxicillin capsules being split open, mixed with water, and given orally to poultry and pigs.

3.3.10 Observations made during interviews

In instances where interviews were being conducted in Kiswahili by the translator, there was opportunity to observe farms and the way that business ran in agrovet shop (as we allowed paying customers priority when they arrived at agrovet shops).

Whilst on farms, photographs were taken of a number of different antimicrobials used in treatment of animals, as antimicrobial packaging is commonly disposed of in waste pile (**Figure 3.5**), for burning, in Busia. Despite the most commonly used antimicrobials being volunteered to the interviewer, as part of the questionnaire study, packaging found on farms suggests some discordance to what is actually be used.

At agrovet shops, illegal practices were witnessed, such as selling single syringes of antimicrobials from an opened bottle of penicillin-streptomycin (**Figure 3.6**). This practice is contravened by Kenyan Law (National Council for Law Reporting, 2012a), but is a common

practice owing to the reduced cost of purchasing a single syringe, opposed to a whole vial of antimicrobials.

Figure 3.5 Image of discarded packaging of antimicrobials on a farm.

Figure 3.6 Image of a single syringe of penicillin-streptomycin being sold at an agrovet shop. This practice is contraindicated by Kenyan Law (National Council for Law Reporting, 2012a).

3.4 Discussion

This study investigated the common knowledge, attitudes, and practices towards AMU and AMR of both antimibocirbial users and prescribers. A questionnaire was used as a framework to interview farmers, agrovets and AHAs across 7 sub-counties in Busia, western Kenya. Questions were designed to specifically assess how antimicrobials are accessed, prescription patterns and knowledge of AMR and withdrawal periods. The findings from this study suggest that there is widespread access to antimicrobials, with and without prescriptions, and that knowledge regarding AMR is generally superficial. This is the first study to collect information regarding access to and understanding of antimicrobials and AMR in western Kenya, and the data presented here are an important set of results which help to address the paucity of data regarding AMU and AMR in this region.

Previous studies conducted in sub-Saharan African countries have identified that one of the main factors causing increasing prevalence of AMR is the indiscriminate use of antimicrobials (Robinson et al., 2017). This is exacerbated by unregulated access to wide varieties of antimicrobials, which do not require prescriptions to be purchased (Kalungia et al., 2016). The patterns of antimicrobial use and the general understanding of them at the community level remains largely unknown, though there is evidence to indicate that the high prevalence of AMR in animals is contributed to by overreliance of antimicrobials in agricultural practices (Marshall and Levy, 2011; Chang et al., 2015b; Hoelzer et al., 2017). In Kenya, people can access human antimicrobials at retail pharmacies without a prescription. Similar practices are common in the livestock sector, in the form of agrovet shops, due to poor regulation (Higham et al., 2016). All participants in Busia county accessed antimicrobials via their local agrovet shops or through AHAs who travelled to farms to treat animals. All the agrovet shops visited in this study (except for two large agrovets which supplied agrovet within Busia) were small privately-owned businesses; there was an absence of larger businesses or chains, suggesting that there is limited opportunity for scalability.

There are many agrovet shops in each sub-County (average: 7) though it is unclear how many farms that these shops cover. Almost half of the visited agrovet shops (several may have not been located or were closed during repeat visits) did not have a license to operate. To legally open an agrovet shop, a license is to be issued by the county government, and to issue a license, a person must present a valid college diploma in a course related to antimicrobial prescription and/or animal health and disease. According to an official investigation, fewer than half of

agrovet staff did not have the required college diploma to legally sell veterinary antimicrobials (National Council for Law Reporting, 2012b) and this agrees with our findings in this study. By law, class 1 poisons (including vaccines and antimicrobials) should only be dispensed against a valid prescription and findings from this study indicate that approximately 40% of antimicrobials were not dispensed against a valid prescription, having been sold by specific request of a farmer. Non-prescription use of antimicrobials is worrying, as farmers may tend to select their favoured products by recognising packaging of previously used antimicrobials. In these instances, the disease being treated may not be susceptible or the drug may be inappropriate for the type of infection. All antimicrobial use creates selective pressures for AMR to develop, thus there is a need for better regulation, in order to reduce inappropriate use. One of the primary considerations for farmers purchasing antimicrobials was cost - as farmers may have limited income, they may wish to only purchase part of an entire course of antimicrobials for animals. Few studies have examined dosing regimens in livestock production in Kenya, though there has been evidence in the past of consistent underdosing in Narok county farmers (Irungu et al., 2007) which is thought to be increasing the prevalence of AMR. Under-dosing is a known contributor of AMR development, as the selective pressures destroy sensitive bacteria, allowing those with higher minimum inhibitory concentrations i.e. more resistant strains, to outcompete others (Roberts et al., 2008).

It has been shown that antimicrobial dispensers play an important role in providing information to the public about human and veterinary antimicrobials, and this has been shown in various sub-Saharan African countries (Kehinde and Ogunnowo, 2013; Kheder and Ayed, 2013; Higham et al., 2016). As agrovets do not usually offer clinical services, they have little actual involvement in the diagnostic process, and may not have any practical experience in livestock health and disease. As this study noted, less than half of agrovet staff had not completed any formal training in livestock health and disease or completed their college diploma. A third of agrovet staff suggested that their main training came from pharmaceutical companies – it is likely that these informal trainings are not impartial and are specific to the antimicrobials being provided by that company. This reveals a significant and concerning gap in impartial training provision for agrovet staff, on the informed and responsible dispensing of veterinary drugs to customers. A number of agrovet staff also suggested that they gave advice regarding antimicrobials from their own experience or from memory; it is possible that due to gaps in their knowledge, they could be spreading inaccurate information to farmers. This can be easily rectified with additional training and implementation of government-regulated antimicrobial prescribing guidelines –

these would mean that all agrovets are confident in suggesting appropriate antimicrobial treatments for farmers. Efforts are ongoing to ensure this happens, but limited resources and lack of personnel to ensure regulations are maintained mean that there has been some issue in targeting those who would benefit most from these suggestions, as well as discussing who could deliver educational programs and who will bear the cost (Government of Kenya, 2017).

In cases where farmers purchase antimicrobials without consultation or with a valid prescription, they make the primary decision when choosing and administering antimicrobials to livestock. The findings in this study echo previous observations regarding the discordance between point-of-sale of veterinary antimicrobials and the advice given during the sale. Two previous studies also demonstrated the lack of diagnostic skill and knowledge of livestock diseases and treatment options amongst farmers and pastoralists (Grace et al., 2009; Jandreau and Berkes, 2016). As this study noted, the majority of farmers estimated animal weight by eye – all antimicrobials should be dosed according to weight, therefore it is important to accurately estimate this. A study examining smallholder farmers and AHAs ability to estimate the weight of animals found that inaccurate estimations were leading to chronic underdosing of antimicrobials (Machila et al., 2008). In that study, 85.7% of cattle were estimated to have their weights underestimated by an average of 46.9% of their true weight. Only 19.0% of cattle had their weights estimated accurately to within ±20% of their true weight by farmers. AHAs in the same study were better at estimating cattle weights – they accurately estimated 76.6% of cattle to within ±20% of their true weight (Machila et al., 2008). This suggests that AHAs are significantly better at estimating the weight of animals, and it is therefore important that they are consulted when treating animals, to ensure the correct dosages are being given to animals. Chronic underdosing can also place enhanced selective pressures on bacteria, enabling resistance to develop more rapidly, however, it is possible to mitigate this issue. By training farmers and AHAs, evidence suggested that both groups estimates of animal weights improved over time, as they received feedback of the true weights of different sizes of cattle (Machila et al., 2008). Similarly, an alternative to estimation, such as using weight tapes for larger animals, may significantly improve the issue with chronic underdosing.

This study (**Chapter 3**) compiled commonly used antimicrobials from a mixture of participant recollection and records kept by farmers, agrovets and AHAs. The most commonly used antimicrobials in livestock treatment included oxytetracyclines, penicillin-streptomycin and sulfa-drugs. According to the literature review (Chapter 1) there are around 24 African countries

which do not have antimicrobial use data; indeed, the majority of use in animal production systems in LMICs is largely undocumented (Schar et al., 2018). The use of antibiotics classed as HPCIAs (EMA, 2015; WHO, 2019b) was extremely limited which is positive to note. This KAP study found no reported use of fluoroquinolones, 3rd+ generation cephalosporins, and only one reported use of colistin. Few people had heard of or recognised any examples of critically important antimicrobials; this is likely due to lack of awareness and available information, given that these drugs are available for purchase in more urbanised areas (Muloi et al., 2019b).

There was a high prevalence of antimicrobial resistance detected amongst faecal *E. coli;* these findings differed from previously published studies, however, suggesting that there is a much lower carriage of tetracycline and sulfa-drug resistant *E. coli* in Kenyan goats (Njoroge et al., 2013) and poultry (Langata et al., 2019), and animals in other African countries such as Zambia (Mainda et al., 2015). Resistance to ampicillin and trimethoprim are also commonly reported in *E. coli* isolated from food-producing animals in sub-Saharan African countries (Kikuvi et al., 2006; Ojo et al., 2010; Donkor et al., 2012). Some AHAs reported that some certificate and diploma courses did not have specific training in prescribing or recommending antimicrobials for specific diseases, beyond broad-spectrum tetracyclines and penicillin-streptomycin. The Kenyan NAP (Fleming Fund, 2019) aims to increase education regarding AMR, as well as reducing inappropriate sale and use of antimicrobials. There has been a correlation between AMU in animals and the occurrence of AMR, and several studies suggest that reducing the use of antimicrobials in animals, was effective at reducing AMR in animals (Dorado-García et al., 2016; McDonnell et al., 2017). However, it remains to be seen if there are direct benefits to reduction of AMU in animals on AMR in humans, though it stands to reason that reducing any reservoirs of AMR bacteria amongst animals (food-producing, and companion animals) may prevent opportunistic colonisation of humans (van Alen et al., 2017).

Many different diseases affecting livestock were reported by all groups. In the majority of cases, treatment was syndromic, without diagnostic support. Three main diagnostic laboratories exist to cover western Kenya – the Busia/ILRI diagnostic laboratory, Kericho regional laboratory and Eldoret regional laboratory. Outside of these three laboratories, there is a lack of well-funded and well-equipped diagnostic services. Due to logistical issues with transporting samples (lack of cold-chain, and distance to these laboratories) as well as long turnaround time and cost involved, this has made farmers and field veterinarians reluctant to submit samples for culture/sensitivity testing to confirm diagnoses, before beginning treatment (Global Antibiotic

Resistance Partnership, 2011). AHAs usually rely on their clinical experience for disease identification, and agrovets typically rely on farmers or more experienced agrovets to report clinical signs. This can lead to misdiagnosis, and lack of confidence in diagnosis can drive the use of empirical, broad-spectrum antimicrobials (which is common practice in Kenya). In a follow-up study, it may provide useful insight to include specific questions into why there is high use of empirical therapies and compare these with the results in this KAP study. Because AMR surveillance has not been previously systematically conducted in Kenya, for many AHAs and agrovets, there is no indication as to why commonly used antimicrobial regimens are becoming less effective against many infections, and this is a concern.

The general knowledge of AMR varied according to farmers, agrovets and AHAs. Initially, few agrovets, AHAs and farmers had a good understanding of what AMR was, but after a definition was provided, many were able to give examples of factors which they thought may contribute to the emergence of AMR. However, this study has indicated that general knowledge regarding AMR is superficial. As noted above, there is insufficient finance and capacity in place to ensure that public health education materials are delivered to antimicrobial prescribers and providers. One major issue around lack of understanding ABR is that participants were unable to distinguish between AMR and treatment failure. For example, AMR could be due to chronic underdosing such is the case where doses incorrect to inaccurate estimation of animal weight (Machila et al., 2008), whereas treatment failure could be due to incorrect diagnosis and provision of an inappropriate (antimicrobial) treatment. Similarly, the disease may not be bacterial in origin. Where treatment failure has occurred, farmers often switch to another class of antimicrobial. This is endorsed by World Organisation for Animal Health (OIE) guidelines for responsible use (OIE, 2017), but as no diagnostics procedures (bacterial culture and antimicrobial sensitivity) are performed, there is often a switch between two classes of broad-spectrum antimicrobials (penicillin-streptomycin and oxytetracyclines), which may not be the most appropriate or effective treatment. There is a high proportion of AHAs and agrovets who provided an alternative antimicrobial to a farmer without obtaining further clinical information regarding the animals, and a quarter of farmers suggested that they requested a different antimicrobial without first consulting an agrovet or AHA. This self-prescription or uninformed prescription could be addressed through reforms of regulation controlling the sales of antimicrobials and introducing dispensing audits (Tangcharoensathien et al., 2018). These audits could potentially feed into the new surveillance systems being implemented as part of the national AMR Action Plan.

Withdrawal periods were also generally not well understood or abided by in some cases. A study conducted in Tanzania also found that depending on the tribe, people were more or less likely to observe withdrawal periods (Caudell et al., 2017). In Kenya, there are regulatory frameworks for residue surveillance in animal products in parts of Kenya, though this is primarily directed towards milk sale (Kosgey et al., 2018). According to this study, some farmers with good knowledge of withdrawal periods still allowed calves and dogs to drink residue-containing milk, believing that this was safe for animals. Evidence investigating the impact of allowing calves to suckle residue-containing milk suggests the changes in faecal microbiota may alter the relative abundance of microbial cell functions (affecting cell signalling and stress responses), which can impact on the selection and dissemination of virulence genes and antimicrobial resistance (Pereira et al., 2018). Other farmers suggested that withdrawal periods only applied to milk or eggs and were unaware that residues may also exist in meat. There may be socio-economic factors e.g. not selling animal products may mean that farmers have no money to feed their families or pay for school fees. Recent studies of the dairy value chain concluded that antimicrobial residues detected in animal products were predominantly due to ignoring withdrawal periods (Orwa et al., 2017).

A limitation of this study was the requirement for travel to farms alongside government representatives and a member of the Busia diagnostic laboratory during this study. It is likely that there was bias in the way that questions were answered by respondents, due to presence of government representatives. For example, farmers would not want to disclose poor or illegal practices, and may have answered the questions differently than if government representatives were not present. Another limitation of this study was language barrier – as more than half of the interviews were conducted in Kiswahili, by a translator, some questions may not have direct equivalence when asked in English. An example of this is the word *dawa* which is a Kiswahili word for 'medicine' – this is commonly used to denote antibiotics, as well as non-antimicrobials such as paracetamol in western Kenya. However, answers provided by participants to many questions were mostly consistent across each sub-county, particularly for antimicrobial purchase, which suggests that accurate insights of KAPs from a variety of antimicrobial users, providers and prescribers from larger and small farms, as well as a wide variety of agrovets shops was gained. Finally, interviews were conducted across Busia, using a stratified approach. This was done as a logistical consideration, as vehicular access was shared between other members of the laboratory group. Stratification assumes that there is homogeneity within each subgroup, however, this was not investigated prior to conducting the study. As several priors which formed

the sample size calculation were unknown, it may be possible that our analyses of the dataset has a higher standard error than if a different sampling strategy (such as optimum allocation) was used. Furthermore, after completing the interviews, no survey weighting was used. As the proportion of participants sampled did not consider the population density within strata, data may need to be weighted sothat downstream analyses have tighterh confidence intervals, and more accurately represent the population. Data weights are also usually used to compensate for non-responses, though there were no instances of questions that had non-responses during interviews. Due to the stratified approach in selecting farms, and lack of weighting given to questionnaire responses, no formal comparison has been made between sub-counties using the interview data. This may have provided additional insight into knowledge, attitudes and practices towards antimicrobials, in different geographical locations and will be considered in future analysis.

There are several matters surrounding the understanding of, and consequences of, the use of antimicrobials in farm animals. Foremost, we determined that a key area to address is to encourage additional training regarding AMU and AMR, to fill in gaps in knowledge of antimicrobial users and providers who prescribe and sell antimicrobials. Training could be reasonably addressed in the short-term, through interactive courses or the introduction of a set of detailed guidelines on antimicrobial prescription and usage. Next, efforts could be increased to reform regulation surrounding antimicrobial use. There are good records in place of antimicrobials sold at agrovet shops and improving this by standardising record-keeping into a computerised system at agrovet or county-government level, would allow for accurate tracking of prescribed and sold antimicrobials and minimise over- and non-prudent use of antimicrobials. A widely accessible treatment plan could be made available as guidelines for treatments of animals; existing projects in western Kenya are seeking to survey the area for several diseases and this will be important in the future for advising policies. With the new National Action plan for AMR, a country-wide surveillance system will help to identify the aetiological basis for many diseases and following this introduction of better diagnostic facilities would allow for microbiological support where first-line treatments are ineffective. This will allow antimicrobial providers and prescribers to initiate more narrow-spectrum therapies, relieving the overreliance on broad-spectrum antimicrobials. In the long term, additional ideas may be implemented to optimise livestock production and better control diseases in rural Kenya, as well as mitigating AMR in both animals and humans. It is vital that the One Health approach is accepted along with the investment of research into the still unknown contribution that

agriculture plays in AMR, to generate a synergistic educational, communication and clinical effort between human and veterinary medicine. If properly implemented, it is expected that the Kenyan National AMR action plan (Fleming Fund, 2019) will do all of these things.

Chapter 4

Patterns of AMR *E. coli* Isolated from Humans, Livestock & Their Shared Environment, on Smallholder Farms in Western Kenya

4.1 Introduction

AMR is having a negative impact on human and veterinary health (Cassini et al., 2019), by increasing treatment failures, morbidity and mortality and increasing the financial burden on healthcare services (O'Neill, 2016). As there is overlap in the antimicrobial classes used in both human and veterinary medicine, it is probable that AMU in animals exacerbates the development of AMR in bacteria acquired by humans, and vice-versa (Woolhouse et al., 2015), though a direct link between AMR in humans as a result of animal exposure is difficult to determine (Muloi et al., 2018). Adjacent to human and veterinary medicine is the selective pressures being placed upon environmental bacteria to develop or acquire AMR genes; this is occurring through leakage of antimicrobial residues and other co-selecting agents such as heavy metals, into the environment. As such, recent research has turned to a 'One Health' approach, to determine the degree to which antibiotic usage in human and veterinary medicine is exacerbating the selection and dissemination of bacterial resistance between humans, animals (Angulo et al., 2004; Carlet, 2012) and the environment (Chang et al., 2015b; Founou et al., 2016).

AMR in humans is thought to have increased rapidly due to widespread and indiscriminate AMU (World Health Organization, 2014). This is having an effect not only specialist pathogens, such as *S. pneumoniae* and *M. tuberculosis,* but also on commensal bacteria including *E. coli* and *K. pneumoniae,* which are increasingly becoming resistant to carbapenems and fluoroquinolones (World Health Organization, 2017). AMR in animals is also rising rapidly due to the reliance on antimicrobials for promotion of animal health, welfare and increased productivity in commercial systems (Hockenhull et al., 2017); there has been recent research to suggest that food-producing animals are an important reservoir of AMR bacteria (Aarestrup, 2015). In the environment, there is a reservoir of AMR in soil bacteria; this is unsurprising as bacteria have been producing natural antibiotics to eliminate competition for more than 30,000 years, and perhaps longer (D'Costa et al., 2011; Perron et al., 2015).

Commensal bacteria such as *E. coli* can cause diarrhoeal disease, and other extraintestinal infections in humans and animals; whilst the diarrhoea is usually self-limiting, the emergence of MDR and ESBL-*E. coli* have led to increased mortality and morbidity in humans and animals (Colomb-Cotinat et al., 2016; van Duin and Paterson, 2016). As *E. coli* is readily isolated from the gastrointestinal tract of different animal species (including cattle, pigs, poultry, small ruminants), humans, and the environment (to a lesser extent), it is a good indicator species for

investigating AMR (van den Bogaard and Stobberingh, 2000; Sanderson et al., 2018). Due to *E. coli*'s ecological niche in the gut, the frequent exposure it receives to various antibiotics puts increased selection pressure on them to acquire antimicrobial resistance (Mubita et al., 2008; Wooldridge, 2012).

Antimicrobial resistance genes are transferred between bacteria by horizontal transfer on mobile genetic elements such as plasmids (Chang et al., 2015a; Ter Kuile et al., 2016). These genes may then integrate into host *E. coli* chromosomes or spread to other commensal or pathogenic bacterial species (El Salabi et al., 2013). Plasmid-meditated ESBL-resistance is of particular concern, as plasmids carrying ESBL genes such as $bla_{\text{CTX-M}}$ and bla_{SHV}, also frequently carry genes conferring resistance to aminoglycosides, quinolones and other antibiotics (Nilsen et al., 2013).

A number of studies suggest that AMR bacteria and resistance genes of farm-animal-origin bacteria can be transferred to humans. There are thought to be three major routes of zoonotic transmission of AMR (though there is little empirical evidence for these) - contamination within the food chain (improper handling, inadequate cooking or storage), through direct contact with animals or their products, including meat and milk, or through contamination of the environment; use of animal faeces as fertiliser and subsequent leaching into water sources may also be a potential pathway for spread (Carattoli, 2008; Wooldridge, 2012; Woolhouse et al., 2015; Muloi et al., 2019b). This is likely to contribute to the emergence of antibiotic resistance among human pathogens (Voets et al., 2013; de Been et al., 2014; Berg et al., 2017; Castellanos et al., 2017). However, the exact role of farm animals in the emergence and dissemination of AMR to humans is still contested (Marshall and Levy, 2011; Woolhouse et al., 2015) and Muloi from above. Many studies have relied on traditional methods such as MLST and macro-restriction pulsed-field gel electrophoresis (which is only useful in indicating clonal dissemination of antimicrobial resistance, rather than the transfer of resistance genes between bacteria). These techniques may or may not have sufficient discriminatory power to infer directionality or provide evidence of transmission (Woolhouse et al., 2015; Muloi et al., 2018).

The dynamics of AMR in developing countries are poorly understood, especially in community settings, due to a sparsity of data on AMR prevalence. Several studies involving pathogenic *E. coli* from sub-Sharan African countries have been conducted, and these report increased isolation of ESBL-producing *E. coli* (Bercion et al., 2009; Sangare et al., 2016;

Musicha et al., 2017) and MDR *E. coli* from various human clinical cases, as well as from animal sources (Ibrahim et al., 2012; Manyahi et al., 2014; Adenipekun et al., 2015; Hassell et al., 2019). However, the reported data is limited to very few countries, due to the absence of surveillance systems (World Health Organization, 2017). Some transmission dynamics studies conducted in SSA countries have shown evidence to indicate the transfer of bacteria between humans, animals and the environment (Rwego et al., 2008; Lupindu et al., 2015), and this study will aim to build on those foundations. The issue with many previous studies investigating transmission is that simply demonstrating similarity of AMR bacteria and/or resistance determinants in humans, animals and the environment does not allow inference on the directionality of transfer. WGS is advantageous when compared to previously used methods as it can provide increased resolution for the study of genetic similarity of bacteria e.g. resistance can be identified on individual plasmids. WGS was not previously widely accessible, due to its high cost; as the technology and methods improve over time, it is becoming a more affordable, and accessible, method. By combining WGS analysis with epidemiological information, such as the prevalence of AMR genotypes and phenotypes, as well as antimicrobial usage statistics, it may be possible to reconstruct the complex transmission dynamics of resistant bacteria and their AMR determinants between human and food animal populations (De Maio et al., 2015; Woolhouse et al., 2015).

The purpose of this study was to assess the carriage of AMR faecal *E. coli* of farmers, livestock and the farmers' living environment by classical microbiological methods; assess the carriage of resistance and virulence genes of faecal *E. coli* isolated from farmers, livestock and the environment; determine relatedness of *E. coli* by constructing phylogenies using WGS, and; to determine risk factors for the carriage of AMR, MDR and ESBL-producing *E. coli*.

4.2 Methods

4.2.1 Study Population and sample size determination

The aim was to sample the faeces of the most commonly owned livestock species on smallholder farms; the animal population consisted of pigs, poultry, sheep and goats, and cattle from smallholder farms in Busia county, western Kenya. The sample size was calculated according to a modified Cochran formula. As little previous work of this nature had been undertaken in the study area, a conservative estimate of the prevalence (*p)* of AMR *E. coli* of 35% was suggested by ILRI scientist Jane Poole, informed by the previously unpublished Urban Zoo project (Muloi et al., 2019b). Using a precision (*d)* of 5% and 95% confidence (standard two-sided 5% significance levels), an estimated 350 animals (*N*) needed to be recruited; this would be the optimal size for the study to assure an adequate power to detect statistical significance or differences in AMR *E. coli* carriage between human, livestock and envrionemtnal populations:

$$N = \frac{Z^2 p(1-p)}{d^2}$$

The number of farms was predetermined from our previous questionnaire study (**Chapter 3**) to be 70 farms (10 farms in each of the seven sub-counties in Busia as the same farms were to be re-visited). The intra-cluster correlation coefficient (ICC) was estimated based on the same previous study (Urban Zoo) to be approximately 0.15 (*p*). The average cluster size was estimated to be approximately 10 (*n*). Thus, the adjusted sample size was 822.5 animals. This would average to approximately 12 samples per each of the 70 farms.

$$DE = 1 + (n-1)p$$

To achieve 12 samples per farm an equal number from each species present on that farm were collected such that the number from each species differed between farms depending on the composition of the species present. e.g. a farm containing cattle and goats only, had 6 samples collected from each of those species. Human participants were selected according to the expectance that 1-3 members of the household engaged with farming would be present on each farm (according to our questionnaire study) – this meant that between 70-210 human samples could be collected.

As most smallholder farms in this area were thought to have a single source of water, 1 water sample per farm was collected from each farm. Similarly, one environmental (boot swab)

sample was collected from each household. A prior control study was performed at the University of Liverpool alongside Professor Nicola Williams. This was used to develop a protocol for use of boot swabs, and methods of growing and isolating bacteria from boot swabs (SK and NJW, unpublished data, 2016). This control study suggested that a diverse array of bacteria could be collected and successfully cultured from a single bootswab following overnight enrichment in buffered peptone water. As such only one boot swab sample per farm was collected.

4.2.2 Data & Sample Collection

Participants were recruited up to three days before the farm visit using GPS coordinates captured during the KAP study (Chapter 3) to locate farms. 27 farms declined to participate in this study, and so 27 new farms were recruited using the previously defined random method. A telephone number was collected at the time of recruitment to give warning the day before arrival at the farm. Immediately prior to sample collection, a questionnaire-based interview was used to obtain data from farmers (**Appendix II, Figure II-iii**); information collected included slaughter practices, personal protection equipment, human and animal illness, antimicrobial usage, and purchase. Samples were collected continuously between 25th April and 7th May 2017. Ethical approval was obtained from the University of Liverpool and ILRI prior to collection of data and samples; all samples were collected after obtaining signed and informed consent from participants.

4.2.2.1 Human Faeces Collection

Human participants were visited up to three days prior to sample collection to explain our intentions and what was required from them. A faecal pot and protective gloves were provided to each participant at the time of recruitment along with instructions on how to collect a non-urine-contaminated sample; participants typically defecated onto a sheet of newspaper and then scooped up a faecal sample (5-40g) with the provided faecal pot (spoon attached to the lid). Participants were informed the night before they were due to be sampled so that they could provide a fresh faecal sample on the same morning the interview and animal samples were collected.

4.2.2.2 Animal Faeces Collection

Up to 12 lots of deposited faeces (i.e. from the ground) from around each farm were collected using a non-random (haphazard) method; faecal deposits were identified according to species

and an equal number of faeces were selected according to the type of species kept on the farm, if they were further than 1m apart from each other. If there were 4 different species, 3 samples from were collected each type of animal. A faecal pot (with spoon attached to the lid) was used to collect between 20-35g of faeces. Poultry faeces were collected by placing a chicken onto newspaper/paper bag and then collecting deposited faeces, or from sampling a chicken house directly.

4.2.2.3 Water and Environmental Samples

The main water source was identified via a questionnaire. 15ml of water was collected in sterile universal tubes from various water sources, including boreholes, wells, rivers, and lakes where there was common grazing ground, collected rainwater and taps.

For environmental sampling, sterile shoe covers (boot swabs) were used to collect bacteria from the floor of participant's homes, where animals were allowed access. Boot swabs were pre-moistened with 100µl of sterile water (to enhance the collection of bacteria from the ground) and then placed over blue overshoes, to prevent contamination of the boot swabs by the investigator's shoes. Investigators then walked along the floor of participant's living spaces, including cooking areas, covering as much floor area as available. Boot swabs were then transferred to sterile ziplock bags and sealed.

4.2.3 *E. coli* Isolation

After faecal, water and environmental sample collection was completed, samples were immediately placed into a cool box for transport back to the lab and processed on the same day. At the lab, initial culture and isolation of bacteria was performed at the Busia/ILRI laboratory (latitude/longitude 0.46416, 34.10670). Approximately 1g of faeces was homogenised separately in 5ml tryptone soy broth (Oxoid, Hampshire, UK). 10ml of each water sample was mixed with 10ml of double concentration (40g/l) buffered peptone water (Oxoid, Hampshire, UK) in a universal tube. 100ml of buffered peptone water was added to a ziplock bag containing boot swabs, shaken vigorously and then 20ml was collcted into a sterile universal tube. All samples were incubated aerobically overnight at 37°C. Following incubation, all samples were streaked onto plain eosin-methylene-blue agar (EMBA) (Oxoid, Hampshire, UK) with a sterile 10µl plastic loop and again incubated overnight under the same conditions. Where *E. coli* was evident morphologically (shiny, metallic green colonies), a full-plate streak was taken with a sterile 10µl plastic loop and transferred to MicroBank™ tubes (Pro Lab diagnostics, Ontario, CA)

and inverted several times to equally cover the beads. Where there was no growth consistent with *E. coli*, the sample was discarded. After several minutes, excess supernatant was removed using a sterile pipette tip and discarded. All tubes were then frozen at -40°C prior to being shipped to the University of Liverpool, on ice packs. All original faecal samples were kept for long-term storage at -40°C. Samples were shipped with appropriate export and import permits.

At the University of Liverpool, samples were resuscitated by streaking one MicroBank™ bead onto a plain EMBA plate and incubating aerobically overnight at 37°C. From this, bacteria were sub-cultured for single colonies onto three EMBA plates, one containing cefotaxime (1µg/ml), one containing ceftazidime (1µg/ml) and one containing no antimicrobials. From the plain EMBA plate, five random picks of colonies that were morphologically consistent with *E. coli* (metallic green, shiny) were selected and sub-cultured onto nutrient agar. From the EMBA plates containing cefotaxime and ceftazidime, one pick was randomly selected for sub-culture.

4.2.4 Antibiotic susceptibility testing

All isolates were sub-cultured onto nutrient agar and subject to antibiotic disc diffusion testing, using a panel of seven antibiotics determined to be common to the study-site, informed by the questionnaire study (**Chapter 3**). Suspensions were made according to the EUCAST guidelines (EUCAST, 2017). MHA plates were inoculated with each isolate and seven antimicrobial discs applied: ampicillin (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), gentamicin (10µg), sulfathiazole (1000µg), tetracycline (30µg) and trimethoprim (5µg). Isolates selected from EMBA plates containing ceftazidime or cefotaxime were subject to double-disc diffusion testing, using three pairs of antibiotic discs containing ceftazidime (30µg), cefotaxime (30µg) and cefpodoxime (30µg) with and without clavulanic acid (10µg). All plates were incubated aerobically overnight at 37°C and zones of inhibition were measured in mm, using a photograph of each plate and FIJI to digitally measure zones of inhibition; all but sulfathiazole and tetracycline zones of inhibition were interpreted according to EUCAST human clinical breakpoints (EUCAST, 2017). Tetracycline was interpreted according to BSAC human clinical breakpoints (BSAC, 2015). Sulfathiazole was interpreted according to a generated TECOFF (**Chapter 2.5.1**), according to the bimodal distribution of zones of inhibition. Suspected ESBL production was confirmed if the zone of inhibition for the cephalosporin with clavulanic acid disc was more than 5mm greater than the zone of its counterpart without clavulanic acid (M'Zali et al., 2000).

4.2.5 PCR Assays

PCR assays targeting the *uidA* gene (McDaniels et al., 1996) were performed as previously described **(Chapter 2.5.3)** on all isolates to confirm if isolates were *E. coli*. Isolates which were confirmed as ESBL-producing by double-disc diffusion test were further analysed for the presence of $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{OXA} and bla_{TEM} genes (Boyd et al., 2004; Dallenne et al., 2010). Isolates with the $bla_{CTX\text{-}M}$ gene were then further analysed to determine the specific CTX-M cluster (group 1, 2 or 9) (Batchelor et al., 2005; Hopkins et al., 2006; Carattoli et al., 2008). PCR-confirmed ESBL-*E. coli* were also tested for the presence of *trpa* and *pabB* genes (Clermont et al., 2009), indicating if they belonged to the ST131 pandemic clone.

4.2.6 Whole Genome Sequencing

Due to funding limitations, only 166 isolates could be selected for WGS, out of a total of 729 cultures. Farms were selected if there were isolates of at least two or more species of animal, one human and (where possible) one environmental sample. Once a list had been compiled fulfilling this criterion, a random selection of antimicrobial-resistant, antimicrobial-sensitive, MDR and ESBL-producing properties were chosen according to a random number generator. This selection was made to ensure comparisons of resistance genes between livestock, humans and the environment could be made.

4.2.6.1 DNA Extraction

DNA was extracted from 24-hour cultures using the QIAamp® DNA Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. To quantify the concentration and purity of DNA, two methods were used: a) Nanodrop spectrophotometer (Thermo Fisher Scientific, Cheshire, UK) was used to analyse the purity of the DNA extraction by assessing the 260/230 and 260/280 wavelength ratios and, b) Qubit Fluorometer (Thermo Fisher Scientific, Cheshire, UK) to analyse the concentration of dsDNA in each sample. All extracts were stored at -20°C before being transferred to Liverpool Centre for Genomic Research for sequencing.

4.2.6.2 DNA Sequencing

The following protocol is provided as is, from the University of Liverpool CGR:

One hundred and sixty DNA samples were submitted for Illumina TruSeq nano libraries preparation. Briefly, 200ng DNA of each sample was sheared with the Picoruptor to generate fragments approx. 550bp. After shearing the samples were cleaned-up with a 1.6x Ampure XP

beads and end-repaired. Following the end repair reaction, the samples were size selected with the sample prep beads and then A-tailed followed by the ligation of Truseq DNA UDI adaptors. These samples were Ampure cleaned twice to remove excess adapter and amplified with 7 cycles of PCR. The libraries were cleaned with equal volume of Ampure XP beads. The quantity and quality of the pools were assessed by Qubit and Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa (KK4854) on a Roche Light Cycler LC480II according to manufacturer's instructions. Briefly, a 20 µl PCR reaction (performed in triplicate for each pooled library) was prepared on ice with 12 µl SYBR Green I Master Mix and 4 µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial concentration determined by the Qubit dsDNA HS Assay Kit). PCR thermal cycling conditions consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds (denaturation) and 60°C for 45 seconds (annealing and extension), melt curve analysis to 95°C (continuous) and cooling at 37°.
and checked with Qubit assay and Bioanalyser chip.

Following calculation of the molarity using qPCR data, template DNA was diluted to 3nM and 5ul of each final pool were denatured for 8 minutes at room temperature using 5µl freshly diluted 0.1 N sodium hydroxide (NaOH) and the reaction was subsequently terminated by the addition of 5ul 0.1M TrisCl pH=8. The final loading concentration of 300pM was reached by adding 35µl exclusion amplification enzyme mix. The libraries were sequenced on 2 lanes of an Illumina HiSeq 4000 platform using sequencing by synthesis (SBS) technology to generate 2 x 150 bp paired-end reads.

4.2.6.3 WGS Workflow

The standard workflow as described in (**Chapter 2.6.1)** was run on all sequences. Briefly, raw paired-end reads were quality controlled using FastQC v0.11.7 (Andrews, 2010) Poor quality reads (score <20) and any detected primers or adapters were removed using Trimmomatic v0.36 (Bolger et al., 2014). Forward and reverse reads were then mapped to a reference *E. coli* genome (*E. coli* K12 MG1655; NCBI Reference Sequence: NC_000913.3 (Blattner et al., 1997), using the Burrow-Wheeler Alignment (bwa mem v0.7.17) (Li, 2013). Once BAM files had been made, a further QC step to check the mean mapping quality scores and coverage in relation to the reference genome was performed with QualiMap2 (Okonechnikov et al., 2015). Any reads which did not map to the reference genome were assembled *de novo* into contigs, using SPADES v3.12.0 (Bankevich et al., 2012). Speciation analysis of those contigs was then performed using Kraken v2.0.7 (Wood and Salzberg, 2014) and an in-house database downloaded from the NCBI

sequence read archive (https://www.ncbi.nlm.nih.gov/sra/). Non-*E. coli* contigs were excluded from analysis. Phylogenetic trees were constructed using IQ-Tree (Nguyen et al., 2015), using 1000 bootstrap replicates and the GTR model Tree topology was checked using Figtree v1.4.4 (Rambaut, 2012) and then annotated using the interactive tree of life (iTOL) v5.0 (Letunic and Bork, 2016). MLST was determined using a batch script which blasted each contig against the *E. coli* #1 schema (https://pubmlst.org/escherichia/) (Jolley and Maiden, 2014) – from this a sequence type (ST) was assigned according to the *adk, fumC, gyrB, icd, mdh, purA* and *recA* loci. *In silico* phylotyping was serotyping were formed using the Clermont Typing tool (Beghain et al., 2018) and SerotypeFinder (Joensen et al., 2015) respectively. As of 2019, the Clement phylotyping tool also identifies the new phylogroup, G (Clermont et al., 2019). Plasmid replicon typing was performed using the PlasmidFinder database using the batch uploading platform (identity ≥90%, coverage ≥60%) (Carattoli et al., 2014).

A brief comparison of methods was made regarding resistance and virulence gene data. Rather than relying on online tools, the most up to date resistance and virulence gene sequences from several different databases were downloaded, and the frequency of detection of common resistance and virulence genes was assessed. Databases were manually compiled on 18th October 2018 and used to screen all reads mapped to the *E. coli* reference strain (U00096.3). Databases downloaded included ResFinder (Zankari et al., 2012), CARD (McArthur et al., 2013), NCBI Antibacterial Resistance Reference gene database (NCBI A Accession: PRJNA313047, ID: 313047), EcOH (Ingle et al., 2015), PlasmidFinder (Carattoli et al., 2014) and VDFB (Chen et al., 2016).

4.2.7 Antimicrobial Resistance Data Analysis

Both questionnaire and microbiological data were entered in a spreadsheet initially (Microsoft Excel 2019, Microsoft Corporation) and reviewed to ensure accurate input. A binary coding system was implemented such that data was either susceptible (0) or resistant (1), using EUCAST human clinical breakpoints (EUCAST, 2017), BSAC human clinical breakpoints (BSAC, 2015) for tetracycline and the previously defined TECOFF value for sulfathiazole (**Chapter 2.5.1**). Descriptive statistics and Chi-squared tests for association were conducted in SPSS v25 (IBM Corp, Armonk, NY).

4.2.8 Questionnaire Data Analysis

Responses given by participants during questionnaire-guided interviews were stored within the AppSheet application until an internet connection was available. Data were then uploaded to a secure server in XLS format for later analysis. Data were imported from the XLS file into Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) and descriptive analysis (frequency tables of participant demographics), was conducted using SPSS Statistics 25.0 (IBM SPSS Statistics for Windows Version 25.0, (New York: IBM Corp).

Open-ended questions were analysed on a question-per-question basis using a thematic approach (Nowell et al., 2017). Briefly, text responses were read twice to ascertain a general comprehension of the responses. A large number of themes were identified and input into an Excel spreadsheet. Once all responses had been categorised, similar themes were condensed together. To ensure reproducibility, these themes were cross-checked by Dr Gina Pinchbeck (University of Liverpool). All data was then imported into SPSS Statistics 25.0 (IBM SPSS Statistics Version 25.0, New York: IBM Corp).

4.2.9 Risk Factor Analysis

Data for risk factor analysis comprised only of sample-level data; risk factor analysis used data from all isolates and the outcome variable was phenotypic resistance to antibiotics following AST testing. From each set of 5 isolates belonging to a primary sample (of which there were n=X), if one isolate carried a specific resistance phenotype, then the sample was classed as having antibiotic resistant *E. coli*. Antibiotic resistance outcomes included: resistance to any of the seven antibiotics tested, MDR (resistance to three or more classes of antimicrobial) and ESBL-producing *E. coli*. Potential risk factors were identified from the questionnaire data obtained concurrently with biological sampling. All but one explanatory variable (**see section 4.3.6.2**) derived from the questionnaire were binary (yes/no). One explanatory variable (farm size) was included as a continuous variable for analysis. Species was kept in all models as a fixed effect.

As data were clustered within farms and within regions, each outcome was examined with a separate multilevel logistic regression model with binomial distribution and logit link function. Each model included region and farm as random effects at level 3 and 2 respectively, to account for clustering. Samples were considered the level one unit of interest. Univariable analysis was conducted on all explanatory variables using individual models for each outcome. All variables

which showed association with the presence of resistant *E. coli* from individual univariable analyses (with a *P*-value <0.25) were considered for incorporation into a final multivariable model for each outcome. Final models were checked for farms which may have had an increased influence upon the mixed effects models. Farm level (2) residuals were plotted against the overall means; where there was any significant difference detected, all data from those farms were meticulously rechecked (and verified by Gina Pinchbeck) for any errors.

Multilevel, multivariable models were constructed using manual backward variable selection; only variables with a Wald *P*-value <0.05, after adjusting for other variables, were retained in the final model. First order interaction terms were tested for biologically plausible variables remaining in the final models. All analyses were conducted in MlWin v3.02 (Centre for Multilevel Modelling, University of Bristol).

As data obtained within this study were clustered within farms, to accurately approximate the prevalence (and confidence intervals) of resistance within this population, the prevalence of each outcome was estimated using separate logistic regression models including farm as a random effect to account for clustering at this level. The adjusted prevalence was calculated using the beta coefficient (β_0) from intercept-only, random effects models constructed for each outcome using the following formula:

$$P_T = \frac{e^{\beta 0}}{1 + e^{\beta 0}}$$

In the case of prevalence estimates, 95% confidence intervals were calculated as a function of the calculated standard error of the beta coefficient from the intercept-only logistic regression model for each outcome.

4.2.10 Maps and Georeferenced Data

Maps were constructed using QGis v3.2 (QGIS Development Team, http://qgis.osgeo.org/). The latitude and longitude of each farm were captured using a mobile phone and Google Maps (Google Map Data 2019, California, USA). All co-ordinates for each farm were then imported into QGIS for mapping. A base layer of Busia county was taken from Google Maps – upon this, Kenya administrative levels 0-2 boundary polygons and line shapefiles were layered, having been acquired from an open-source database hosted by the Humanitarian Data Exchange (https://data.humdata.org/). Map data is updated annually and as such, the borders of sub-

counties have moved slightly since the start of this study. Ten farms per sub-county were sampled, but according to generated maps e.g. **Figure 4.1**, some sub-counties appear to be over or underrepresented. This is due to map boundaries changing over time. For all resistance analysis, farms were considered to be part of the same sub-county as when first recorded.

4.3 Results

4.3.1 Samples Collected

In total 840 faecal samples from pigs, poultry, sheep, goats and cattle, from 70 farms were collected for this study, as well as 130 faecal samples from human participants, 1 water sample, and 1 environmental boot swab (70, each) were collected from each household. The total number of animals representing each sample was: cattle, n=208; pig, n=58; poultry, n=199; sheep and goats, n=96.

4.3.2 Prevalence of Antibiotic-Resistant *E. coli*

E. coli was successfully cultured from 726 human, animal, and environmental samples and *E. coli* with resistance to at least once class of antibiotic was isolated from 692 (95.3%) samples. The sample prevalence of resistance to each of the tested antibiotics, as well as MDR and ESBL-producing *E. coli* are detailed in **(Table 4.1)**. The total prevalence of resistance was adjusted for clustering, using intercept-only models.

MDR was isolated from 53.9% of samples with the largest proportion from sheep and goats and environmental sources. The largest proportion of ESBL *E. coli* was isolated from water samples, though this was a small sample size of water (n=15) and environment (n=20). Tetracycline and trimethoprim were the two most common antimicrobial resistance phenotypes detected in all sample groups. Fluoroquinolone and aminoglycoside resistance were the least prevalent amongst all sample groups.

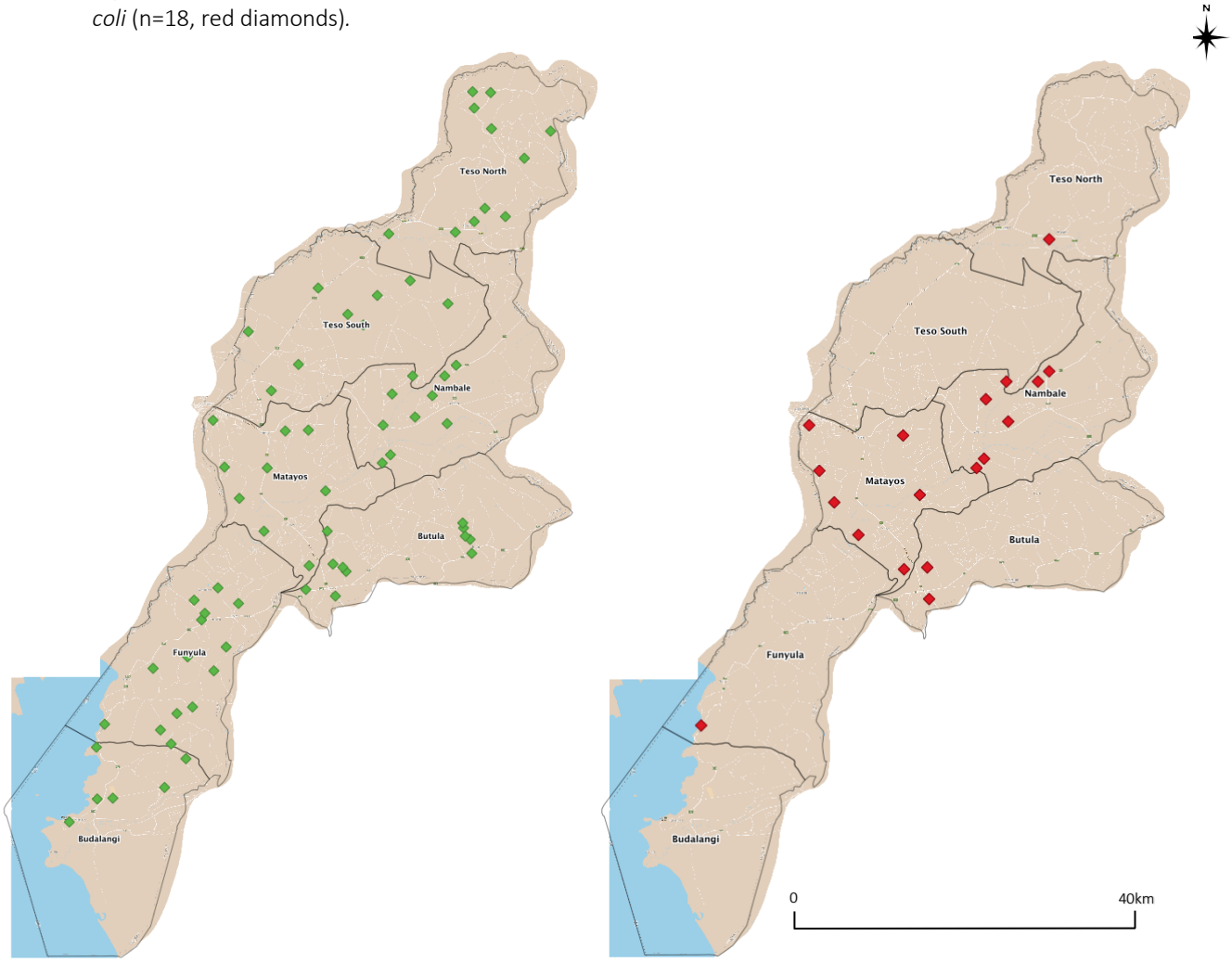
All farms contained *E. coli* with resistance to at least one antimicrobial. The locations of farms with *E. coli* containing MDR and ESBL-producing *E. coli* are shown in (**Figure 4.1**). Twenty-nine samples (4.0%) had *E. coli* which were sensitive to all tested antibiotics. MDR *E. coli* was found in at least one sample on all visited farms; 320 samples (44.1%) had *E. coli* which were resistant to 3 classes, 60 samples (8.3%) to 4 classes, 9 samples (1.2%) to 5 classes and 3 samples (0.4%) were resistant to 6 classes of antibiotic. No samples were resistant to all 7 classes of antibiotic tested. ESBL-producing *E. coli* was found across Busia county, though there were two sub-counties with a higher prevalence - Nambale (central) and Butula (south-east). Chi-squared tests showed a significant association between region and prevalence of MDR (p=0.006), and prevalence of ESBL-producing *E. coli* (p=0.01).

Table 4.1 The proportion of human, animal and environmental-origin *E. coli* with resistance to at least one antibiotic, MDR resistance and ESBL-production. Proportions are of *E. coli* were successfully cultured, i.e. 726 samples. Below each proportion are 95% CIs. Total column was adjusted for clustering. Numbers indicate prevalence and 95% CIs in brackets. Prevalence are colour coded from green (low) to red (high).

Antimicrobial Resistance	Cattle n=208	Pigs n=58	Poultry n=199	Sheep/Goats n=96	Humans n=130	Water n=15	Environment n=20	Total n=726
Ciprofloxacin	4.3% (1.6 – 7.1)	1.7% (0.0 – 5.0)	3.5% (0.9 – 6.1)	5.2% (0.8 – 9.6)	2.3% (0.0 – 4.9)	0.0% (0.0 – 20.0)	0.0% (0.0 – 20.0)	3.70% (2.2 – 6.3)
Chloramphenicol	4.3% (1.6 – 7.1)	8.6% (1.4 – 15.8)	4.5% (1.6 – 7.4)	11.5% (5.1 – 17.9)	9.2% (4.2 – 14.2)	0.0% (0.0 – 20.0)	25.0% (6.0 – 44.0)	6.8% (4.9 – 9.5)
Gentamicin	2.9% (0.6 – 5.2)	1.7% (0.0 – 5.0)	2.0% (0.1 – 4.0)	4.2% (0.2 – 8.2)	7.7% (3.1 – 12.3)	0.0% (0.0 – 20.0)	0.00% (0.0 – 20.0)	3.6% (2.5 - 5.3)
Tetracycline	88.5% (84.2 – 92.3)	93.1% (86.6 – 99.6)	82.4% (77.1 – 87.7)	93.8% (89.0 – 98.6)	93.1% (88.7 – 97.5)	100% (80.0 – 100)	100% (80.0 – 100.0)	89.2% (83.0 - 93.4)
Sulfathiazole	59.8% (53.1 – 66.5)	69.0% (57.1 – 80.9)	65.3% (58.7 – 71.9)	71.9% (62.9 – 80.9)	84.6% (78.4 – 90.8)	66.7% (42.9 – 90.6)	90.0% (76.9 – 99.9)	69.4% (60.6 – 76.9)
Ampicillin	57.9% (51.2 – 64.6)	55.2% (42.4 – 68.0)	58.3% (51.5 – 65.2)	67.7% (58.4 – 77.1)	66.2% (58.1 – 74.3)	73.3% (50.9 – 95.7)	65.0% (44.1 – 85.9)	60.9% (56.0 – 65.6)
Trimethoprim	62.7% (56.1 – 69.3)	69.0% (57.1 – 80.9)	67.8% (61.3 – 74.3)	74.0% (65.2 – 82.8)	79.2% (7.9 – 19.7)	66.7% (42.9 – 90.6)	85.0% (69.4 – 99.9)	71.0% (62.4 – 78.3)
MDR (n=371)	52.2% (45.4 – 59.0)	51.7% (38.8 – 64.6)	53.8% (46.9 – 60.7)	65.6% (56.1 – 75.1)	58.5% (50.0 – 67.0)	46.7% (21.5 – 72.0)	60.0% (38.5 – 81.5)	53.9% (48.7 – 59.1)
ESBL (n=103)	14.4% (9.6 – 19.2)	15.5% (6.2 – 24.8)	13.6% (8.8 – 18.4)	18.8% (11.0 – 26.6)	14.6% (8.5 – 20.7)	46.7% (21.5 – 72.0)	10.0% (0.0 – 23.2)	14.8% (11.1 – 19.5)

When divided according to individual sources, different patterns of resistance were observed (**Figure 4.2**). Overall, the most common resistance phenotype among *E. coli* was tetracycline-sulfathiazole-ampicillin-trimethoprim, which was found in 38.6% of all the samples. Tetracycline resistance was found in all five of the most common resistance phenotypes. Tetracycline only resistance was the most common resistance pattern in cattle. Three of the four most common resistance phenotypes were MDR.

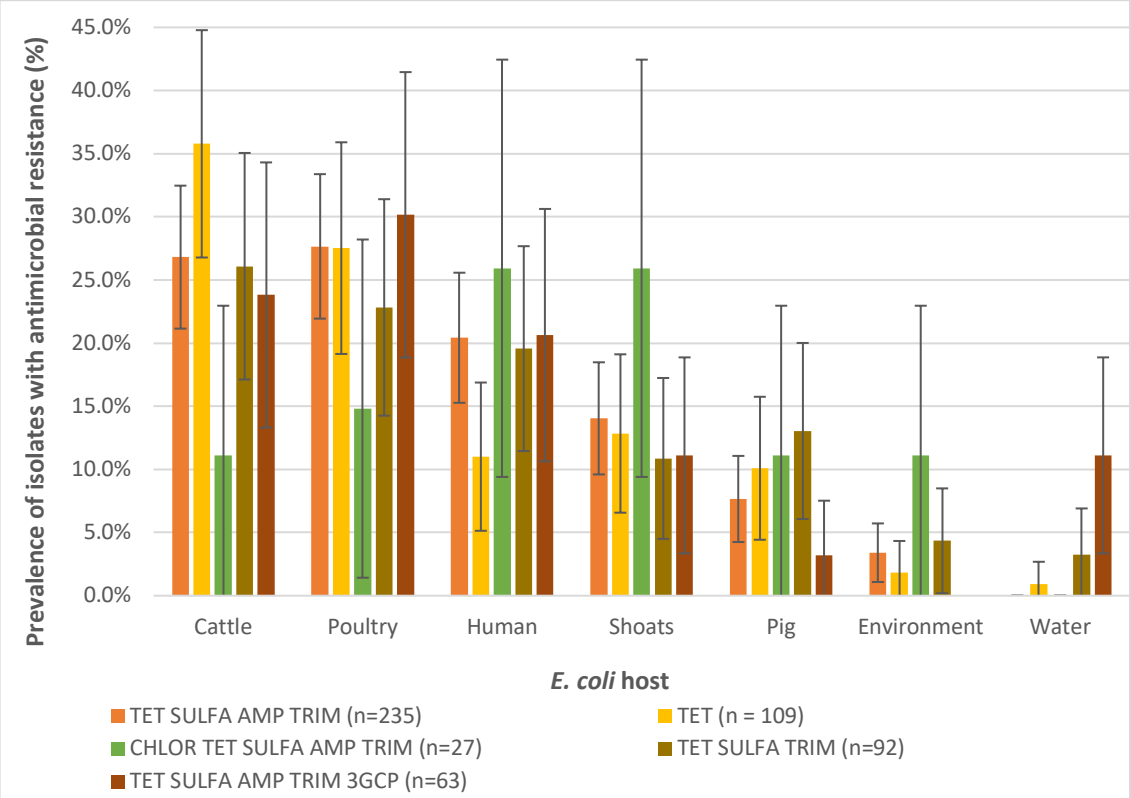
Figure 4.1 Geographical distribution of a) all farms (n=70, green diamonds) and b) farms with ESBL-producing *E. coli* (n=18, red diamonds).



4.3.3 Whole Genome Sequence Analysis

Following mapping of reads to the reference genome, a further QC step was undertaken to assess the mapping quality and depth/coverage of genomes. Of the 166 sequenced samples, 150 mapped genomes (representing 150 original samples) were considered for downstream analysis, as 16 samples were disregarded due to poor coverage across sites indicating resistance determinants (0-10x). Of those 150 genomes, 24 belonged to human-derived *E. coli* isolates, 118 from animal and 8 from the environment.

Figure 4.2 The four most commonly identified antimicrobial resistance phenotypes, divided by individual animal, human and environmental origin. The figure reflects the overall proportion of isolates displaying a specific antimicrobial resistance phenotype; 726 unique *E. coli* isolates were examined. Bars indicate 95% CIs.



TET = Tetracycline, **SULFA** = Sulfathiazole, **TRIM** = Trimethoprim, **AMP** = Ampicillin, **CHLOR** = Chloramphenicol, **3GCP** = 3rd Gen Cephalosporin. 3GCF non-specific, as determined by double-disc diffusion test.

4.3.3.1 Comparison of Resistance Gene Methods

A comparison of the most prevalent resistance genes from each database was compiled **(Table 4.2).** There were differences in the number of results returned by each method tested; ResFinder v2.2 returned 55 unique genes, ResFinder custom-database 18th October 2018 (termed version 'S') returned 58 genes, NCBI AMR database returned 73 unique genes, and the CARD database, which reports each constituent of a gene family separately (e.g. one entry for each of the four *str* fimbriae) returned over 1600 unique resistance-associated genes. However, for the most commonly identifiable and reported resistance genes in the literature, there was good similarity of fosfomycin, macrolides, phenicols, quinolones and sulfonamides resistance genes between each ResFinder iteration and the NCBI database. Where there were any differences in the frequency of genes reported by each database, the average difference between databases was 0.85% (i.e. one count of a gene).

There were some small discrepancies found between databases with regards to aminoglycosides, tetracyclines, trimethoprim and β-lactams and the MDR gene *mdf(A)*. The ResFinder vS database underreported the presence of aminoglycoside *aadA1* compared to all other databases. The NCBI AMR gene database did not record any instance of the MDR gene *mdf(A),* despite the other databases finding a high proportion of this gene. Only the NCBI AMR gene database identified the *qacEΔ1* gene, conferring resistance to ethidium bromide, antiseptics and disinfectants (Kazama et al., 1998) as an AMR gene. The prevalence of the tetracycline resistance *tet(A)* gene was reported differently by all databases, notably most frequently in the v2.2 database, but less frequently in the ResFinder vS database. In the CARD database, *tet(A)* was reported in only 12.7% of genomes, compared to an average of 68.5% in the other databases. Similarly, *tet(B)* was not reported at all in CARD, despite being reported in all other databases. The trimethoprim resistance gene *dfrA1* was also not reported in the CARD database, despite being recorded in all others. Finally, the NCBI AMR gene database recorded a family of chromosomally encoded, class C β-lactamases, which were not found in the other comparison databases.

From this comparison, there was close similarity between both iterations of ResFinder, with some minor differences in aminoglycosides and *mdf(A)* gene reporting. The NCBI database detected on average more β-lactamase genes and was the only database to contain the *qacEΔ1* resistance gene. The CARD database was the most divergent - it underreported or omitted fosfomycin, all phenicol, quinolone, tetracycline and trimethoprim genes, despite a manual check of the database showing that it contained these genes. Despite underreporting numerous major genes, the CARD database identified the most resistance determinants, though many of these included gene regulators, activators, and sensors, making it difficult to compare to ResFinder and NCBI. It is possible there was some alternative naming used in the CARD database. As such, minor discrepancies between reporting of genes between each database can alter the presented results. As much of the existing literature uses ResFinder preferentially, the work presented here is based on those databases.

Initial analysis was performed with the most up-to-date version of the ResFinder database (generated on 18th October 2018). To date (April 2020), there have been numerous updates to the ResFinder programme, which have included updates of *bla*$_{CARB}$ and *bla*$_{FRI}$ genes, updates to the fusidic acid, tetracycline, beta-lactamase and quinolone databases. These updates have not largely altered the results published in this thesis.

Table 4.2 Most commonly identified resistance genes from whole genome sequencing of 150 *E. coli* isolates from animal, human and environmental samples from 42 farms. Five different databases were used to screen sequences for resistance genes – the prevalence in the dataset, according to each of the databases is shown here. Resistance genes are shown on the same row, with the associated prevalence of that gene according to that database.

ResFinder v2.2 (Sept16)			ResFinder vS (Oct18)			NCBI AMR Genes			CARD Database		
Gene	**Ab Class**	**%**	**Gene**	**Ab Class**	**%**	**Gene**	**Ab Class**	**%**	**Gene**	**Ab Class**	**%**
aph(6)-Id	aminoglycoside	82.2	*aph(6)-Id*	aminoglycoside	79.3	*aph(6)-Id*	aminoglycoside	79.3	*aph(6)-Id*	aminoglycoside	79.3
aph(3'')-Ib	aminoglycoside	80.9	*aph(3'')-Ib*	aminoglycoside	69.3	*aph(3'')-Ib*	aminoglycoside	68.7	*aph(3'')-Ib*	aminoglycoside	69.3
aadA1	aminoglycoside	34.2	*aadA1*	aminoglycoside	14.0	*aadA1*	aminoglycoside	34.0	*aadA1*	aminoglycoside	34.0
fosA	fosfomycin	7.2	*fosA*	fosfomycin	7.3	*fosA*	fosfomycin	6.7	-	-	-
fosA7	fosfomycin	2.0	*fosA7*	fosfomycin	2.0	*fosA7*	fosfomycin	2.0	*fosA7*	fosfomycin	2.0
fosA3	fosfomycin	1.3	*fosA3*	fosfomycin	1.3	*fosA3*	fosfomycin	1.3	*fosA3*	fosfomycin	1.3
mph(A)	macrolides	6.6	*mph(A)*	macrolides	6.7	*mph(A)*	macrolides	6.7	*mph(A)*	macrolides	6.7
mdf(A)	MDR	94.7	*mdf(A)*	MDR	80.7	-	-	-	*mdf(A)*	MDR	80.7
catA1	phenicols	7.2	*catA1*	phenicols	7.3	*catA1*	phenicols	7.3	-	-	-
catA2	phenicols	2.0	*catA2*	phenicols	2.0	*catA2*	phenicols	2.0	-	-	-
cmlA1	phenicols	0.7	*cmlA1*	phenicols	1.3	*cmlA1*	phenicols	1.3	-	-	-
qnrS1	quinolones	19.1	*qnrS1*	quinolones	19.3	*qnrS1*	quinolones	19.3	*qnrS1*	quinolones	19.3
qnrB19	quinolones	2.6	*qnrB19*	quinolones	2.7	*qnrB19*	quinolones	2.7	-	-	-
oqxA	quinolones	1.3	*oqxA*	quinolones	1.3	*oqxA*	quinolones	0	*oqxA*	quinolones	1.3
-	-	-	-	-	-	*qacEΔ1*	ethidium bromide	44.7	-	-	-
sul2	sulfonamides	82.2	*sul2*	sulfonamides	82.7	*sul2*	sulfonamides	82.7	*sul2*	sulfonamides	82.7
sul1	sulfonamides	41.4	*sul1*	sulfonamides	41.3	*sul1*	sulfonamides	40.0	*sul1*	sulfonamides	40.0
sul3	sulfonamides	3.3	*sul3*	sulfonamides	3.3	*sul3*	sulfonamides	3.3	*sul3*	sulfonamides	3.3
tet(A)	tetracycline	71.1	*tet(A)*	tetracyclines	66	*tet(A)*	tetracyclines	66.0	*tet(A)*	tetracyclines	12.7
tet(B)	tetracycline	13.2	*tet(B)*	tetracyclines	12.7	*tet(B)*	tetracyclines	12.7	-	-	-
tet(D)	tetracycline	2.0	*tet(D)*	tetracyclines	2.0	*tet(D)*	tetracyclines	2.0	*tet(D)*	tetracyclines	10.0
dfrA14	trimethoprim	44.1	*dfrA14*	trimethoprim	44.7	*dfrA14*	trimethoprim	44.7	*dfrA14*	trimethoprim	44.7
dfrA1	trimethoprim	33.6	*dfrA10*	trimethoprim	29.3	*dfrA1*	trimethoprim	34.0	-	-	-
dfrA7	trimethoprim	11.8	*dfrA7*	trimethoprim	12.7	*dfrA7*	trimethoprim	12.0	*dfrA7*	trimethoprim	12.0
$bla_{\text{TEM-1B}}$	β-lactams	67.8	$bla_{\text{TEM-1B}}$	β-lactams	68.7	$bla_{\text{TEM-1B}}$	β-lactams	68.7	$bla_{\text{TEM-1B}}$	β-lactams	70.7
$bla_{\text{CTX-M-15}}$	ESBL	21.7	$bla_{\text{CTX-M-15}}$	ESBL	22.0	$bla_{\text{CTX-M-15}}$	ESBL	22.0	$bla_{\text{CTX-M-15}}$	ESBL	22.0
$bla_{\text{ACT-4}}$	β-lactams	2.0	$bla_{\text{ACT-4}}$	β-lactams	1.3	$bla_{\text{ACT-4}}$	β-lactams	1.3	$bla_{\text{ACT-4}}$	β-lactams	1.3
-	-	-	-	-	-	$bla_{\text{EC-18}}$	β-lactams	41.3	-	-	-

4.3.3.2 Genetic Background of Isolates

To determine the genetic background of the *E. coli*, all 150 isolates were assigned a Multilocus Sequence Type (MLST) and Clermont phylogroup. Molecular serotyping was also performed. A comprehensive list of all isolates and the data discussed in the following sections can be found in **[Appendix II, Table II-iv]**.

A maximum-likelihood phylogenetic tree (constructed with 832038 SNPs) (**Figure 4.3**) of all isolates shows the distribution of human, animal, and environmental *E. coli*. There were no apparent clustering by species or location for most of the sampled animal species, human and environmental *E. coli*, suggesting a high diversity of *E. coli* across the entire county. Of note were two clades which indicated an extremely high degree of genetic similarity, suggesting two separate clonal groups, belonging to ST2852 and ST196. This is discussed further in section **4.3.3.6**.

The results of MLST assignment indicated that the 150 human, animal and environmental genomes belonged to 44 different STs. 25 STs were represented by a single sequence only, and 49 genomes could not be assigned a ST as they carried at least one novel allele, not included in the database. The most commonly identified STs included ST196 (8.9%, n=13), ST2852 (6.8%, n=10), ST10 (4.8%, n=7) and ST155 (3.4%, n=5). The distribution of these STs between groups can be seen below in (**Figure 4.4**). 32 STs were only found in livestock and 7 STs were only found in humans. There was only one ST common to all three sample groups (humans, livestock, and environment): ST196, the other most common STs were only found in individual groups (not all three). Of note, two clades of extremely low genetic variation (fewer than 300SNPs) discovered during phylogenetic tree construction belonged to two STs – the first low genetic variation group (comprised of animal and environmental isolates only) belonged to ST2852; the second low genetic variation group (comprised of human, animal and environmental isolates) belonged to ST196. Two further small clades were identified with the same degree of low genetic varitions, and these each had three genomes in their clades. These belonged to ST46 and ST1421.

All sequences were phylotyped *in silico*, according to Clermont's phylogrouping (Beghain et al., 2018; Clermont et al., 2019). Several isolates could not be phylotyped *in silico* and were reported as 'unknown' (**Figure 4.5**). Phylogroup B1 was the overall most common in livestock (49.2%,

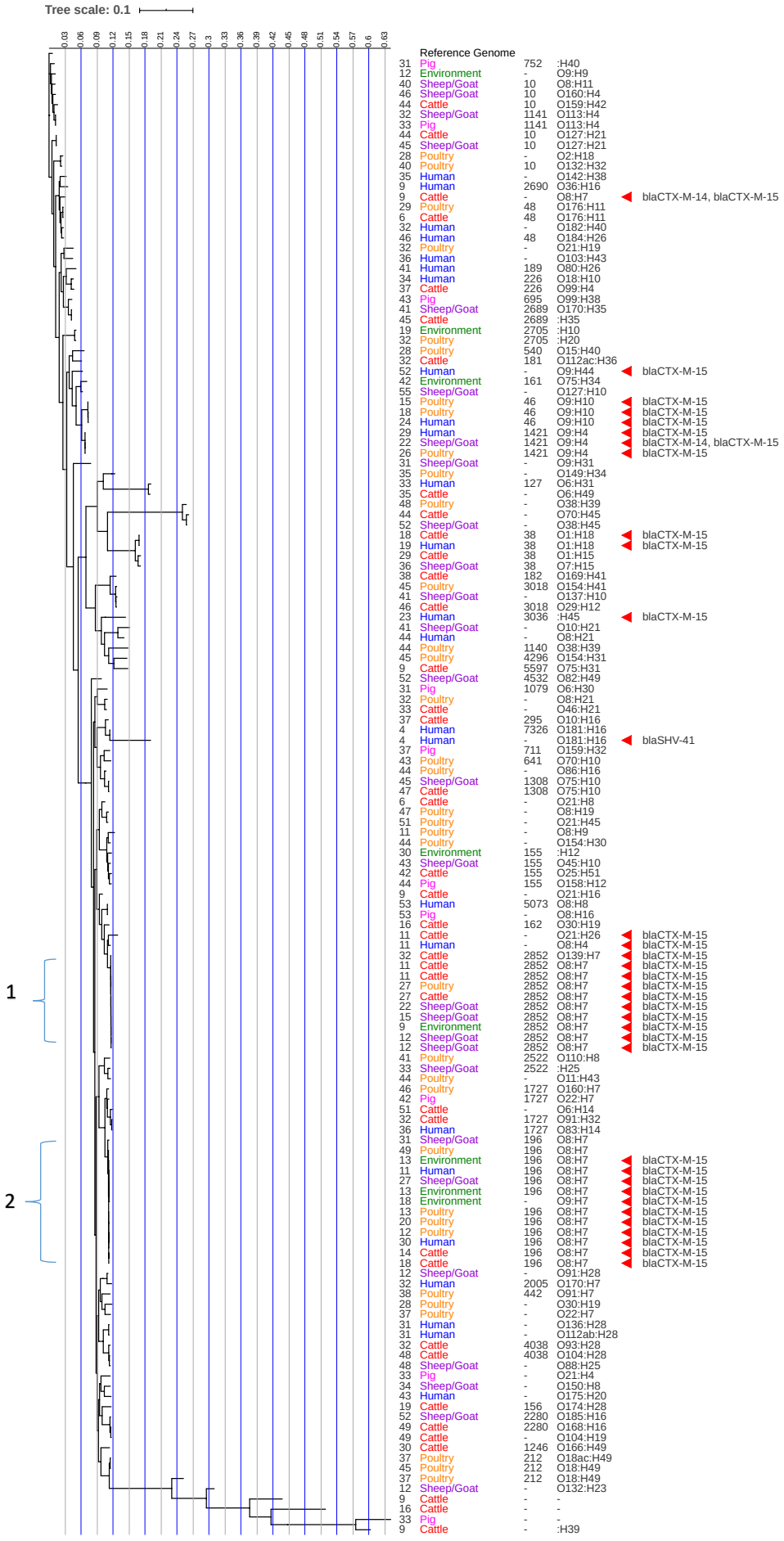


Figure 4.3 Maximum likelihood phylogenetic tree (using 832038 SNPs) of *E. coli* (n=150) isolated from humans, animals and the environment. From left to right, text to the right of the tree indicates: farm number; genome host; MLST; serotype. Red triangle indicates ESBL-producing *E. coli*. ESBL genes are to the right of each triangle. Tree scale is indicated by the coloured grid. Two clonal clades are highlighted parentheses and the numbers 1 and 2. An extended diagram showing bootstrap values can be found in (Appendix II, Figure II-iii).

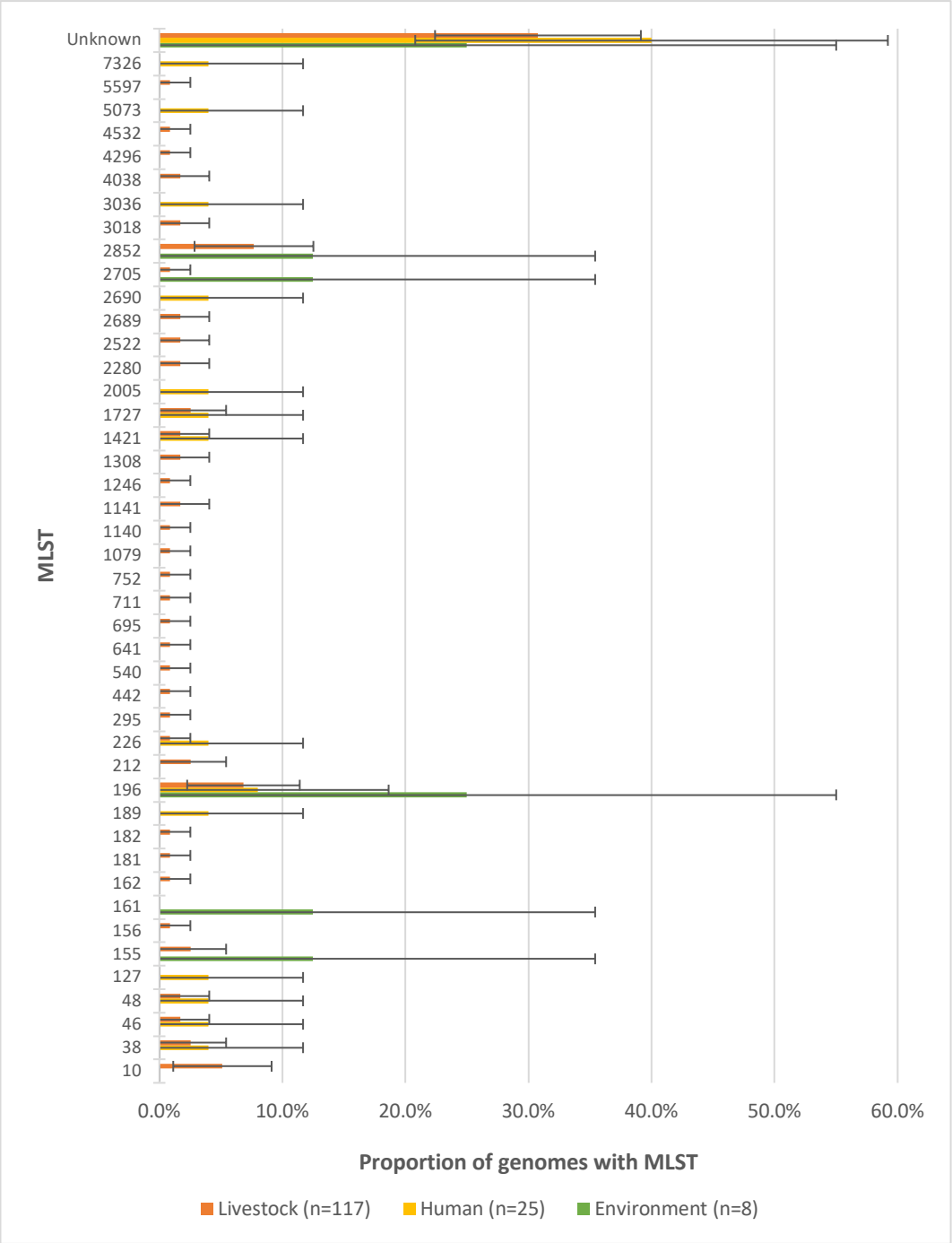


Figure 4.4. Distribution of multilocus sequence types by host group. Error bars are 95% CI.

n=61), human (44.0%, n=9) and environmental (75.0%, n=5) isolates. There was some variation in the detected phylogroups; environmental isolates consisted of phylogroups B1 and A (with one unknown). Human isolates consisted primarily of B1, with a secondary majority of A (32.0%, n=8), one (4.0%, n=1) (each) of phylogroups B2, C, D and E (and 16.7%, n=4) and unknown. Isolates from livestock also were primarily phylogroup B1 (49.2%, n=58) and A (16.9%, n=20). Livestock isolates also had 5.9% (n=6) phylogroup D and 9.3% (n=10) phylogroup E. Surprisingly,

one livestock isolate was also classified as the newly discovered phylogroup G (1.7%, n=1) which is associated with high virulence and AMR potential (Clermont et al., 2019). Surprisingly, this isolate only (F41G4A) only carried two AMR genes and 5 virulence factors. This was rechecked using a lower threshold for % identity (≥75%, instead of ≥90%) which revealed that this isolate actually carried 10 resistance genes (potentially across multiple reads, hence the lower identity).

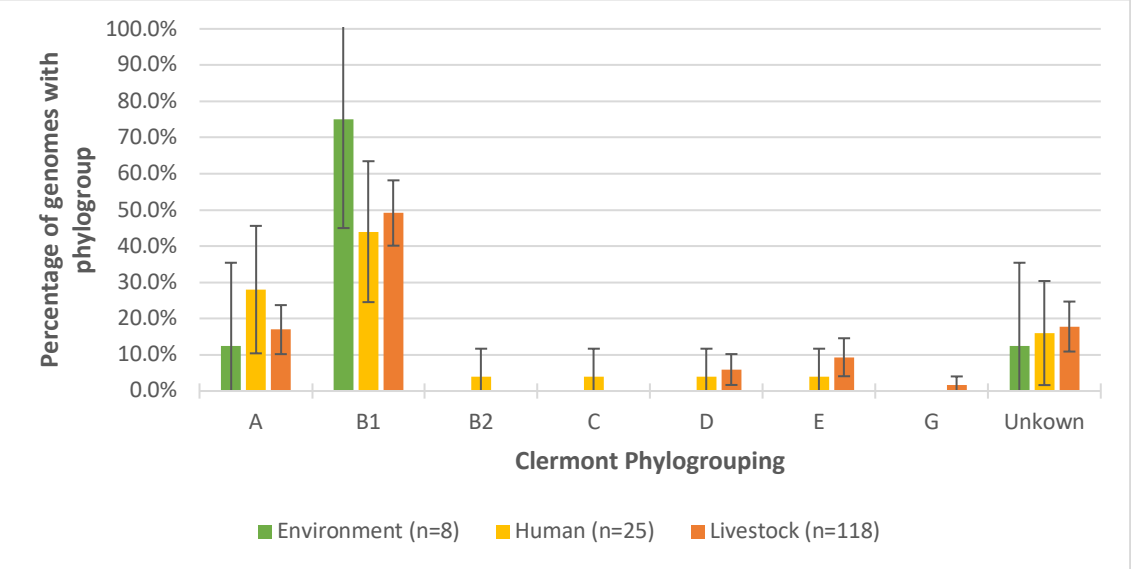


Figure 4.5 Distribution of phylogroups of *E. coli* isolated from humans, livestock and the environment on 70 different farms in western Kenya. Error bars are 95% CI.

All isolates were serotyped *in silico* to assign lipopolysaccharide (O) and flagellar (H) surface antigen groups (Joensen et al., 2015). Of the 150 samples, 3 isolates were not assigned either an O or H grouping, and 8 isolates were only assigned an H grouping. There were 107 uniquely characterised O:H groupings across all three groups of humans, animals, and environmental isolates. The most prevalent O antigens were O8 (21.7%), O9 (7.2%) and O21 (3.6%). The most prevalent H antigens were H7 (20.5%), H10 (8.2%) and H4 (6.2%). The most prevalent combination serotype was O8:H7 (14.6%, n=22). All O8:H7 isolates were all MDR, carrying combinations of β-lactamase, fluoroquinolone, sulfonamide, tetracycline, aminoglycoside, and trimethoprim resistance genes. All isolates in the two clades (marked 1 and 2) (**Figure 4.3**) belonging to ST196 and ST2852, were also serotype O8:H7.

4.3.3.3 Resistance Genes

Using the most up to date ResFinder database (compiled in October 2018), all detected resistance genes were tabulated, and the prevalence of each gene determined according to group (human, individual animal species and environment). The most commonly identified

resistance genes which covered two or more of the host groups were plotted on (**Figure 4.6**) and compared.

A total of 60 unique resistance genes were detected in 149 of the sequenced genomes. All genomes carried more than one resistance gene. The most commonly identified resistance genes overall included to aminoglycosides, the bla_{TEM} beta-lactamase (ampicillin), multi-drug transporters (macrolides-lincosamides-streptogramin), sulfonamides and tetracyclines. There was no statistically significant difference ($p>0.05$, Kruskal Wallis) in the carriage of 12 of the 18 most prevalent resistance gene between host groups i.e. genes which were carried by each of the four animal species, environment, and humans. There were some genes which were absent or differed signifincatly in their carriage, and these are highlighted in the text below.

There were two common β-lactamase resistance genes carried amongst the sequenced isolates. The more common of the two was $bla_{TEM\text{-}1B.}$ This is extremely common in Gram-negative bacteria and confers up to 90% of reported ampicillin resistance in *E. coli*; the gene was highly prevalent in all 6 groups. The other commonly carried resistance gene was $bla_{CTX\text{-}M\text{-}15}$, an important extended-spectrum β-lactamase, associated with multi-drug-resistance. This gene was carried by all animal species except for pigs, as well as in humans and environmental sources.

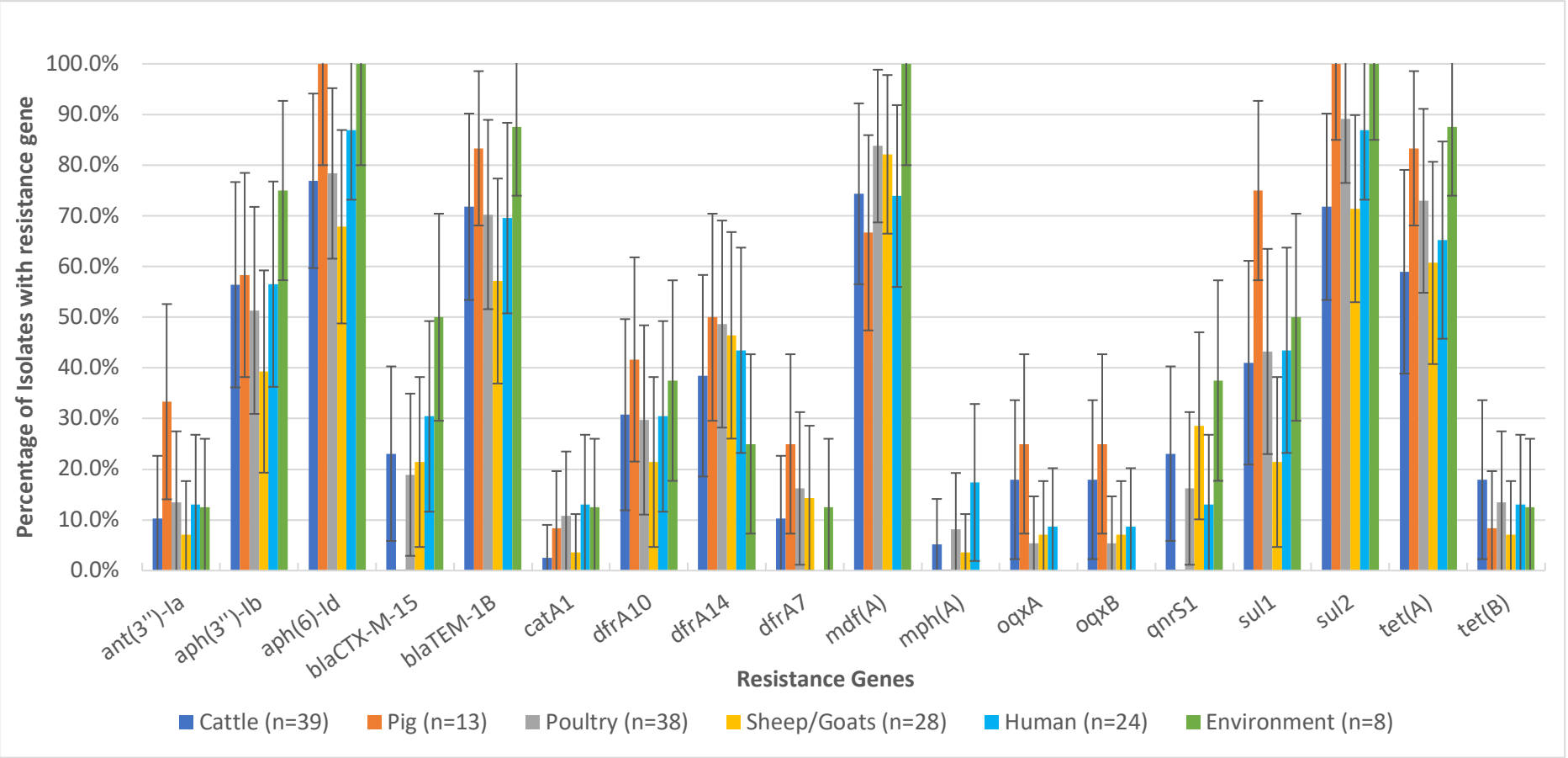
Relatively few isolates were found to carry a chloramphenicol resistance gene.

Three trimethoprim resistance genes were prevalent amongst all isolates – *dfrA7, dfrA10* and *dfrA14*. The dfrA7 resistance gene was carried by isolates in all groups, except for humans. The other two genes were carried by isolates in all groups. The *dfreA14* gene was the most prevalent, followed by *dfrA10* and *dfrA7.*

The multi-drug efflux gene, *mdf(A)* was isolated in a large proportion of all human, animal, and environmental-origin *E. coli.* The pattern observed is similar to the tetracycline resistance genes, *tet(a)* and *tet(b),* as the *mdf(A)* gene is part of the same major facilitator superfamily (MFS). The higher prevalence of *tet(A)* suggests that this is the dominant tetracycline resistance gene, conferring much of the tetracycline resistance in all groups.

There was a low prevalence of resistance genes for quinolones and fluoroquinolones. The three most prevalent resistance genes are all plasmid-mediated resistance genes which provide low-

Figure 4.6 The proportion of isolates carrying a selection of the 18 most common resistance genes, divided according to source/species. Error bars indicate 95%CI. Resistance genes with less than 5% prevalence or found in single groups/species only, were excluded from this figure.



Resistance genes: aminoglycoside: ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id; **ESBL**: *bla*$_{CTX-M-15}$; **non-ESBL**: *bla*$_{TEM-1B}$; **phenicol**: catA1; **trimethoprim**: dfrA10, dfrA14, dfrA7; **multi-drug exporter**: mdf(A); **macrolide**: mph(A); **fluoroquinolone**: oqxA, oqxB, qnrS1; **sulfonamide**: sul1, sul2; **tetracycline**: tet(A), tet(B).

level resistance but may integrate with the host chromosome which results in mutations than can confer higher-level resistance. The two resistance genes *oqxA* and *oqxB* are part of the *oqxAB* gene complex, which encodes for the OqxAB pump; this confers low-level resistance to ciprofloxacin and cross-resistance to trimethoprim.

Neither of these resistance genes were found in any environmental isolates but were found in low numbers in both human and animal bacteria. The *qnrS1* gene is also plasmid-mediated and confers low-level resistance to quinolones such as nalidixic acid. No *qnrS1* was carried by pig isolates, but this gene was more prevalent than *oqxAB* in all groups.

There was a high prevalence of two sulfonamide resistance genes in all groups. In poultry, sheep/goats, humans and the environment, significantly more *sul2* (Mann-Whitney U, $p<0.01$) was carried compared with *sul1*, and there was only a single (pig) isolate which was found to carry the *sul3* gene (not shown on graph). In all groups, the patterns of *sul* resistance gene prevalence in the respective groups was identical, albeit *sul2* having a higher prevalence.

Finally, the *mph(A)* gene which inactivates macrolides (preferentially inactivating erythromycin, telithromycin and roxithromycin) was found in a low proportion of isolates. No *mph(A)* was carried by pig or environmental isolates; the largest proportion was carried by human isolates.

As the ResFinder database only contains a curated list of acquired AMR genes, a search for point mutations was made using the PointFinder tool (Zankari et al., 2017). In total, only 11 genomes were found to have point mutations which conferred resistance to one or more of nalidixic acid, ciprofloxacin and streptomycin. Eight of the point mutations were found in genomes of *E. coli* from livestock and three were from humans. No point mutations were found in environmental *E. coli* genomes. In all three humans, a mutation in *gyrA* (all S83L) was present, in one human there was also two *parC* mutations (S57T and S80I). In livestock, two genomes had point mutations *parC* gene and four had mutations in the *parE* gene (I355T), and three genomes also had *gyrA* mutations (S83L and D87N). Additionally (not seen in human isolates), there was a single livestock genome which had a mutation in the 16S ribosomal RNA gene (rrnaB) which conferred resistance to streptomycin.

4.3.3.4 Virulence Genes

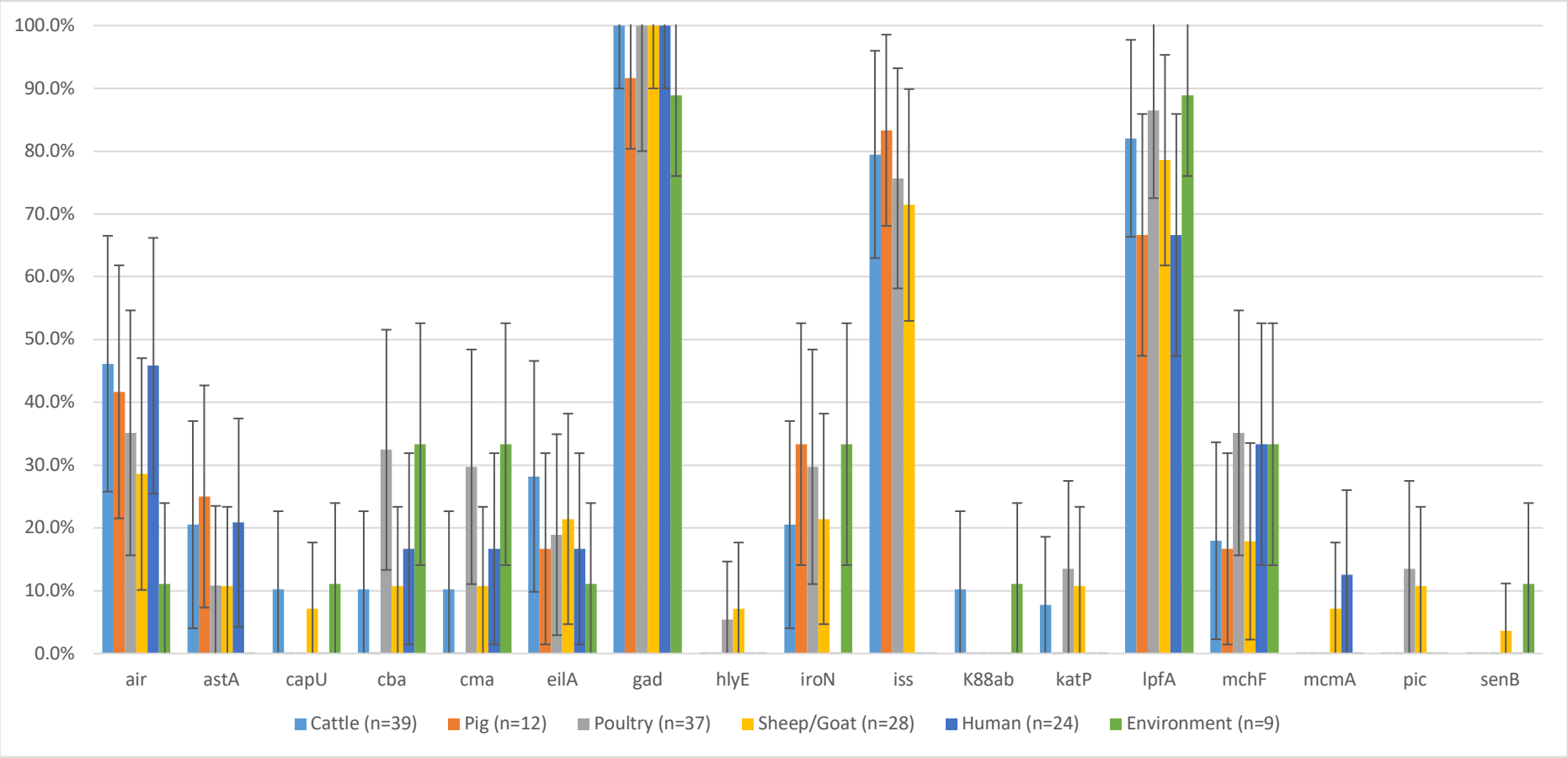
A total of 49 unique virulence genes were identified in all sequenced genomes. Four isolates carried no virulence genes. A brief description of the function of the most common virulence genes is described in (**Table 4.3)**. As with the resistance genes, the most commonly identified virulence genes which spanned at least two groups were assessed for comparison of prevalence (**Figure 4.7**) using the Kruskal Wallis test.

Table 4.3 Brief description of the function of the most commonly carried virulence genes from a population of 145 animal, human and environment-derived *E. coli* isolates. Original references documented in (Table 1.1).

Virulence Gene	Role	Function
gad	Glutamate decarboxylase	Converts glutamate to GABA; maintains intracellular pH when cells when traversing stomach acid – aids in colonisation.
iss	Protectin	Associated with exPEC strains; increased serum survival associated with serum resistance.
lpfA	Adhesin	Encodes for chaperone-usher fimbriae used in adhering to gut wall.
mchF	Antibacterial Peptide	Produces bacteriocin peptide, microcin. Compete with enteric pathogens by mimicking siderophores
iroN	Siderophore	Scavenges iron from mineral phases of soluble iron complexes for growth and maintenance.
cma	Toxin	Toxin active against various Enterobacteriaceae – causes lysis of cells and murein degradation.
astA	Toxin	Produces EAST1 toxin resulting in diarrhoea in host organisms.
eilA	Regulator	Transcription regulator of pathogenicity island SPI1. Activates expression of invasin genes.

The most commonly identified virulence genes overall included *gad*, *iss*, *lpfA*, *air* and *mchF*. In addition to these five, only one other virulence gene was carried by all groups of isolates – *eilA*. Most other virulence genes identified in the genomes were limited to two or three groups of isolates, with no apparent pattern – e.g. *pic* and *hylE* were only found at low prevalence in poultry and shoat isolates, whilst *K88ab* was only found in human and environmental isolates. Pig isolates seemed to have the lowest virulence gene diversity compared to other groups.

Figure 4.7 The most prevalent virulence genes carried by *E. coli* from all groups and species. Error bars indicate 95%CI. There was no significant difference between the carriage of virulence genes between any source. The 20 most prevalent virulence genes were tabulated and those which covered at least two groups or species were plotted in this figure. A short explanation of virulence genes can be seen in (**Table 1.1** and **Table 4.3**).



There were some common virulence gene patterns amongst the genomes – the most frequently occurring combinations of genes included *gad-iss-IpfA* (23.5%, n=35), *gad-IpfA* (12.1%, n=18) and *cma-gad-iroN-iss-IpfA-mchF* (10.7%, n=16).

Overall, three isolates carried more than ten virulence genes: two human isolates: F4H2A (21 genes), F33H2A (17 genes) and one cattle isolate: F35C3E (18 genes); these isolates carried the largest number of virulence genes and were each MDR, however, there was no discernible pattern between the number of resistance and number of virulence genes carried. Despite carrying the highest number of virulence genes, these isolates carried ten, four and four resistance genes respectively; many other isolates carrying fewer virulence genes (e.g. 2) carried up to and including 5 or more resistance genes.

4.3.3.5 Plasmids

Mobile genetic elements such as plasmids are known to carry both resistance and virulence genes. These plasmids can disseminate through *E. coli* populations by horizontal transfer, conferring AMR to previously antimicrobial sensitive bacteria. Considering the high prevalence of MDR *E. coli* amongst these isolates (**Chapter 4**), this could be due to the co-transfer of multiple AMR genes on plasmids.

14 different plasmid replicon types were detected in all three groups (livestock, humans and environment, **Figure 4.8**). When combined by replicon type, the most prevalent plasmid replicons were IncFIB, followed by IncFII and then IncQ; these three types collectively accounted for approximately 66% of the plasmid replicon types detected across all genomes. All the other replicon types were found at a low prevalence in human and environmental isolates. In animal isolates, replicon type IncY was found in 11.3% (n=33) of isolates, but the remainder of replicon types were also found at low prevalence.

Of note was the p0111 plasmid replicon, which was found in the genomes of all three groups. Humans carried significantly more of this plasmid ($p<0.01$, Mann-Whitney U test), compared to livestock and the environment. There was only one other significant difference in the carriage of plasmids between groups: humans also carried significantly more ($p<0.01$) IncFII (pRSB107) than livestock. There was no other significant difference in the carriage of plasmids in instances where one plasmid was found in all three groups.

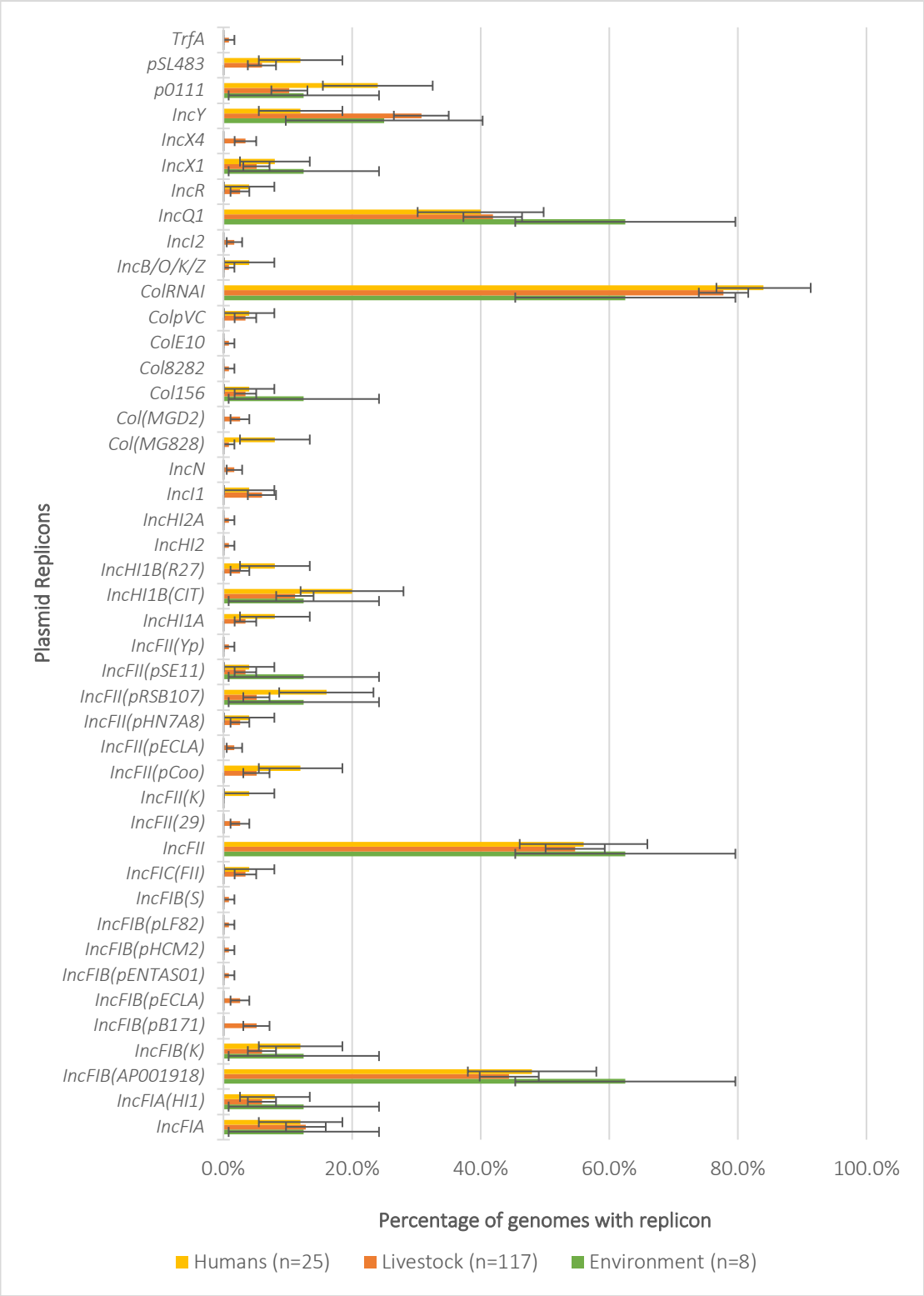


Figure 4.8 Distribution of different plasmid replicon types according to host. Bars are 95% CI.

Analysis of the distribution of AMR genes carried in relation to plasmid replicons types showed that most resistance genes co-occurred with ColRNAI, IncFII, IncFIB, IncQ1 and p0111 plasmids. IncFII and IncFIB replicons were also often co-carried, suggesting that both replicons may have

been be carried on a single plasmid. There was a significant association between the presence of ColRNAI and p0111 plasmids, and carriage of mdf(A), sul2, tet(A), aph(3'')-Ib, aph(6)-Id and dfrA1 (χ^2 = 8.5, p0.002, Chi-squared test).

To further imply co-carriage of multiple AMR genes, several isolates carrying the same plasmid replicon profiles **(Table 4.4)** showed similarities in the virulence and resistance genes carried by the bacterium. For isolates found on the same farm **(Table 4.5)**, there was a higher likelihood of observing similarities between resistance and virulence genes, then there was on isolates between farms.

Table 4.4 Corresponding virulence and resistance genes carried by three groups of isolates with the same plasmid replicon profiles.

Isolate	Species	Resistance Genes	Virulence	Plasmids
F41G3E	Sheep/Goat	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA1 dfrA14 mdf(A) qnrS1 sul1 sul2 sul3 tet(A)*	*capU iss lpfA*	IncFII IncFIB
F37P3B	Poultry	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*gad lpfA*	IncFII IncFIB
F41P1E	Poultry	*fosA7 mdf(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB
F32P1A	Poultry	*aadA1 aph(3'')-Ib aph(6)-Id dfrA1 dfrA14 mdf(A) sul2 tet(A)*	*gad lpfA*	IncHI1B
F32C1A	Cattle	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA7 mdf(A) sul1 sul2 tet(A)*	*gad lpfA*	IncHI1B
F28P2A	Poultry	*aph(3'')-Ib aph(6)-Id dfrA14 mdf(A) sul2*	*gad iss lpfA*	IncHI1B
F38C3A	Cattle	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA7 mdf(A) sul1 sul2*	*air eilA f17A f17G gad iss*	IncI1
F32C5A	Cattle	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA7 mdf(A) sul1 sul2 tet(A)*	*gad iss lpfA*	IncI1
F32P3A	Poultry	*aph(3'')-Ib aph(6)-Id dfrA14 mdf(A) sul2 tet(A)*	*gad iss lpfA*	IncI1

Table 4.5 Two examples of virulence and resistance genes carried by *E. coli* isolates from humans and animals and the environment, on the same farm ((F)arm 11 and (F)arm 12).

Isolate	Host	Resistance Genes	Virulence Genes	Plasmids
F11C4E	Cattle	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 dfrA14 mdf(A) qnrS1 sul1 sul2 tet(A) tet(B)*	*air celb cma eilA gad iha iroN iss lpfA mchF*	IncFIA IncFIB IncFII IncFIB IncY IncB/O/K/Z
F11P3E	Poultry	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA8 mdf(A) sul2 tet(A) tet(B)*	*air astA eilA gad ireA iroN iss lpfA mchF*	IncFII IncFIB
F11H3CZ	Human	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F11H3A	Human	*aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *blaOXY-2-1 bla*$_{TEM-1B}$ *dfrA14 fosA7 mdf(A) qnrS1 sul1 sul2 tet(A)*	*astA capU eilA gad iss K88ab lpfA*	IncFII IncI1 IncFIB IncFII IncX1 IncY
F11C4CZ	Cattle	*aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY

Isolate	Host	Resistance Genes	Virulence Genes	Plasmids
F11C3CZ	Cattle	*aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F12S2C	Sheep/Goat	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA1 dfrA14 dfrA7 mdf(A) sul1 sul2 tet(A) tet(B)*	*air astA capU eilA iss lpfA nfaE*	IncFIB IncFII
F12HOUSEA	Environment	*aph(3'')-Ib aph(6)-Id bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul2 tet(B)*	*capU gad iss lpfA nfaE*	IncFII IncFIB
F12S2B	Sheep/Goat	*aph(3'')-Ib aph(6)-Id blaACT-4 dfrA14 fosA mdf(A) qnrE1 sul2*	*gad iss lpfA*	IncFII IncFIB IncFII IncFIB IncFIA IncFIB
F12P3CZ	Poultry	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB
F12S1CX	Sheep/Goat	*aph(3'')-Ib aph(6)-Id bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY

4.3.3.6 Extended-Spectrum Beta Lactamases

ESBLs are usually associated with MDR bacteria and as such their proliferation within communities (and hospitals) is as an important threat to global health. To highlight the potential clonal nature of specific clusters of isolates carrying AMR genes conferring the ESBL phenotype, a separate phylogenetic tree was constructed **(Figure 4.9)** and isolates were tabulated to assess their resistance profiles **(Table 4.6)**. Due to funding limitations, only a subset of isolates could be sequenced, thus, a number of additional analyses of non-sequenced ESBL isolates were undertaken by PCR (section **4.3.3.7**).

Phylogenetic analysis indicates four distinct clades (marked 1-4 on **Figure 4.9**) which are very closely related (based on 238092 SNP with an average branch length of <0.0007, this suggest that they are fewer than 167 SNPs different). Interestingly, one isolate from clade one (F18HouseCX) and two (F32C3A) do not share the same serotype, despite having identical MLST and plasmids carried.

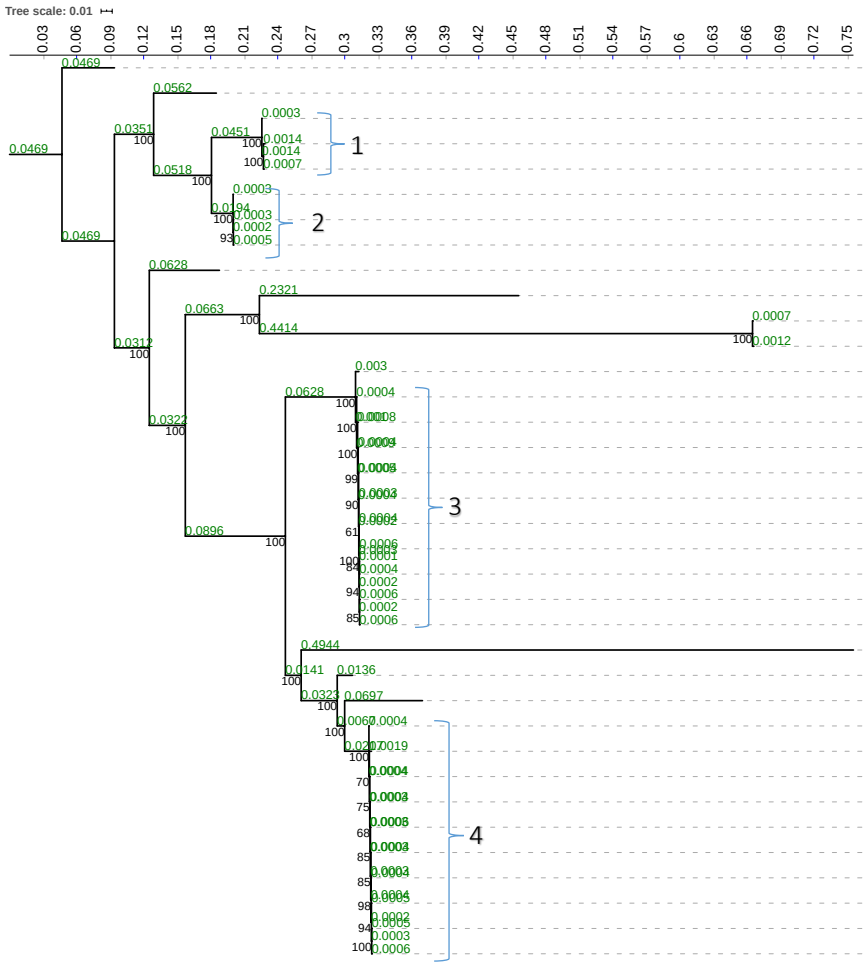
Clade two primarily consisted of livestock-associated *E. coli* genomes and a single environmental genome. Clade one was much more diverse, with ESBL-producing *E. coli* being isolated from all three host groups (human, livestock and environment), on multiple farms across multiple sub-counties. Whilst *bla*$_{CTX-M-15}$ was the major ESBL gene carried within all of these genomes, there was also detection of a two *bla*$_{CTX-M-14}$ (one human isolate and one cattle, which were co-carried alongside *bla*$_{CTX-M-15}$) and a single *bla*$_{SHV-41}$ (isolate from a sheep/goat *E. coli*) from separate farms.

Whilst not an ESBL, of note was that none of the genomes was found to carry *bla*$_{CMY}$ AmpC β-lactamase resistance gene, but a single isolate carried a *bla*$_{DHA-1}$.

Alongside genes conferring the ESBL phenotype, mobile genetic elements typically carry aminoglycoside and quinolone resistance genes also. Interestingly, a number of isolates carrying *bla*$_{CTX\text{-}m\text{-}15}$ in conjunction with an IncY plasmid (13 isolates) carried quinolone resistance genes (QnrS/B) but did not carry any aminoglycoside resistance genes. Those isolates carrying *bla*$_{CTX\text{-}M\text{-}15}$ in association with an IncFII plasmid (16 isolates) almost always (14 isolates) carried an aminoglycoside resistance gene (*aadA1*), but only 5 of those isolates also carried any quinolone resistance genes. Also interesting was the number and diversity of virulence genes carried by ESBL isolates – those with the IncY replicon type carried 3 or fewer virulence genes, whilst those with the IncFII replicon type always carried 5 or more. Several unschemed plasmid replicons were also detected, including IncX and IncQ. One ESBL isolate was not found to carry any plasmids, despite being MDR, this was rechecked using a lower threshold for identification of plasmids (of ≥75%). After using a lower threshold for detection, plasmids were discovered. Irrespective of the plasmid replicon type, all but a single isolate carrying an ESBL gene were MDR. The majority of those MDR phenotypes were similar and reflected the overall most common resistance phenotype (TET-SULFA-AMP-TRIM) and the most common 3GC phenotype (TET-SULFA-AMP-TRIM-3GC).

Twenty of the O8:H7 isolates also carried the *bla*$_{CTX\text{-}M\text{-}15}$ gene. The majority of these were isolated from animal-origin *E. coli.* There was a significant association (p=0.003, Chi-squared test) with carriage of both *bla*$_{CTX\text{-}M\text{-}15}$ and serotype O8:H7 - 13/20 isolates with this combination of genes were found in the same sub-county. The most commonly associated STs associated with ESBL-producing *E. coli* were ST196 and ST2852. When examine alongside the phylogenetic tree, due to the high degree of similarity in carriage of plasmids, resistance genes, and low genetic diversity between each genome, there is strong evidence to suggest that the dissemination of these four groups of *E. coli* carrying *bla*$_{CTX\text{-}M\text{-}15}$ may be clonal. Each of the suggested clonal groups were isolated predominantly from Nambale and Busia sub-counties **(Figure 4.9).** Besides ST196 and ST2852, there were 4 other STs (ST36, ST1421, ST3036 and ST38) associated with carriage of *bla*$_{CTX\text{-}M\text{-}15}$, though these were proportionately less representative of this population sample. There were some differences in the resistance genes carried by isolates within each ST, though the virulence genes in both groups were identical. One isolate carrying *bla*$_{SHV\text{-}41}$ also had an unknown ST but carried many resistance and virulence genes.

Figure 4.9 Maximum likelihood tree (1000 bootstraps (black text)) of genomes carrying AMR genes *bla*$_{SHV-41}$, *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$, which confer the ESBL genotype (n=35). Based on 238092 SNPs compared to reference strain *E. coli* K12 MG1655. All *E. coli* was isolated on MHA containing ceftazidime (CZ) or ceftriaxone (CX). Four distinct genetically non-diverse clades are indicated with bracketed numbers 1-4. These all have branch lengths of lengths of <0.002 (green text). Root is the reference strain (U00096.3).



Sample ID	Sub-county	Genome Host	ST	Serotype	Plasmid Replicons
U00096.3					
F52H3C	Teso North	Human	-	O9:H44	IncX1
F15P5CX	Nambale	Livestock	46	O9:H10	IncFIA IncFIB IncFII IncN IncQ1
F18P2CX	Nambale	Livestock	46	O9:H10	IncFIA IncFIB IncFII IncN IncQ1
F24H2CX	Busia	Human	46	O9:H10	IncFIA IncFIB IncFII IncN IncQ1
F29H2CZ	Busia	Human	1421	O9:H4	-
F22G1CZ	Busia	Livestock	1421	O9:H4	IncHI2A IncHI2
F26P2CZ	Busia	Livestock	1421	O9:H4	-
F9C4CX	Bumala	Livestock	-	O8:H7	IncY
F23H3CZ	Busia	Human	3036	:H45	IncFII IncY
F18C6CX	Nambale	Livestock	38	O1:H18	IncY
F19H3CX	Nambale	Human	38	O1:H18	IncY
F18HOUSECX	Nambale	Environment	196	O9:H7	IncFII IncFIB IncQ1
F13HOUSECZ	Nambale	Environment	196	O8:H7	IncFII IncFIB IncQ1
F27G2CX	Busia	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F11H3CZ	Bumala	Human	196	O8:H7	IncFII IncFIB IncQ1
F13P3CX	Nambale	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F13WATERCZ	Nambale	Environment	196	O8:H7	IncFII IncFIB IncQ1
F20P5CZ	Nambale	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F12P3CZ	Nambale	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F30H2CZ	Busia	Human	196	O8:H7	IncFII IncFIB IncQ1
F14C3CZ	Nambale	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F18C1CX	Nambale	Livestock	196	O8:H7	IncFII IncFIB IncQ1
F4H2C	Bumala	Human	-	O181:H16	IncFII IncFIB IncR
F11H3A	Bumala	Human	-	O8:H4	IncI1 IncFIB IncFII IncX1 IncY
F11C4E	Bumala	Livestock	-	O21:H26	IncFIA IncFIB IncFII IncY
F11C3CZ	Bumala	Livestock	2852	O8:H7	IncY
F32C3A	Budalangi	Livestock	2852	O139:H7	IncY
F27P7CX	Busia	Livestock	2852	O8:H7	IncY
F11C4CZ	Bumala	Livestock	2852	O8:H7	IncY
F27C1CX	Busia	Livestock	2852	O8:H7	IncY
F22G1CX	Busia	Livestock	2852	O8:H7	IncY
F15S2CX	Nambale	Livestock	2852	O8:H7	IncY
F9WATER	Bumala	Environment	2852	O8:H7	IncY
F12G2CX	Nambale	Livestock	2852	O8:H7	IncY
F12S1CX	Nambale	Livestock	2852	O8:H7	IncY

Table 4.6 Details of WGS-only *E. coli* isolates carrying the *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15 \text{ and }}$ *bla*$_{SHV-41}$ ESBL resistance genes. Results are clustered by ST, then plasmid replicon type and resistance genes.

Sample ID	Host	Location	ST	*Resistance Genes*	*Virulence Genes*	Plasmid Replicon Type
F19H3CX	Human	Nambale	38	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrB19 qnrS1 sul2 tet(A)*	*eilA gad iss*	IncY
F18C6CX	Cattle	Nambale	38	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*eilA gad iss*	IncY
F15P5CX	Poultry	Nambale	46	*aadA5 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *catA1 dfrA17 mdf(A) mph(A) qnrS1 sul1 sul2 tet(A) tet(B)*	*cba cma gad mchF*	IncFIA IncFIB IncFII IncN IncQ1
F18P2CX	Poultry	Nambale	46	*aadA5 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *catA1 dfrA17 mdf(A) mph(A) qnrS1 sul1 sul2 tet(A) tet(B)*	*cba cma gad mchF*	IncFIA IncFIB IncFII IncN IncQ1
F24H2CX	Human	Busia	46	*aadA5 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *catA1 dfrA17 mdf(A) mph(A) sul1 sul2 tet(B)*	*cba cma gad mchF*	IncFIA IncFIB IncFII IncN IncQ1
F27G2CX	Shoat	Busia	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) qnrS1 sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F12P3CZ	Poultry	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F13HOUSECZ	Environment	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F13P3CX	Poultry	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F13WATERCZ	Environment	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F14C3CZ	Cattle	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F20P5CZ	Poultry	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1

Sample ID	Host	Location	ST	*Resistance Genes*	*Virulence Genes*	Plasmid Replicon Type
F30H2CZ	Human	Busia	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F18HOUSECX	Environment	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) qnrS1 sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F11H3CZ	Human	Bumala	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F18C1CX	Cattle	Nambale	196	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 mdf(A) sul1 sul2 tet(A)*	*astA cma gad iroN iss lpfA mchF*	IncFII IncFIB IncQ1
F26P2CZ	Poultry	Busia	1421	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrB19 qnrS1 sul2 tet(A)*	*gad*	-
F29H2CZ	Human	Busia	1421	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad*	-
F22G1CZ	Shoat	Busia	1421	*aadA1 aadA2 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-14}$ *bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *cmlA1 dfrA14 fosA3 mdf(A) qnrB19 qnrS1 sul2 sul3 tet(A)*	*gad*	IncHI2A IncHI2
F11C3CZ	Cattle	Bumala	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F11C4CZ	Cattle	Bumala	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F12G2CX	Shoat	Nambale	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F12S1CX	Shoat	Nambale	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F15S2CX	Shoat	Nambale	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F22G1CX	Shoat	Busia	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY

Sample ID	Host	Location	ST	*Resistance Genes*	*Virulence Genes*	Plasmid Replicon Type
F27C1CX	Cattle	Busia	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F27P7CX	Poultry	Busia	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F32C3A	Cattle	Budalangi	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad iss lpfA*	IncY
F9WATER	Environment	Bumala	2852	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 mdf(A) qnrS1 sul2 tet(A)*	*gad lpfA*	IncY
F23H3CZ	Human	Busia	3036	*aph(3'')-Ib aph(6)-Id blaCTX-M-82 blaDHA-1 dfrA17 mdf(A) mph(A) qnrB4 sul1 sul2 tet(A)*	*gad iha mchB mchC mchF*	IncFII IncY
F4H2C	Human	Bumala	Unknown	*aadA1 aph(3'')-Ib aph(6)-Id bla*$_{SHV-41}$ *dfrA15 fosA mdf(A) oqxA oqxB sul1 tet(A)*	*aaiC aap aar aatA agg3B agg3C agg3D agg5A aggR astA gad lpfA mchB mchC mchF mcmA ORF3 ORF4 pic sat sepA*	IncFII IncFIB IncR
F11C4E	Cattle	Bumala	Unknown	*aadA1 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA1 dfrA14 mdf(A) qnrS1 sul1 sul2 tet(A) tet(B)*	*air celb cma eilA gad iha iroN iss lpfA mchF*	IncFIA IncFIB IncFII IncY
F11H3A	Human	Bumala	Unknown	*aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *blaOXY-2 bla*$_{TEM-1B}$ *dfrA14 fosA7 mdf(A) qnrS1 sul1 sul2 tet(A)*	*astA capU eilA gad iss K88ab lpfA*	IncI1 IncFIB IncFII IncX1 IncY
F52H3C	Human	Teso North	Unknown	*aadA5 aph(3'')-Ib aph(6)-Id bla* $_{CTX-M-15}$ *blaTEM-33 dfrA14 dfrA17 sul2 tet(A)*	*gad iss lpfA*	IncX1
F9C4CX	Cattle	Bumala	Unknown	*aph(3'')-Ib aph(6)-Id blaACT-7 bla* $_{CTX-M-14}$ *bla* $_{CTX-M-15}$ *bla*$_{TEM-1B}$ *dfrA14 fosA3 mdf(A) qnrB19 qnrS1 sul2 tet(A)*	*gad lpfA*	IncY

4.3.3.7 PCR characterisation of ESBL Isolates

In addition to those isolates which were sequenced, 88 additional isolates (confirmed by ESBL double-disc diffusion) were also examined by PCR. All isolates were tested for the carriage of bla_{CTX-M}, bla_{SHV} and bla_{OXA} and $bla_{TEM.}$ No isolates carried bla_{SHV} genes, but 26 isolates (29.5%) carried bla_{OXA}. All 88 isolates carried a bla_{CTX-M} gene. All 88 isolates also carried $bla_{TEM-1B.}$ A further PCR confirmed that 7 isolates (10.2%) belonged to group CTX-M-2 and 81 isolates (89.8%) belonged to group CTX-M-1 (the most common of which could have been $bla_{CTX-M-1}$ or $bla_{CTX-M-15}$). Due to the high prevalence of carriage of $bla_{CTX-M,}$ all isolates were subject to another PCR assay to determine if they were the pandemic clone, ST131. Five isolates were found to carry both the *trpa* and *pabA* genes, confirming them as belonging to the pandemic clone ST131. These isolates were carried by animals and humans on four different farms across the region. Two isolates were found on the same farm (belonging to a human and a goat) and the other three isolates were carried by one human, one pig and one cattle on different farms, but clustered in one sub-county. No isolates belonging to the ST131 clone were isolated from the environment.

4.3.4 Questionnaire Data

4.3.4.1 Slaughter Practices and Personal Protection Equipment (PPE)

On-farm slaughter of animals was common in small-holders within Busia county. At the time of the questionnaire, 90.0% (n=63) of farmers reported slaughter of one or more animals in the previous twelve months. Of these, the majority were poultry (87.1%), all of which were for home consumption (100.0%). During the slaughter process, almost none of the farmers (97.1%) indicated they wore any kind of PPE.

4.3.4.2 Animal Sales

Animal sales were conducted by more than half of the interviewed farmers (57.1%, n=40). During the twelve months prior to the questionnaire, the most common animals to be sold or traded were cattle (65.0%) and poultry (40.0%). Most commonly, potential buyers or other farmers looking to trade would purchase directly from the farm (72.5%), or farmers would sell animals at a farmer's market (15.0%) or, very uncommonly, directly to a butcher (2.5%).

4.3.4.3 Understanding of Antimicrobials, Resistance & Withdrawal Periods

Participants were asked numerous questions regarding what they understood by the terms 'antimicrobial', 'withdrawal periods' and 'antimicrobial resistance'. In contrast with the previous

questionnaire, these terms were generally not well understood **(Table 4.7**). Several farmers confused antimicrobials with a type of disease. The majority of farmers did not understand or recognise the term 'antibiotic or antimicrobial resistance' and only a small minority understood or recognised that antibiotic treatment in animals required them to adhere to a withdrawal period.

When the question was rephrased as "How did you know how long to wait after treating with antibiotics, before consuming, slaughtering or selling animal products?" more than one-third of farmers (35.7%, n=25) informed us that a doctor had told them how long to wait before they could safely sell animal products or consume them.

4.3.4.4 Disease Profiles and Disposal of Infected Animals

Animal deaths in the twelve months preceding the follow-up questionnaire were quite common, 61.4% of farmers (n=43) reported no deaths in their animals, meaning that animals had died on 27 farms. A total of twelve diseases were reported, each affecting less than 5 animals, including fowlpox (8.6%, n=6), infected wounds (4.3%, n=3) and foot and mouth disease (2.9%, n=2). The questionnaire did not contain a question to quantify the diseases associated with animal deaths. Of those animals that died, many farmers chose to bury the animal (47.1%, n=33) on their property, however, some farmers opted to consume the deceased animal (12.9%, n=9). The remaining farmers buried animals some distance from their farm. A large majority of animals had developed some clinical signs of gastrointestinal disease within 3 months of the questionnaire. Common clinical signs reported in animals included diarrhoea (78.6%), bloody diarrhoea (38.6%), weight loss (55.7%) and general weakness.

4.3.4.5 Animal Treatments

In the prior three months, approximately half of farmers said they had purchased antibiotics (52.9%, n=37) for the specific treatment of animals. Many farmers reported they had not used antibiotics (42.9%, n=29) in the 3 months prior to the questionnaire. The majority of farmers purchased antimicrobials directly from AHAs (81.4%, n=57) or from agrovets (52.9%, n=37), or a combination of the two.

Table 4.7 Volunteered responses to questions asked regarding understanding of antimicrobials during the pre-sampling questionnaire

Question	Responses	Farmer	
		Number (n)	Percentage (%)
In your own words, can you describe what an antibiotic is?	A disease	29	41.4%
	A drug	10	14.3%
	Don't Know	10	14.3%
	Treatment for bacterial infection	10	14.3%
	Treat (nonspecific) disease	6	8.6%
	Prophylactic drug	2	2.9%
	A Painkiller	1	1.4%
	Treatment for viral infection	1	1.4%
In your own words, can you describe what you understand by 'antibiotic resistance'?	Don't Know	57	81.4%
	The antibiotic has no effect - treatment failure	5	7.1%
	Due to noncompliance with dosage	3	4.3%
	Confusion with "tolerance"	1	1.4%
	Due to extended use	1	1.4%
	Short reoccurrence period	1	1.4%
	The antibiotic has no effect - confused with antimalarial	1	1.4%
	The antibiotic has no effect - requires vaccination	1	1.4%
In your own words, can you describe what an 'antibiotic withdrawal period' is?	Don't Know	50	71.4%
	Time to wait after treatment before consuming milk only - unknown time	5	7.1%
	Time to wait after treatment before consuming any products - incorrect time	4	5.7%
	Time to wait after treatment before consuming meat or milk only	3	4.3%
	Knows, but ignores withdrawal period	2	2.9%
	Time to wait after treatment before consuming any animal products - correct time	2	2.9%
	Time to wait after treatment before consuming milk only	1	1.4%
	Time to wait before consuming milk - gives to animals during withdrawal period	1	1.4%
	Time to wait until last dose given	1	1.4%

The most common antimicrobials used in treating animals included oxytetracycline (62.2%, n=23) and penicillin-streptomycin (29.7%, n=11) and sulfonamides (5.4%, n=2). When using injectable antibiotics (including penicillin-streptomycin and oxytetracycline), more than half of farmers used the same needle/syringe for all animals and all antimicrobials (55.7%, n=39). About two-thirds washed the needle and syringe between animals and when changing antimicrobials (65.0%, n=26).

Farmers often opted to treat single animals when they became ill (57.1%, n=40), opposed to the entire herd, however, less than half of farmers decided to separate the ill animal (42.9%, n=40) from the rest during treatment. Vaccinations were given to animals shortly after birth or purchase (60.0%, n=42) in more than half of cases, though usually only to poultry and cattle.

4.3.4.6 Human Health & Hygiene Practices

No large livestock were allowed access to the inside of the housing (living spaces/kitchen etc.) however, all farmers noted that poultry had access, and regularly did enter the households.

Almost all farmers indicated that their toilet facilities were deep pits, located outside of the house, on the farm (97.1%, n=68). Defecation almost always occurred in the latrine, except in some cases where defecation would occur whilst attending to crops on the farm (5.7%, n=4), due to the distance from the household. The interviewer noted that in most cases, the latrine was in close proximity to animal shelters, or in areas where animals could wander freely (97.1%, n=68).

Most farmers indicated that they have almost daily contact i.e. 6-7 days per week, with any sort of animal on their farms (92.8%, n=65). However, hygiene practices reported were good, 71.4% of farmers (n=50) stated that they washed their hands after touching animals; 90.0% of farmers washed their hands with soap and water before eating and/or cooking meals (n=63), and 88.6% of farmers washed their hands after using the latrine (n=62). Wastewater, used for handwashing, cooking and feeding animals was not routinely treated (14.3%, n=10) before discarding onto the farm.

Farmers also reported various gastrointestinal illnesses within the previous 3 months. These included diarrhoea (54.3%, n=38), abdominal pain (22.9%, n=16), typhoid (14.3%, n=10) and general nausea (10.0%, n=7). Of these people, 91.5% (n=43) opted to purchase some treatment, mainly from a doctor (67.4%) or from a hospital (23.3%). Of those who sought treatment, 30 farmers received antibiotics and 1 received proton-pump inhibitors. Antibiotics reported included metronidazole (41.9%, n=13), ciprofloxacin and amoxicillin (both 16.1%, n=5), Augmentin (amoxicillin + clavulanic acid) (6.5%, n=2) and ceftriaxone, cephalexin, and erythromycin (all 3.2%, n=1). Ten farmers (32.3%) did not recall what they were prescribed.

4.3.5 Risk Factor Analysis

4.3.5.1 Univariable Analysis

Initial univariable analysis revealed 22 potential explanatory variables for inclusion in multilevel, multivariable models ($p<0.25$) of the 6 resistance outcomes - multi-drug resistance (MDR), extended-spectrum β-lactamases (ESBL), tetracycline (TET), sulfathiazole (SULFA), ampicillin (AMP) and trimethoprim (TRIM) resistance. However, only a small number of significant

(P<0.05) variables were identified **(Table 4.8)**. There were no variables that were consistently significant across all resistance outcomes, though several explanatory variables were associated with more than one outcome. As the prevalence of resistance to 3 of the resistance outcomes (chloramphenicol, gentamicin, and ciprofloxacin) was less than 10% of tested samples these were not included in these analyses.

To avoid multicollinearity, tests for correlation (Pearson's correlation coefficient, correlation matrices) between explanatory variables were examined. There were no highly correlated variables (<-0.3 or >0.3) detected which were used in any models.

4.3.5.2 Multivariable Analysis

Mixed effect logistic regression models were undertaken with each of the 6 resistance outcomes. Individual sample level outcomes were clustered within farms and the region (sub-county). Species was included in each model as a fixed effect. The MDR and AMP outcomes each had two significant explanatory variables (p<0.05) which were the farm also having a positive environmental ESBL sample and use of penicillin-streptomycin in the previous 3 months. The SULFA, TET and TRIM outcomes each had a single explanatory variable: farm size for SULFA and animals vaccinated for TET and TRIM **(Tables 4.9** and **4.10)**. The ESBL outcome had no statistically significant explanatory variables. For those models which had multiple explanatory variables, there were no significant interactions identified.

Antibiotic use in the last 3 months was associated with two outcomes.

Animals being vaccinated when born or acquired was also associated with two outcomes – with increased risk of tetracycline resistance and trimethoprim resistance.

After allowing for clustering within farms there were few significant differences between species; humans were significantly at higher risk of MDR compared to cattle and humans and sheep and goats were at higher risk of sulphonamide and trimethoprim resistance.

From the mixed effects models, the variances at the sub-county level were generally small, apart from tetracycline. There was greater evidence of within-farm clustering, particularly with respect to ESBL and tetracycline outcomes. Farm level residual plots for all resistance outcomes (**Appendix II, Figure II-iii**) showed that 7 and 2 farms, respectively, were significantly different

from the overall mean. Secondary examination of the data from those outlying farms showed that they all contained at least one sample which was positive for ESBL and tetracycline resistance. There was a low prevalence of ESBL resistance, so it is plausible that some farms with positive ESBL samples may be outliers. However, there was a high prevalence of tetracycline resistance, and so it is unclear why the variance at farm level was found to be disproportionately high. All data from all farms was rechecked before accepting the model and validated independently by Gina Pinchbeck.

Table 4.8 Univariable analysis of explanatory variables, for inclusion in multivariate models of risk factors for carriage of AMR or ESBL-producing *E. coli* in human, animal and environmental populations.

Explanatory Variables		MDR	ESBL	TET	SULFA	AMP	TRIM
Species	Cattle						
	Human						
	Poultry						
	Pig						
	Sheep/Goats						
Environmental Sample MDR	No						
	Yes						
Environmental Sample ESBL	No						
	Yes						
Gender	Female						
	Male						
Age	18-24						
	25-44						
	45-64						
	<65						
Farm Size	Mean						
Knowledge of Antibiotics	Don't Know						
	Good						
	Bad						
Knowledge of Antibiotic Resistance	Don't Know						
	Good						
Knowledge of Antibiotic Withdrawal Periods	Don't Know						
	Some						
	Good						
	Actively Ignores						
Animal had Mastitis Last 12 months?	No						
	Yes						
Animal had Diarrhoea in the last 12 months?	No						
	Yes						
Animal had diarrhoea in the last 3 months?	No						
	Yes						
Animal had Bloody diarrhoea in the last 3 months?	No						
	Yes						
Animals Vaccinated (when acquired/born)?	No						
	Yes						
When ill, treat all animals or single?	Single Animal						
	Whole Group						
Separate Ill animals?	No						
	Yes						
Bought Tetracycline in the last 3 Months?	No						
	Yes						
Bought Penicillin-streptomycin in the last 3 months?	No						
	Yes						
Used Tetracycline in the last 3 months?	No						
	Yes						
Used Penicillin-streptomycin in the last 3 months?	No						
	Yes						
Use the same needle/syringe for all antibiotics/animals?	No						
	Yes						
Main water source	Taps						
	Borehole/Well						
	River/Lake						
	Rainwater						

MDR = multi-drug resistance, **ESBL** = extended-spectrum β-lactamases, **TET** = tetracycline, **AMP** = ampicillin, **TRIM** = trimethoprim, **SULFA** – sulfathiazole, **CHLOR** = chloramphenicol. Grey shading: p<0.25, *Bla*ck shading: p<0.05.

Table 4.9 Mixed effect logistic regression models of risk factors associated with multi-drug resistance, ESBL production and ampicillin resistance in *E. coli* isolated from faeces of animals and humans, and *E. coli* isolated from water and environmental sources in Busia, Kenya.

		MDR					ESBL					AMP				
Variable	**Category**	**β**	**SE**	**OR**	**95%CI**	***p***	**β**	**SE**	**OR**	**95%CI**	***p***	**β**	**SE**	**OR**	**95%CI**	***p***
Species	Cattle	(ref)	-	-	-	-	(ref)	-	-	-	-	(ref)	-	-	-	-
	Human	0.699	0.239	2.011	1.258-3.215	**0.003**	0.076	0.359	1.079	0.534-2.18	0.832	0.343	0.239	1.409	0.882-2.251	0.151
	Poultry	0.191	0.207	1.21	0.806-1.817	0.357	-0.13	0.324	0.878	0.465-1.658	0.689	0.059	0.207	1.061	0.707-1.593	0.774
	Pig	-0.108	0.315	0.898	0.484-1.665	0.732	0.24	0.476	1.271	0.501-3.23	0.614	-0.074	0.309	0.928	0.506-1.702	0.81
	Sheep/goats	0.463	0.262	1.589	0.951-2.654	0.077	0.484	0.379	1.622	0.772-3.409	0.202	0.467	0.268	1.595	0.943-2.697	0.081
Environment ESBL	No	(ref)	-	-	-	-	-	-	-	-	-	(ref)	-	-	-	-
	Yes	3.139	1.102	23.084	2.664-200.01	**0.004**	-	-	-	-	-	0.343	0.239	17.35	2.127-141.562	**0.008**
Used Penicillin-streptomycin in the last 3 months?	No	-	-	-	-	-	-	-	-	-	-	(ref)	-	-	-	-
	Yes	0.561	0.195	1.753	1.197-2.567	**0.004**	-	-	-	-	-	0.536	0.187	1.71	1.184-2.468	**0.004**
Variance Estimate	Region	0.015(0.036)					0.035(0.158)					0.023(0.041)				
	Farm	0.150(0.104)					1.207(0.427)					0.068(0.090)				

***P*-values** in bold are statistically significant (p<0.05) values from the Wald Chi squared test; **OR** = Odds Ratio; **SE** = Standard Error; **95%CI** = 95% Confidence Intervals. **Variance Estimate** = Variance (Standard Error).

Table 4.10 Mixed effect logistic regression models of risk factors associated with sulfathiazole, tetracycline and trimethoprim resistance in *E. coli* isolated from faeces of animals and humans, and *E. coli* isolated from water and environmental sources in Busia, Kenya.

		SULFA					TET					TRIM				
Variable	**Category**	**β**	**SE**	**OR**	**95%CI**	***p***	**β**	**SE**	**OR**	**95%CI**	***p***	**β**	**SE**	**OR**	**95%CI**	***p***
Species	Cattle	(ref)	-	-	-	-	(ref)	-	-	-	-	(ref)	-	-	-	-
	Human	1.47	0.308	4.351	2.379-7.958	**<0.001**	0.541	0.491	1.718	0.656-4.5	0.271	1.178	0.300	3.247	1.803-5.846	<0.001
	Poultry	0.341	0.225	1.406	0.905-2.184	0.13	-0.521	0.346	0.594	0.302-1.17	0.132	0.327	0.229	1.387	0.886-2.171	0.153
	Pig	0.447	0.356	1.564	0.779-3.14	0.209	0.682	0.683	1.978	0.519-7.539	0.317	0.311	0.357	1.365	0.677-2.751	0.384
	Sheep/goats	0.712	0.298	2.038	1.135-3.658	0.017	0.871	0.581	2.389	0.764-7.467	0.134	0.668	0.304	1.95	1.074-3.539	**0.028**
Farm Size	Mean	0.012	0.006	1.012	1.001-1.023	**0.033**						-	-	-	-	-
Animals Vaccinated	No	-	-	-	-	-	-	-	-	-	-	(ref)	-	-	-	-
	Yes	-	-	-	-	-	0.9	0.452	2.46	1.014-5.97	**0.047**	0.015	0.006	1.016	1.003-1.028	**0.012**
Variance Estimate	Region	0.259(0.198)					0.688(0.572)					0.266(0.203)				
	Farm	0.469(0.186)					1.187(0.524)					0.471(0.189)				

***P*-values** in bold are statistically significant (p<0.05) values from the Wald Chi squared test; **OR** = Odds Ratio; **SE** = Standard Error; **95%CI** = 95% Confidence Intervals. **Variance Estimate** = Variance (Standard Error).

4.4 Discussion

This study investigated the carriage and prevalence of AMR *E. coli* associated with humans, animals, and their shared environments in a smallholder crop-livestock system in western Kenya, part of the wider Lake Victoria crescent ecosystem. *E. coli* was isolated from the faeces of farmers and four species of farm animal commonly kept in the region, as well as from the living and cooking areas of farmers' houses, and the main water source at each farm. The potential risk for the transmission of antimicrobial-resistant bacteria between animals, or animal food products and humans in sub-Saharan Africa has been highlighted before, however, from this region, no data on the spread of AMR determinants between human and animal reservoirs have been published (Alonso et al., 2017). The results of this study indicate a high prevalence of AMR, as well as a high level of diversity in the *E. coli* isolated, within and between hosts, according to their genetic makeup and carriage of virulence and resistance genes. This the first study investigating the prevalence of faecal-carriage of antibiotic-resistant *E. coli* in farm animals, farmers, and their associated environments, in western Kenya. Busia is a relatively poor county which borders Uganda to the west. A large proportion of people were traditional subsistence, small-holder farmers, but there has been a recent shift towards business-oriented farming. As such, more farmers are increasing the number of livestock they own.

There was a high prevalence of AMR *E. coli* isolated from the faeces of humans and animals, as well as from the environment. More than half of all isolates were MDR (53.6%, 95% CI 50.0% – 57.2%) which is a higher prevalence that is reported in studies in other Kenyan sub-counties. In Kitale sub-country, AMR *E. coli* in human clinical gastroenteritis cases showed a lower prevalence of MDR *E. coli* (42.2%) (Kipkorir et al., 2016) than our human population estimate of 58.5% (95% CI 54.9% - 62.1%). Conversely, another study examining *E. coli* in the gut of Ugandan pastoralists, showing that approximately 57.0% of isolates were MDR (Stanley et al., 2018), similar to our estimate. Human isolates in this study were also found to carry a high proportion of trimethoprim and sulfathiazole-resistant *E. coli*, with a prevalence of 84.6%, which is higher than in a parallel study conducted in Nairobi, who reported a rate of 46.7% MDR amongst humans (Muloi et al., 2019b). Use of trimethoprim and sulfa drugs as prophylaxis in persons infected with HIV (approximately 32% according to Kwena et al) may account for a proportion of the described resistance, as Busia county and other coastal areas are considered to be a high-risk area for HIV transmission (Kwena et al., 2019).

There was also a high prevalence of AMR-*E. coli* found in food-producing animals. There was moderate to high prevalence of resistance to tetracycline, sulfathiazole, ampicillin, and trimethoprim, in all animals however as with the human population, there was a lower prevalence to ciprofloxacin, chloramphenicol and gentamicin. Sheep and goats in this study had higher carriage of MDR compared to non-human species (though this was not significantly different). The high prevalence of MDR and AMR-*E. coli* in goats and sheep is difficult to explain; deposited faeces from cattle and poultry were typically collected from across the entire farms, however, in the case of goats and sheep and pigs, faeces were usually limited to enclosures or pens. Goats and sheep are not always limited to pens though, and during the day may migrate across shared grasslands or within the farm to graze. Other studies suggest that animals raised in poor hygienic conditions (e.g. cramped in small areas) were more likely to carry AMR *E. coli* (Rehman et al., 2014; Islam et al., 2016), and pens on some of the visited farms were heavily littered with shoat faeces. A similar pattern was found in Ugandan livestock – *E. coli* from goat faeces showed a higher proportion of sulfa-drug resistance (87.5%) than in cattle, pigs or poultry (Okubo et al., 2019).

In groundwater and surface water sources (including boreholes, wells and rainwater) in Isiolo county in Kenya, *E. coli* was isolated from 22.9% and 36.8% of sources, respectively (Onyango et al., 2008). This rate of isolation of *E. coli* (21.4%), in our study was similar, even though most of the *E. coli* was isolated from wells and boreholes. No phenotypic resistance was identified to ciprofloxacin or gentamicin in water or environmental boot swab isolates, although sample sizes were small; in other studies, boot swabs are routinely taken from animal enclosures when sampling (Nilsson et al., 2014; Gundran et al., 2019), however, our study opted to take swabs from the inside of houses, to specifically asses if bacteria were being brought in via humans or animals entering households. Resistance to chloramphenicol was the most prevalent in environmental samples (25.0%), though due to the low number of samples cultured confidence interval are wides. Although humans and poultry were the only two populations to have access to the insides of houses, there was no inflated prevelance of chloramphenicol resistacne in humans or poultry, compared to other hosts. There was a similar proportion of AMR *E. coli* in water isolates as there was in humans and animals, however isolation of *E. coli* from water (and the environment) was relatively low. These findings do still suggest that contamination of water in boreholes and wells may be occurring and that environmental sources present a risk of exposure. This is documented in the literature and is usually attributed to organic and faecal pollution of water sources; antimicrobial residues in faeces may be leaching into the soil, which

in turn leach into boreholes (Chen et al., 2017; Manyi-Loh et al., 2018; Cycoń et al., 2019); during the wet season in western Kenya, the water table may be close to the surface (Ogege. J, 2001).

There were 43 different resistance phenotype patterns identified from all groups. The most common phenotype (TET-SULFA-AMP-TRIM) represented approximately one-third (32.4%) of all isolates (20.4% in humans, 15.0% to 30.0% in animal species, and 3.4% in environmental). Differing farm management practices and geographical locations have been shown to influence genetic diversity and the presence of AMR genes in commensal *E. coli* in pigs (Leistner et al., 2013), thus it is likely that this occurs in other animal populations too. High frequencies of tetracycline, streptomycin and trimethoprim/sulfamethoxazole resistance have been increasingly described in *E. coli* of animal origin, especially from poultry and pigs (Badi et al., 2018), but there is less chloramphenicol resistance being reported. Environmental factors such as selection pressure caused by administration of antimicrobials may lead to upregulation of previously silenced genes, which results in phenotypic resistance to these antimicrobials (Card et al., 2013). Use of chloramphenicol in food-producing animals was banned in Kenya in 1994, according to the European Decision 2003/181/EC) (Wesongah et al., 2012), though it is possible that they are still being used privately, or the genes are remnants of high fitness-cost effectiveness. Indeed, from (**Chapter 3**) there were no reported sale or by proxy, use, of chloramphenicol to treat animals, neither were there any packaging observed in waste piles. Despite this, it is still avaial for purchase in some LMICs, without prescription, for use in animals. It has been shown that chloramphenicol resistance genes are transmissible on plasmids between *E. coli* in Kenyan farm animals (Kikuvi et al., 2007).

As part of this study, several major databases were compared to determine the concordance of detected resistance and virulence genes in *E. coli* genomes. There was some concordance in commonly identified resistance genes between three different databases – ResFinder, NCBI and CARD, but there were also genes reported at vastly different frequencies, e.g. no detected chloramphenicol genes when using the CARD database, and no multi-drug efflux exporters (*mdf(A)*) reported by the NCBI database. One possible explanation for the discordance found between the investigated methods is that despite downloading the most recent databases for each method, those databases may not have contained identical genes. This may have led to false-negative WGS predictions. The need to continuously update curated resistance genes databases is a difficult challenge for bioinformaticians; indeed, as WGS and NGS become more common, it is likely that many novel mutations will be identified within resistance genes.

Alternatively, some discordance could be attributed to the specific methods used by each piece of software. Programs such as ResFinder use a method based on assembling WGS reads and then using *BLA*ST to identify resistance genes (Zankari et al., 2012). By using this approach, it is possible that the prevalence of genes can be underreported where they are split across multiple assembled contigs, or in some cases, Blast may have a higher chance of detecting one of multiple closely related genes. The takeaway message from this comparison was that there is a trade-off between the required sensitivity and specificity to accurately detect genes and their variants. For instances where there was large discordance (such as detection of the *mdf(A)* gene), it may be appropriate in future studies to combine the curated databases used in each of the described methods, and then screen assemblies or mapped reads to this database.

Phylogenetic reconstruction of genomes showed that a diverse array of STs and serotypes was dispersed amongst the isolates. The most prevalent ST was ST196 which has been linked to *E. coli* isolated from human faeces, livestock and food, which carry $bla_{\text{CTX-M-15}}$, in studies in Germany and Cambodia (Fischer et al., 2014; Stoesser et al., 2015). Isolates belonging to ST196 showed highly similar resistance patterns and formed their own clade with almost no genetic distance (less than 150 SNPs) between them on a phylogenetic tree (**Figure 4.6**). Due to their low genetic variation and close distance on phylogenetic trees, it is likely that some of these have since acquired additional resistance or virulence genes, explaining their slight variation. This shows evidence of sharing of bacteria between hosts but does not help to infer directionality. The second most common ST was ST2852 (10 isolates), and these isolates also formed a distinct clade on a phylogenetic tree (**Figure 4.6**), with low genetic difference. All ST2852 isolates carried identical virulence and resistance genes, as well as the same plasmid replicon type (IncY), indicating these isolates may be clonal in nature, and are being disseminated amongst humans, cattle, sheep, goats, poultry, and their shared environments. As with isolates reported in various farm animals in Tanzania (Seni et al., 2016b), these clones all carried the $bla_{\text{CTX-M-15}}$ resistance genes, as well as those encoding aminoglycoside, sulfa-drugs, trimethoprim, fosfomycin and tetracycline resistance.

These findings suggest that multiple clones (or extremely closely related bacteria) of ST196 and ST2852 may be circulating in the community, likely as intestinal commensals. Due to the carriage of $bla_{\text{CTX-M-15}}$ and numerous resistance genes, they warrant further investigation to determine their origin. By merging the WGS data collected as part of this study (**Chapter 4**) and using additional WGS from other studies (if available) within western Kenya, it may be possible to try

to map the evolution of the resistance genes over time using Bayesian analysis of molecular sequences, with a relaxed clock model.

Three additional STs were also found, including ST10, ST155 and ST38. ST10 is found in a broad range of hosts and has been described as a pandemic ExPEC lineage (Manges et al., 2019) being recorded most third-most frequently (after ST131 and ST69) in human clinical isolates, as well as meat products and food animals (Oteo et al., 2009; Cortes et al., 2010; Cohen Stuart et al., 2012; Peirano et al., 2012). The ST10 *E. coli* isolated from animals were all (but one) MDR; these isolates carried large numbers of resistance genes (up to 12), but none carried an ESBL resistance gene. Isolates also carried many virulence genes (up to 21). ST155 has also been associated with human (ExPEC infections) and animals worldwide (Maluta et al., 2014; Matamoros et al., 2017). Isolates belonging to this ST were isolated from both environmental and animal sources, and were all (but one) MDR, carrying up to 13 resistance genes. None of these isolates carried an ESBL resistance gene. Finally, ST38, a successful enteroaggregative *E. coli* associated with urinary tract infections (Chattaway et al., 2014), was found in cattle, goats, poultry and humans. Two of these isolated also carried $bla_{\text{CTX-M-15}}$ gene, but very few EAEC-associated virulence genes, suggesting that these isolates were not EAEC.

The majority of *E. coli* isolates belonged to phylogroups A, B1, B2 and D; these strains vary in their phenotypic and genotypic characteristics, ecological niche, and propensity to cause disease (Tenaillon et al., 2010). The majority of isolates which could be phylotyped, belonged to groups A and B1; in tropical climates and LMICs, human commensals are predominantly A and B1 (Escobar-Paramo et al., 2004) whereas animal commensals are usually B1 (Carlos et al., 2010). This fits with our findings and indicates that there may be some association between phylogenetic groups and host species. It has been suggested in the literature that extraintestinal pathogenic strains of *E. coli* mainly belong to phylogroups B2 and to a lesser extent, D (Mora et al., 2009; Shaer et al., 2018). As relatively few of the isolates were found to be non-A or B1, it can be assumed that the sample consisted largely of commensal and non-pathogenic *E. coli*. Phylogroup E was detected in livesotkc isolates only, though there appears to be little literature which describes the association between phylogroup E and potential pathogenic or non-pathogenic strains, thus there is uncertainty as to whether there is any relevance in this finding. One isolate of the newly discovered phylogroup G was also found during re-scanning of all reads using a newer ClermonTypying database (February 2020). Phylogroup G are isolates with high resistance and virulence potential (Clermont et al., 2019), and indeed this isolate carried a large

number of resistance (10) and virulence (8) genes. As this re-analysis was performed some time (almost two years) after the original results were compiled, this highlights the need to re-evaluate historic data, to assess if existing epidemiological assumptions are still correct. The presence of a new phylogroup G isolate could be worrisome for public health if it is able to spread, or indeed acquire additional AMR genes, such as multiple ESBL genes.

All isolates were also serotyped – there was a wide diversity of serogroups represented in this dataset, with 111 unique O:H groups reported. By itself, the most common O antigen was O8; O8 antigens are commonly associated with ETEC, which causes serious foodborne infections in humans (Kaper et al., 2004). The most common H antigen was H7, which is famous for its association with O157:H7, a foodborne STEC strain which causes severe bloody diarrhoea and kidney failure (Lim et al., 2010). The most commonly occurring serotype was O8:H7 and this was found predominantly in animal isolates. All O8:H7 isolates were MDR, carrying combinations of β-lactamase, fluoroquinolone, sulfonamide, tetracycline, aminoglycoside, and trimethoprim resistance genes. Most isolates identified in the large ST2962 and ST196 clades were also serotype O8:H7. Isolates carrying $bla_{\text{CTX-M}}$ genes, in association with serotype O8:H7 have also been described in low prevalence on Japanese Dairy farms (Ohnishi et al., 2013). The number of resistance genes carried by these bacteria is worrying, as there is potential for these to rapidly disseminate to other animals, humans, and parts of the country.

Isolates to be sequenced were pseudo-randomly selected as previously described. Analysis of resistance genes carried by all isolates showed a high diversity carried by *E. coli.* It was typical for several resistance genes to be found together – usually, *aph(6)-1d*/*aph(3")-1b* were found together with sulfonamide, tetracycline and trimethoprim resistance genes, *sul2*/*sul1*, *tet(A)*/*tet(B)* and *dfrA*, respectively. It is likely that many of these resistance genes are carried on the same or similar plasmids, as many of the isolates carried IncFII or IncFIB plasmids. Low carriage of *mph(A)* (6.6%) is representative of the relatively low use of macrolides, but appears to be lower than the average carriage found by Nguyen et al, in five sub-Saharan African countries including Niger and Senegal (Phuc Nguyen et al., 2009). The proportion of *dfrA* genes also appeared to be dissimilar from the rates in neighbouring Tanzania, where significantly higher proportions (73.7%) of *dfrA* genes encoding trimethoprim resistance in commensal *E. coli* isolated from cattle and humans were reported (Galdiero et al., 2016), compared to our study (<50%). Fosfomycin resistance genes, *fosA* were discovered in only 7.2% of isolates – this is likely because antimicrobials containing fosfomycin were the most expensive type of product,

according to our prior questionnaire data (**Chapter 3**); and only approximately 8% of agrovets stated that they regularly sold fosfomycin-containing products. The low prevalence of fosfomycin resistance gene detection is in line with published figures for *E. coli* genomes (4.6%) (Ito et al., 2017). Where resistance genes were carried by all groups of isolates, there were almost no significant differences between their carriage. This suggests that patterns of AMR genes are similar between humans, animals, and the shared environment in this population.

Alongside resistance genes, 49 unique virulence genes were carried by *E. coli*. There were no discernible patterns between the number of virulence genes carried, between animal species, humans, and the environment either between farms or within farms. Interestingly, even animals which were kept in the same housing or enclosures were found to carry different virulence genes. On one farm an *E. coli* isolated from a cow carried 18 virulence genes (F35C3E), and a human isolate and a poultry isolate carried only three and six virulence genes, respectively. Another isolate carried 21 virulence genes (F4H2C), as well a large number of resistance genes, including bla_{SHV-41}. To our knowledge, this genotype, in association with a large number of virulence genes has not previously been reported in the literature, and may be of importance if it continues to spread. The three most commonly carried virulence genes carried by all groups include *gad*, *iss* and *IpfA*. The *gad* gene (encoding glutamate dehydrogenase) was the most prevalent virulence gene identified, with 92.8% of isolates carrying it. *Gad* is highly prevalent in both pathogenic and non-pathogenic *E. coli* isolated from both humans and animals; it is the most efficient acid resistance system in *E. coli (Large et al., 2005)* and is vital in maintaining physiological pH when passing through highly acidic environments, such as the stomach. The *iss* gene encodes proteins which are involved in increasing serum survivability and resistance to serum complement. They were first described in the ColV plasmid and are typically associated with APEC and UPEC (Nolan et al., 2003; Askari Badouei et al., 2015). Whilst particularly prevalent amongst our poultry samples (71.4% carriage), *iss* was also commonly detected in all other animal species, as well as in humans. Interestingly, 25.9% of isolates carrying *iss* also carried *iroN* which is a siderophore, important for scavenging iron from host tissues. This is also associated with ExPEC (including APEC), NMEC and UPEC from numerous species (Ewers et al., 2007; Najafi et al., 2019). Finally, the *IpfA* gene, carried by 68.4% of isolates, encodes for long polar fimbriae which are involved in increased adhesion to epithelial cells (Toma et al., 2006) and resulting diarrhoea caused by EHEC and EPEC (Afset et al., 2006; Dogan et al., 2012).

IncF plasmids routinely carry both virulence and resistance genes, and a number of *E. coli* pathotypes are characterised by the carriage of toxins, adhesins and siderophores that are encoded by IncF plasmids (Johnson and Nolan, 2009). The most commonly identified plasmid and replicon type was IncFIB, which is sporadically reported in the literature, and is thought to have resulted from recombination events between other IncFII plasmids (Coque et al., 2008b; Partridge et al., 2011). IncFII plasmids were the second most commonly carried plasmid, followed by IncQ. Plasmids belonging to the IncQ incompatibility group are able to replicate in a broad range of bacterial hosts (Rawlings and Tietze, 2001), but are not widely reported in the literature in association with *E. coli*. IncF plasmids have previously been identified as carrying virulence genes and they have also been associated with carriage of $bla_{\text{CTX-M-15}}$ that is often associated with $bla_{\text{TEM-1}}$, $_{\text{bla}}$*OXA-1*, and *aac(6')-Ib-cr* resistance genes (Carattoli, 2009). Indeed, the results from analysis of plasmids found circulating amonst *E. coli* from humans, livestock and the nvnonrment fit these findingds. 17 isolates from human, cattle, poultry, sheep, goats and the environment carried a combination of IncFIA, IncFIB and IncFII plasmids, as well as $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$, but *aph(3'')-Ib* and *aph(6)-Id* instead of *aac(6')-Ib-cr.* In northern Tanzania, AMR *E. coli* carrying IncF plasmids was also isolated from drinking water sources (Lyimo et al., 2016) with the same resistance profile as in our study, at similar rates (16% vs our study 18.1%), as well as in faecal samples collected from healthy people in Nigeria (14.0%) (Inwezerua et al., 2014).

For more than 10 years, CTX-M-producing *E. coli* have been spread all over the world as colonisers of the gut in livestock and wildlife animals as well as in humans or as a causative agent of various infections. Within the sample population, plasmid-associated resistance to cephalosporins was mediated primarily by the production of an ESBL (CTX-M), rather than p*AmpC* enzymes. This is in line with prevalence data from a number of Tanzanian, Kenyan and Ugandan studies examining predominant ESBL-encoding genes (CTX-M, TEM and SHV), albeit in clinical settings (Sonda et al., 2016). In non-African studies, there is also clear evidence to show that there is a predominance of ESBL-mediated resistance overproduction of *pAmpC* enzymes (Potz et al., 2006; del Castillo et al., 2013; van Hoek et al., 2015). The results presented in our study are also comparable to studies investigating faecal carriage of ESBL-producing *Enterobacteriaceae* in healthy individuals and community patients in the UK and Northern Europe, as well as animals in China, which identify $bla_{\text{CTX-M}}$ as the most prevalent ESBL variant in these populations (Rao et al., 2014; Valenza et al., 2014; Reuland et al., 2016). Globally, $bla_{\text{CTX-M-15}}$ has been linked to the dissemination of the pandemic *E. coli* ST131 clone, named as it has

spread to many different countries, particularly amongst humans, but also increasingly reported in animals, worldwide (Ewers et al., 2010; Schembri et al., 2015; Chiluisa-Guacho et al., 2018). However, we did not detect any ST131 isolates in our sequenced genomes. Due to the limitations in the number of isolates that could be sequenced, all non-sequenced ESBL isolates (confirmed with double-disc diffusion) were also assessed for *trpa* and *pabA* genes via PCR, indicating if they were ST131. Five isolates were identified as being ST131:O25b:FimH30 – the pandemic clone. Two of these came from a single farm, from a human and goat, and three others were found in humans and animals across three other farms; all isolates had the same resistance phenotype (TET-SULFA-AMP-TRIM). This is a serious publich health threat, as the ST131:O25b:FimH30 clone carries a large numer of AMR genes and has high virulence potential.

A large number of other STs are associated with the transmission of $bla_{\text{CTX-M}}$ e.g. ST10, ST38, ST46, ST196, ST131, ST405 etc. (Canton and Coque, 2006; Hernandez and Gonzalez-Acuna, 2016). As well as ST196, and ST2852, 4 additional STs were associated with carriage of $bla_{\text{CTX-M}}$ genes; these were ST38, ST46, ST1421 and ST3036. Whilst ST3036 appears to be a novel ST, ST1421 has been associated with another CTX-M, $bla_{\text{CTX-M-64}}$, in a German patient with a UTI (Pfeifer et al., 2018). The isolate associated with ST1421 (F22G1CZ) carried by a goat, carried both $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and 14 additional resistance genes, suggesting that this may be a particularly resistant strain. Whilst there was no documented use of cephalosporins, they may have been administered prior to our study, or without the knowledge of those interviewed farmers, as a concurrent study conducted in Nairobi appears to suggest a somewhat higher use of cephalosporins (Muloi et al., 2019a).

By collectively assessing the resistance genes, virulence genes, plasmids and MLST of the sequenced isolates, there is evidence of potential transmission of AMR *E. coli* between humans, animals, and the environment. There were many similarities in AMR genes between humans, livestock and the environment, and comparison of these demonstrated that those three groups have overlapping AMR gene communities. There are complex interactions between humans, livestock and the environment which could help to explain this. In rural areas such as Busia, there is a high proportion of direct contact with livestock, consumption of livestock products, and a shared environment which receives both human sewage and manure from livestock. It is therefore possible that acquisition of AMR from a common source (the environment) could be playing some role in the similarities between AMR gene profile in all groups. Indeed, the presence of two different (large) highly similar (potentially clonal) groups (and two smaller ones)

dispersed throughout the population do suggest that there is transmission of AMR *E. coli* occurring between hosts.

Questionnaire results were in-line with previous findings (**Chapter 3**), specifically, the majority of farmers slaughtered animals on their premises for familial consumption, sale to neighbours or to butchers. Most commonly traded animals were cattle and poultry, which coincides with the most commonly kept animals on the visited smallholder farms. During slaughter, almost no farmers wore PPE, which may contribute to the spread of AMR-bacteria via interaction with contaminated blood, bodily fluids, and viscera. It has been seen in an examination of slaughterhouse conditions in Busia and neighbouring counties that the combination of poor knowledge of zoonotic transmission of disease, lack of PPE and the slaughter of sick animals may all contribute to the spread of AMR between humans and animals (Cook et al., 2017).

Relatively few participants of this questionnaire had a good understanding of AMR and withdrawal periods; approximately 40% of participants thought that antimicrobials were a disease, 82% did not know what antibiotic resistance was and 70% did not know what an antibiotic withdrawal period was. We suggest that the reason for this difference in knowledge between the two time points is attributable to the lack of sub-county veterinary officer present in follow-up visits. Speculation suggests that when these questions were first asked (**Chapter 3**), veterinary officers may have over-explained the concept to farmers when translating into Kiswahili due to differences in language equivalency. Alternatively, the presence of government officials could have biased recruits responses as they would not wish to divulge incorrect or illegal practices for fear of punishment. This highlights the difficulties of conducting KAP studies in multiple languages and reinforces the idea that all interviewers must be briefed appropriately, and be intimately familiar with the study and its outcomes. This will prevent the asking of leading questions to participants, and ensure there is continuity between interviews conducted in English and Kiswahili.

Farmers reported several gastrointestinal illnesses which may have had a bacterial cause, including diarrhoea and abdominal pain. Farmers also specified that typhoid was a clinical sign. Relatively few farmers treated their gastrointestinal diseases with antimicrobials, but of those who did, common treatments included antiprotozoals, metronidazole, 3rd generation cephalosporins, fluoroquinolones and penicillins. Many of these were obtained from a doctor or hospital. There was little reported use of cephalosporins and fluoroquinolone for human

therpaies, which is in-line with the observed prevalence of resistance to these antimicrobials; human isolates were found to have a low prevalence of ESBL and ciprofloxacin resistance. Hygiene practices were generally good. Only a small proportion of farmers did not wash their hands prior to cooking and eating, and the majority washed their hands after using the latrine, thus limiting the risk of ingesting zoonotic bacteria. The high prevalence of AMR observed in human isolates highlights the need for continued monitoring, and may be explained by (i) the high antibiotic usage in humans (Kariuki and Dougan, 2014) though there is little correlation between AMU and AMR; or ii) frequent contact between humans and livestock. However, as suggested by the mixed effects modelling, AMU was seen as a significant ($p>0.005$) factor for AMR in some cases. This suggests the need to reduce the number of antimicrobials used or prescribed by hospitals or doctors in the area.

Mixed effects regression models identified several factors associated with the investigated resistance outcomes. Use of penicillin-streptomycin was a significant risk factor; as previously explored, there is chronic underdosing, overdosing and potentially inappropriate use of antimicrobials in LMICs, as well as Kenya. Numerous studies have highlighted that when antimicrobials are purchased without a prescription, variable concentrations of antibiotics are given to animals (Mitema et al., 2001b; Irungu et al., 2007; Global Antibiotic Resistance Partnership, 2011) which increases selection pressure for AMR. Whilst there appears to be a preference for oxytetracycline use, there is also high use of penicillin-streptomycin. Where an environmental sample was also an ESBL-producer, this was also a risk factor for MDR bacteria in humans and animals. Surprisingly, the ESBL outcome did not have any significant variables implicating its risk. A study conducted by Dohman et al also suggests that current or recent AMU was not significantly associated with isolation of ESBL-*E. coli* on pig farms, but use of cephalosporins sometime in the past increased the risk of sampling ESBL-producing *E. coli* from pigs (Dohmen et al., 2017). As there was no documented usage of 3rd generation cephalosporins on farms in this study, it is possible that the same phenomenon is occurring in this setting. However, the number of ESBL-producing *E. coli* in this study was low compared to others, and so low power may have had some impact on the ability to detect risk factors.

For the ampicillin resistance outcome, use of penicillin-streptomycin was also a risk factor. There are numerous described examples in the literature of one antimicrobial potentially driving cross-resistance to another (Langsrud et al., 2004; Horinouchi et al., 2017; Adamus-Bialek et al., 2019) due to carriage of multiple AMR genes on plasmids. Within this study (**Chapter 4**) a number of

resistance genes were found to be carried by each isolate, and *bla*$_{TEM-1B}$ was highly prevalent in the study population. This gene was suggested to have originated in food-producing animals and transmitted to other *E. coli* by HGT of virulence and antibiotic resistance genes – more importantly, this gene confers resistance to ampicillin (Johnson and Nolan, 2009; Rebbah et al., 2018). Increasing farm size was associated with increased sulfathiazole resistance. This may be due to larger farms using greater quantities of antimicrobials to treat infections, thus transmission is enhanced in larger farm sizes. In poultry, sulfa-drugs are routinely used for treatment, as well as for growth promotion in the form of sulfadiazine and sulfadimidine (Sasanya et al., 2005; Mubito et al., 2014). As the preferred mode of delivery of antimicrobials for poultry is in communal water sources, the dosing may be incorrect and lead to a higher concentration of circulating sulfonamides in both the water and animals. It would therefore be difficult to ascertain the true dose of the antibiotic ingested by each animal. For tetracycline and trimethoprim resistance, animals being vaccinated was a significant risk factor. It is unclear what vaccinations were used or whether antibiotic use is lower or higher in vaccinated animals in Kenya, as in other populations. Future studies should also look at the vaccination status of animals and the understanding that people have regarding vaccination; according to the questionnaire study (**Chapter 3**) there was confusion between vaccinations and injectable antibiotics. This could mean that there is a higher use of antibiotics than is reported with some confusion between vaccines and antibiotic therapies.

Within this population, the proportion of variance attributed to region (sub-counties) ranged from 0.015 to 0.688. This is expected, as the animals, humans and environments encountered within each sub-county were likely to be more similar than in other sub-counties (the size of sub-counties ranges from approximately 10-20km across). This could be due to clustering of farm management practices, which may be marginally different, according to the dominant tribe in the sub-counties e.g. Teso (in Teso North and South) or Luhya (In the other 5 sub-counties). However, despite stratifying farms by sub-county, no formal weighting was used to correct the responses to questionnaire data. As such tribe could not be used as a factor in any formal comparisons, and only speculation can be made on whether that contributed to variance. Similarly, as no other studies have been conducted in this area examining differences in farm management practices by tribe, there is no data to compare to. The variance attributed to farms varied for different model outcomes, there were two large (and two smaller) identified clonal groups associated with ESBL carriage, though unlike non-clonal groups of isolates, the spread of these isolates was often within an entire sub-county, not limited to individual farms.

There were several limitations to this study; the questionnaire focused primarily on current practices, though a number of questions also asked about historical exposure and use of antimicrobials i.e. last 3 or last 12 months. Validation of the information provided was often not possible as record-keeping was found to be largely incomplete, and there was no corroboration from treating AHAs or veterinary officers. Furthermore, when constructing the multivariable models, there was a low prevalence of AMR for some outcomes, so these were omitted. This study shows that the scale of sampling should be increased in order to identify a larger number of AMR isolates so that risk factor analysis can be undertaken for all outcomes. In future studies, the estimated prevalence of carriage of AMR *E. coli* from this study can be used in future sample size calculations.

Regarding the use of WGS to infer sharing of AMR genes, all reads were mapped directly to a single reference genome, which despite being a common practice in viral genomics, will likely have caused us to miss a significant proportion of diversity by forcing alignment to a single *E. coli* genome. In future studies, rather than using a reference genome to map all reads to, it would be more appropriate (and yield additional relevant variant data) if all contigs were constructed *de novo,* and then scaffolds are used as a backbone to which reads could be mapped. Furthermore, as samples were only collected at a single time point, and in the case of animals, from deposited faeces, the true diversity of all animal *E. coli* was not sampled. Longitudinal sampling of the study site may allow for the construction of time-dependent Bayesian phylogenies (using relaxed clock models) which could allow for inference of directionality between humans, livestock and the environment (by measuring the variation in SNPS over time), opposed to only observing AMR gene similarities. Despite collecting many environmental samples using boot swabs, rather than limiting the study to human living areas, future studies should also examine the *E. coli* found on farms (not just human living areas), as only a relatively small proportion of *E. coli* were cultured from these boot swabs. Despite these limitations, the data presented here is an important set of results in an otherwise data-poor research landscape, and they will help guide future surveillance activities in this region.

Within this study, the prevalence of AMR in commensal enteric *E. coli* in humans and animals was investigated, as well as the *E. coli* isolated from environmental and water sources. The genetic make-up for a subset of these isolates was assessed and two major genetically similalr (potentially clonal) groups (and two smaller genetically similar groups) associated with carriage of ESBL resistance genes ($bla_{\text{CTX-M-15}}$) were found. This has implications for public and animal

health, and efforts should be directed to determining the origin and transmission of these genes. These data clearly show that in this complex ecology, sharing of bacteria is occurring and there is potential for the emergence of AMR strains of both human and animal health relevance. Several risk factors for the carriage of AMR and ESBL *E. coli* have been identified in human, animal and environmental populations in western Kenya, and these indicate that there is increased risk of AMR owing to AMU. Limitations on the access to antimicrobials for animals, and increased diagnostic capabilities could help to determine resistance patterns prior to treatment, and guide appropriate therapies, reducing reliance on empirical broad-spectrum therapies. The high prevalence of AMR, MDR and ESBL-producing *E. coli* reported in this study is of great concern; the high diversity of resistance and virulence genes found in commensal *E. coli* could potentially be disseminated to other commensal and pathogenic bacteria such as *Salmonella spp.* which may spread through the food chain. We suggest that there is urgent need to monitor AMR bacteria such as *E. coli* in all sectors, notably in food-producing animals, in humans and the environment they share, in order to better understand the evolution and transmission dynamics of resistant bacteria in this area.

Chapter 5

Antibiotic Resistance Patterns and Characterisation of faecal *E. coli* Isolated from Slaughterhouse Workers in Western Kenya

5.1 Introduction

E. coli is a commensal bacterium found amongst the intestinal microflora of humans and a wide variety of animals. It is a zoonotic bacterium which is important in both human and veterinary health, due to the risk of transmission between the two host groups. Whilst many *E. coli* are benign, there are highly virulent and enteropathogenic livestock-associated strains, such as O157:H7, which can be transmitted to humans (Belanger et al., 2011; Carrie-Ann et al., 2017; Sarowska et al., 2019). Slaughterhouse workers have extremely close contact with animals, specifically faeces and internal organs, during the slaughter process, and as such, they may represent key contact points for bacterial transmission. Previous studies have identified links with the zoonotic spread of livestock-associated bacteria during the slaughter process (Mulders et al., 2010; Gilbert et al., 2012), indicating that workers may be at higher risk of occupational acquisition of pathogenic *E. coli.* Of the *Enterobacteriaceae*, *E. coli* is extremely common, and pathogenic strains associated with MDR and ESBL-producing resistance genes are considered to be highly infectious, with the potential for rapid transmission, particularly between humans (Pitout and Laupland, 2008). As such *E. coli* (specifically carbapenem and 3rd-generation cephalosporin-resistant) is recognised by the WHO as a bacterium to guide research, discovery, and development of new antibiotics (WHO, 2017a) to attempt to limit some of the dangers of acquiring AMR *E. coli*.

E. coli ST131 is associated with the worldwide spread of the $bla_{\text{CTX-M-15}}$ ESBL resistance gene, and has a strong association with MDR, including to fluoroquinolones (Johnson et al., 2010) and more recently, strains have been identified which are resistant to carbapenems (Peirano et al., 2014). The presence of ST131 is considered to be a public health threat, and indeed, there is potential for the spread of ESBL-producing *E. coli* from animal carcasses to slaughterhouse workers from animal carcasses (Bardon et al., 2013) to humans during the slaughter process.

Foodborne *E. coli* is one subset of bacteria highlighted by the WHO Foodborne Disease Burden Epidemiology Reference Group as being a global public health concern due to their role in meat contamination and food-borne disease transmission (Hoffmann et al., 2017), particularly amongst beef (cattle) and other small ruminants. Several public health concerns are linked to slaughter processes in sub-Saharan African countries. Inadequate infrastructure, poor hygiene, lack of ante- and post-mortem inspection, and inadequate

training can result in meat contamination, allowing for the transmission of pathogens along the supply chain (Herenda et al., 1994; Heinz, 2008; Mekonnen Haileselassie et al., 2013; Basulira et al., 2019). In Ethiopia and Uganda, bovine tuberculosis, toxoplasmosis and porcine cysticercosis have all been detected post-inspection, corroborating that the meat inspection process may be inadequate (Muwonge et al., 2012; Gebremedhin et al., 2014; Thomas et al., 2016). Indeed in a study examining the working conditions and practices of slaughterhouses in Kenya, some workers (9%) admitted to slaughtering sick animals, exposing both themselves and potential consumers to zoonotic diseases (Cook et al., 2017) and indicating that inspection was inadequate. This may also be due to lack of regulation enforcement as approximately 93% of slaughter slabs were inspected daily by meat inspectors, but only 7% performed an antemortem examination of animals (Cook et al., 2017).

In western Kenya, Cook et al (2017) conducted a thorough investigation of the working conditions and practices of slaughterhouses. A number of potential risk factors for the spread of zoonotic disease were identified, for example, few slaughterhouses (3%) had access to running water and many did not have appropriate sanitation facilities such as toilets (12%) or soap (64%) for handwashing. Few workers wore personal protective equipment (PPE), such as aprons or gloves (32%), important for preventing interaction with blood and viscera during the cleaning and splitting of carcasses. In almost all slaughterhouses, a practice of 'batch slaughtering' was followed, slaughtering, bleeding, skinning, and evisceration were all performed in the same area, on the ground (Cook et al., 2017). This practice is also reported in other EU countries, such as southern Ireland (Wheatley et al., 2014). Furthermore, 25% of workers had reported being injured at work and 8% had an open wound during the study. Open wounds, linked with the lack of PPE is a significant risk for bacterial transmission (Cook et al., 2017).

The situation described is generally indicative of a low level of hygiene in these key nodes of the food chain. Inadequate training in food safety and slaughter practices resulted in higher bacterial loads of *E. coli*, *S. aureus* and *B. cereus* on slaughterhouse meat in Ethiopia (Mekonnen Haileselassie et al., 2013). Similarly, poor training and education of meat inspectors have been associated with an increased risk of foodborne pathogens in Kenya (Kariuki et al., 2013). Finally, there has been a clear link to the lack of PPE worn by

slaughterhouse workers and the contraction of brucellosis in Uganda and Tanzania (Swai and Schoonman, 2009; Nabukenya et al., 2013).

There are three classes of slaughterhouses (A, B and C) in Kenya, according to the size and whether meat is intended for local consumption or transport out of the community (Cook et al., 2017). Slaughterhouses are sub-divided into ruminant (cattle, sheep/goats) or pig-only slaughterhouses, to respect the Muslim community. Many slaughterhouses in rural areas are informal, unregulated and commonly referred to as 'slaughter slabs'. These facilities are usually privately owned and rented to butchers who employ their own team of slaughter workers (Kagira and Kanyari, 2010; Roesel and Grace, 2014). It is plausible that slaughterhouse workers can act as 'sentinels' for emerging zoonotic diseases (Abu-Elyazeed et al., 1996; Rabinowitz et al., 2009; Nguku et al., 2010; Cook, 2015).

Three main types of slaughterhouse were considered in this study, those which slaughtered cattle only, cattle and sheep and goats, and pigs only. The purpose of this study was to assess the carriage of AMR *E. coli* in the faeces of slaughterhouse workers from 93 slaughterhouses in Busia county and to assess the carriage of resistance and virulence genes of *E. coli* by WGS, with a specific focus on the carriage of MDR and ESBL-producing *E. coli.*

5.2 Methods

All methods in this chapter prior to bacterial culture of *E. coli* and WGS were conducted by Elizabeth Cook and colleagues (ILRI, Nairobi) during the collection of the original samples; slaughterhouse locations and details of recruitment and questionnaire results are published in the manuscript 'Working conditions and public health risks in slaughterhouses in western Kenya' (Cook et al., 2017) and described in brief here. Further information was obtained directly by personal communication. Informed consent collected by Cook et al. at the time of the study, allowed subsequent re-use of archived specimens for further research. This chapter makes use of those original samples.

5.2.1 Study Population and Recruitment

A census of slaughterhouses was undertaken in Busia and three other surrounding counties (Bungoma, Kakamega and Siaya) to quantify the total number of official slaughterhouses in each county. Data were provided by county directors of veterinary services, who were responsible for delegating meat inspections. All consenting slaughterhouses were recruited for the study (n=142). In slaughterhouses with 12 workers or less, all consenting participants were recruited. In slaughterhouses with more than 12 workers, a random selection of 12 consenting participants from the workers present on the day were sampled.

5.2.2 Sample Collection

Samples and questionnaire data were collected between February and October 2012. Participants were asked if they were willing to provide a stool sample and informed consent was obtained from all participants individually; those who were amenable were provided with a stool pot with an integrated spoon in the lid. Participants were instructed on how to collect a large teaspoon of a sample, which was not contaminated with urine or had been in contact with the ground. After receiving stool samples, two cotton swabs were inserted into the stool, removed, and examined to ensure they had come into contact with stool. Swabs were then immediately inserted into a tube of Cary Blair transport medium (Transwab®, Medical Wire, Wiltshire). Samples were placed in a cool box and transported back to the lab for initial processing. The original study was undertaken with full informed consent and ethical approval from the Kenya Medical Research Institute (SCC Protocol 2086).

5.2.3 Faecal Culture

Faecal samples were processed at the time of collection before they were provided for use in this study. Briefly, a swab was removed from Cary Blair transport medium and incubated overnight in selenite F broth (Oxoid, Hampshire, UK), at 37°C. The following day, a sterile loopful of broth was inoculated onto one MacConkey agar plate and one Xylose lysine deoxycholate (XLD) agar plate; these were then incubated overnight at 37°C. Following incubation, plates were assessed for fermentation and hydrogen sulphide production. A nutrient agar plate was used to sub-culture colonies from both the XLD and MacConkey plates. *E. coli* was identified according to morphology. Colonies were then frozen at -40°C in tryptone soya broth (TSB) broth containing 20% glycerol in Busia, before being shipped to Nairobi for long-term storage at -80°C.

5.2.4 Sample Acquisition and Shipping

A 1ml aliquot was taken from each of the located samples from a -80°C long-term storage freezer in Nairobi, transferred to labelled cryovials, and shipped on ice back to the University of Liverpool for culture, antibiotic resistance testing and WGS (on a subset). All samples from Busia sub-county were located, but those from surrounding sub-counties (Bungoma, Siaya and Kakamega) were not all located in freezers. As such, comprehensive resistance analysis (as performed in (**Chapter 4**) were performed on *E. coli* isolated from Busia slaughterhouse workers, only.

5.2.5 *E. coli* Culture at Liverpool University

At the University of Liverpool, samples were resuscitated by streaking one 5µl loopful of the aliquot onto a plain EMBA plate and incubating aerobically overnight at 37°C. From this, bacteria were sub-cultured for single colonies onto three EMBA plates, one containing cefotaxime (1µg/ml), one containing ceftazidime (1µg/ml) and one containing no antimicrobials. From the plain EMBA plate, five random picks of bacterium that were morphologically-consistent with *E. coli* (green metallic, shiny) were selected and sub-cultured onto nutrient agar. From the EMBA plates containing cefotaxime and ceftazidime, one pick was randomly selected for sub-culture.

5.2.6 Antibiotic Resistance Testing

1247 isolates from 447 slaughterhouse workers were sub-cultured onto nutrient agar and subject to disc diffusion testing, using a panel of seven antibiotics. Suspensions were made according to EUCAST guidelines (EUCAST, 2018). MHA plates were inoculated with

each isolate and seven antimicrobial discs applied: ampicillin (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), gentamicin (10µg), sulfathiazole (1000µg), tetracycline (30µg) and trimethoprim (5µg). Isolates selected from EMBA plates containing ceftazidime or cefotaxime were subject to double-disc diffusion testing (M'Zali et al., 2000), using three pairs of antibiotic discs containing ceftazidime (30µg), cefotaxime (30µg) and cefpodoxime (30µg) with and without clavulanic acid (10µg). All plates were incubated aerobically overnight at 37°C and zones of inhibition were measured in mm. Suspected ESBL production was confirmed if the zone of inhibition for the cephalosporin with clavulanic acid disc was more than 5mm greater than the zone of its counterpart without clavulanic acid (Giske CG, 2013).

5.2.7 PCR Assays

PCR assays targeting the *uidA* gene (McDaniels et al., 1996) were performed as previously described **(Chapter 2.5.3)** on all isolates to confirm that they were *E. coli*.

5.2.8 Whole Genome Sequencing

Whole Genome sequencing service was provided by MicrobesNG (http://www.microbesng.uk). All isolates representing *E. coli* with unique MDR or ESBL phenotypes were selected for sequencing. In total, 188 isolates were selected for sequencing and this subset was chosen primarily due to funding limitations, and the desire to investigate only isolates which were MDR or ESBL-producing *E. coli*.

5.2.8.1 DNA Extraction

All frozen isolates were resuscitated from -80°C using the previously described methods. Isolates were provided to MicrobesNG in barcoded MicroBank™ tubes for sequencing. Briefly, isolates were cultured on nutrient agar and incubated overnight at 37°C. The following day, one 5µl loopful of each culture was added to each tube and sent to MicrobesNG.

5.2.8.2 DNA Sequencing

The MicrobesNG laboratory performed the following steps and provided this protocol as-is:

Three beads were washed with extraction buffer containing lysozyme and RNase A, incubated for 25 min at 37°C. Proteinase K and RNaseA were added and incubated for 5

min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer.

DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: 2ng of DNA instead of 1ng were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.

Reads were then downloaded from the MicrobesNG server, and the same WGS workflow as previously defined (and briefly redefined below **5.2.9.2**) was performed.

5.2.9 Data Analysis

5.2.9.1 Antibiotic Resistance Data

Microbiological data were entered in a spreadsheet initially (Microsoft Excel 2016, Microsoft Corporation) and reviewed to ensure accurate input. A binary coding system was implemented such that data indicated an isolate that was either susceptible (0) or resistant (1), using EUCAST human clinical breakpoints (EUCAST, 2018) for all antimicrobials, except for tetracycline and sulfathiazole. Tetracycline was based upon BSAC human clinical breakpoints (BSAC, 2015) and sulfathiazole was based on the distribution of zones of inhibition, as described in (**Chapter 2.5.1)**. Descriptive statistics and Chi-squared tests for association were conducted in SPSS v25 (IBM Corp, Armonk, NY).

5.2.9.2 WGS Workflow

The standard workflow as described in (**Chapter 2.6.1)** was run on all sequences. Briefly, raw paired-end reads were quality controlled using FastQC v0.11.7 (Andrews, 2010) Poor quality reads (score <20) and any detected primers or adapters were removed using Trimmomatic v0.36 (Bolger et al., 2014). Forward and reverse reads were then mapped to a reference *E. coli* genome (*E. coli* K12 MG1655; NCBI Reference Sequence: NC_000913.3 (Blattner et al., 1997), using the Burrow-Wheeler Alignment (bwa mem

v0.7.17) (Li, 2013). Once BAM files had been made, a further QC step to check the mean mapping quality scores and coverage in relation to the reference genome was performed with QualiMap2 (Okonechnikov et al., 2015). Any reads which did not map to the reference genome were assembled *de novo* into contigs, using SPADES v3.12.0 (Bankevich et al., 2012). Speciation analysis of those contigs was then performed using Kraken v2.0.7 (Wood and Salzberg, 2014) and an in-house database downloaded from NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/sra/). Non-*E. coli* contigs were excluded from analysis. Phylogenetic trees were constructed using IQ-Tree (Nguyen et al., 2015), using 1000 bootstrap replicates and the GTR model Tree topology was checked using Figtree v1.4.4 (Rambaut, 2012) and then annotated using the interactive tree of life (iTOL) v5.0 (Letunic and Bork, 2016). MLST was determined using a batch script which scanned each contig against the *E. coli* #1 schema (https://pubmlst.org/escherichia/) (Jolley and Maiden, 2014) – from this a sequence type (ST) was assigned according to the *adk, fumC, gyrB, icd, mdh, purA* and *recA* loci. *In silico* phylotyping was serotyping were formed using the Clermont Typing tool (Beghain et al., 2018) and SerotypeFinder (Joensen et al., 2015) respectively. Plasmid replicon typing was performed using the PlasmidFinder database using the batch uploading platform (identity ≥90%, coverage ≥60%) (Carattoli et al., 2014). For detected ST131 isolates, *fim* typing was undertaken using a custom script to blast genomes for *fimA* and *fimH* genes. Literature was then searched to match the fimtype to known ST131 isolates.

5.2.10 Maps and Georeferenced Data

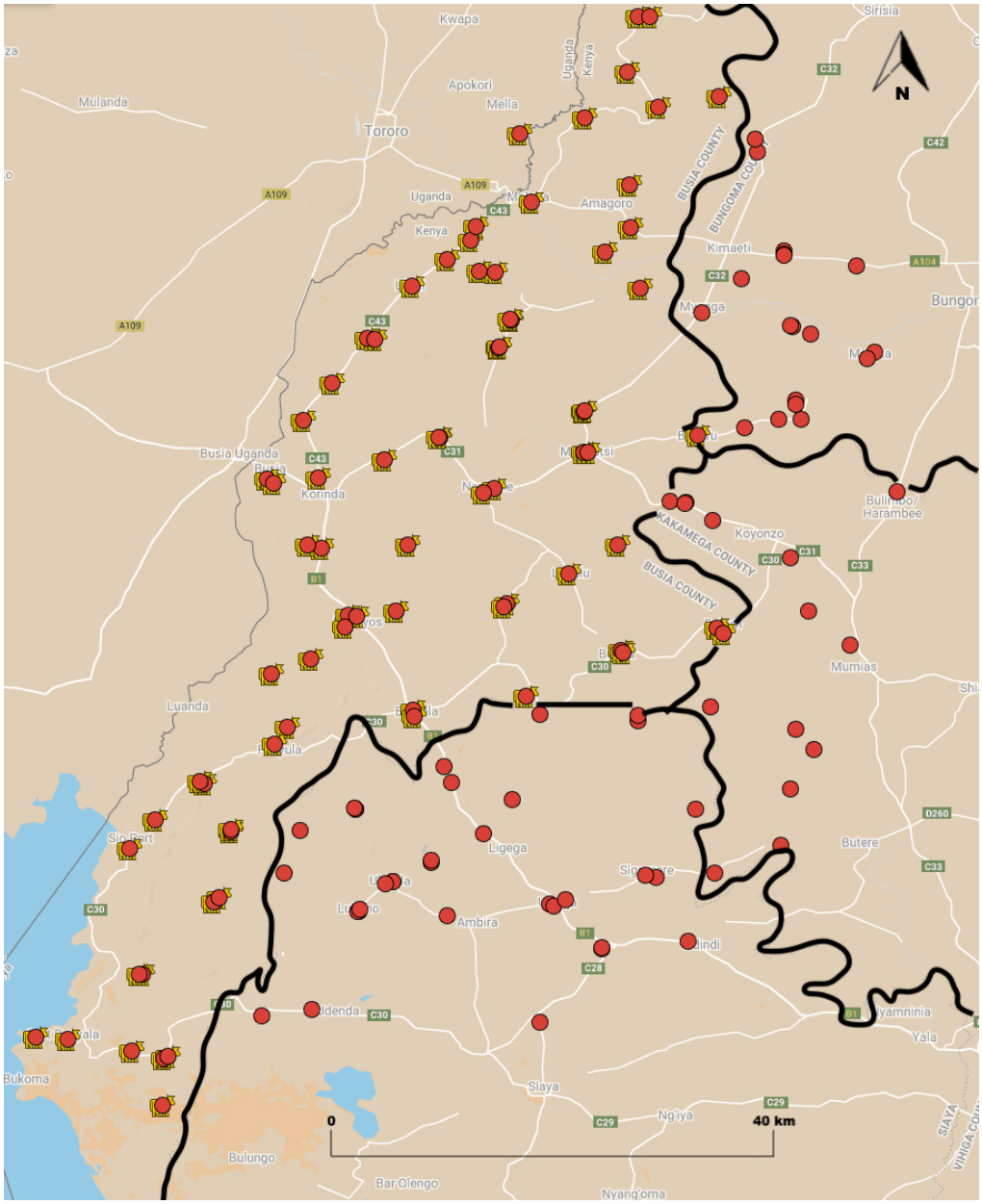
Maps were constructed using QGIS v3.2 (QGIS Development Team, http://qgis.osgeo.org/). Geographic co-ordinates of each slaughterhouse had been captured with a portable GPS device (Garmin eTrex®) during the original study. A base layer of Busia county was taken from Google Maps – upon this, major western Kenyan towns, and cities (boundary polygons and line shapefiles) were layered. These were acquired from an open-source database hosted by the Humanitarian Data Exchange (https://data.humdata.org/). Latitude and longitude co-ordinates for each slaughterhouse were imported into QGIS and maps generated.

5.3 Results

5.3.1 Samples Collected

In total, 1245 *E. coli* isolates were cultured from the faeces of 447 slaughterhouse workers, from 93 different slaughterhouses in Busia, (and neighbouring) Bungoma, Siaya and Kakamega counties (**Figure 5.1**). The original study collected information and samples from 738 slaughterhouse workers from 142 slaughterhouses, our sample size only contains samples from Busia county, however.

Figure 5.1 Geographical distribution of all slaughterhouses where samples were collected, and questionnaires given in the original study conducted by Cook et al (2017). Each individual slaughterhouse is identified with a red circle. Samples which contributed to this study are indicated with a yellow cattle icon (all slaughterhouses in Busia country, n=93)). Counties are (clockwise from most westerly) Busia, Bungoma, Kakemega and Siaya. Map base layer from Google Maps (2017).



5.3.2 Types and Spread of Slaughterhouses

Slaughterhouses were spread across 15 different townships, within 4 main sub-counties. Slaughterhouses were divided according to the type of animals that were slaughtered there: only pigs, only cattle or cattle, sheep, and goats. The largest number of slaughterhouses were in Bumala, Teso and Busia township, all within Busia county. There were variations in the types of slaughterhouse found in each township, in Busia there was predominantly slaughter of pigs only; this was the only township where the number of pig slaughterhouses outnumbered ruminant slaughterhouses. In many townships (7/15), cattle-only were the most common type of slaughterhouse, followed by cattle and shoat slaughterhouses (6/15).

5.3.3 Prevalence of AMR *E. coli*

E. coli was successfully cultured from 447 human faecal samples; 38.0% (n=170) from workers in cattle only slaughterhouses, 32.4% (n=145) from workers from cattle and shoat slaughterhouses and 29.5% (n=132) from workers from pig-only slaughterhouses. Twenty-two samples (4.9%) had completely sensitive *E. coli* isolates. Four hundred and twenty-five samples had isolates which were resistant to at least one class of antimicrobial. The sample prevalence of resistance to each of the tested antibiotics, as well as MDR and ESBL-producing *E. coli* are detailed in **(Table 5.1).** There were 205 (61.5%) MDR *E. coli* isolate-containing samples. Of these 80 MDR samples (17.9%) had *E. coli* which were resistant to 3 classes of antibiotic, 140 (31.3%) to 4 classes, 40 (8.9%) to 5 classes, 12 (2.7%) to 6 classes, and 3 (0.7%, extensively drug resistant (XDR) had isolates resistant to all 7 tested antimicrobials. The largest proportion of MDR *E. coli* (3 or more classes) were isolated from humans working in mixed ruminant slaughterhouses (cattle, sheep and goats). ESBL-*E. coli* was found in 46 samples (10.3%), the largest proportion of which were isolated from humans working in pig-only slaughterhouses. Isolates resistant to between 5 and 7 classes of antimicrobial were evenly spread across humans working in cattle and shoat, and cattle-only slaughterhouses. All three XDR isolates were isolated from humans working in pig only slaughterhouses. Additionally, two of the XDR Isolates were also ESBL producers.

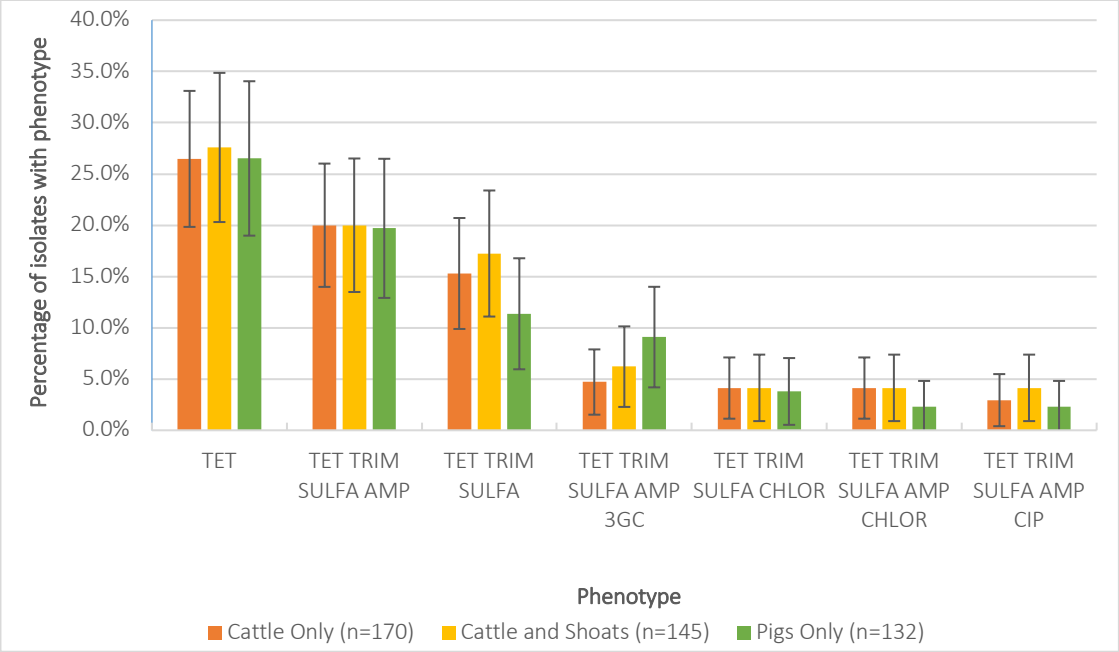
The 7 most common phenotypic resistance patterns were similar between workers working at each of the three types of slaughterhouse **(Figure 5.2).** Overall, the most common resistance phenotype was tetracycline (TET) only, which was observed in approximately a quarter of all samples. Tetracycline resistance featured in nine of the ten

most common resistance phenotypes. Many of the most common phenotypes were MDR, the TET-AMP-SULFA-TRIM phenotype was the most common for MDR isolates. All 46 ESBL isolates were also MDR and were also commonly associated with TET-SULFA-AMP-TRIM phenotypes. Cattle and shoat workers had the highest proportion of MDR isolates, but pig-only workers had the highest proportion of ESBL isolates. Relatively few phenotypes contained ciprofloxacin resistance, but these were generally associated with an MDR phenotype.

Table 5.1 Proportion of AMR, MDR and ESBL-*E.coli* found in 447 samples. All isolates cultured from human faeces from slaughterhouses slaughtering cattle and sheep/goats, cattle only or pigs only. Brackets beneath each proportion indicate 95% CI.

Antimicrobial Resistance	Cattle and Sheep and goats (n=145)	Cattle Only (n=170)	Pigs Only (n=132)	Total (n=447)
Tetracycline	99.3% (97.9 - 100.7)	90.6% (85.8 - 95.3)	85.6% (79.9 - 91.3)	86.2% (80.6 - 91.8)
Trimethoprim	69.0% (61.4 - 76.5)	60.6% (52.6 - 68.5)	60.6% (52.7 - 68.6)	59.3% (51.3 - 67.3)
Sulfathiazole	64.8% (57.1 - 72.6)	59.4% (51.4 - 67.4)	59.9% (51.9 - 67.8)	57.4% (49.4 - 65.4)
Ampicillin	45.5% (37.4 - 53.6)	38.2% (30.3 - 46.2)	47.7% (39.6 - 55.9)	40.7% (32.7 - 48.7)
Chloramphenicol	11.7% (6.5 - 17)	10.6% (5.6 - 15.6)	9.1% (4.4 - 13.8)	9.9% (5 - 14.8)
Ciprofloxacin	10.3% (5.4 - 15.3)	7.7% (3.3 - 12)	6.8% (2.7 - 10.9)	7.8% (3.4 - 12.2)
Gentamicin	4.8% (1.3 - 8.3)	3.5% (0.5 - 6.5)	6.1% (2.2 - 9.9)	4.4% (1.1 - 7.7)
MDR (n=275)	63.5% (55.6 - 71.3)	42.0% (34.6 - 49.4)	49.2% (41.1 - 57.4)	61.5% (57.0 - 66.0)
ESBL (n=46)	11.8% (6.6 - 17.1)	6.5% (2.8 - 10.2)	13.6% (7.8 - 19.5)	10.3% (7.5 - 13.1)

Figure 5.2 The most commonly identified antimicrobial resistance phenotypes, divided by slaughterhouse type. The figure reflects the overall proportion of isolates displaying a specific antimicrobial resistance phenotype; 477 unique *E. coli* isolates (from 477 samples) were examined. Phenotypes with a proportion of <5% were excluded from this figure. Error bars are 95% CI.



TET = Tetracycline, **SULFA** = Sulfathiazole, **AMP** = Ampicillin, **TRIM** = Trimethoprim, **GENT** = Gentamicin, **3GC** = 3rd Generation Cephalosporin

5.3.2 Whole Genome Sequencing

The rationale for this study was to investigate carriage of MDR and ESBL-producing *E. coli* from the slaughterhouse study, to assess if there was specific sharing of multiple resistance genes between slaughterhouse workers. Due to financial restrictions, 188 isolates were randomly selected (using random number generation) selected for sequencing. Of those, 187 isolates were successfully sequenced and passed QC.

Following a comparison of resistance gene methodologies outlined in (**Chapter 4**), the same databases were used to assess carriage of resistance (ResFinder, October 2018) (Zankari et al., 2012) and virulence (VirulenceFinder, October 2018) (Joensen et al., 2014). In total, the following number of isolates from humans working in different slaughterhouses were sequenced: mixed ruminant (cattle, sheep and goats) n=74, cattle only, n=64 and pigs only, n=49.

5.3.2.1 Genetic Background of Isolates

Following the initial QC of reads, mapping to the reference genome (U00096.3) and subsequent QC of the BAM files (assessing mapping quality scores and mean coverage across the genome), 187 genomes (of 188) were included in the construction of a maximum-likelihood phylogenetic tree (**Figure 5.3**) based on 832038 SNPs. Onto this, results of MLST assignment and serogrouping was layered, to assess similarities and genetic variance between isolates.

As with the sequences in (**Chapter 4**) there was a wide distribution of interspersed *E. coli* from workers at different types of slaughterhouse, with no specific clustering by type of slaughterhouse. This suggests a high degree of diversity of *E. coli* amongst slaughterhouse workers across Busia county. Several distinct clades associated with specific multi-locus sequence types can be seen on the phylogenetic tree.

A ST was successfully assigned to 135 DNA sequences; 52 sequences could not be assigned an ST, as one or more loci did not match perfectly to previously registered MLST alleles. These could be a novel arrangement which are not yet in the database, as for each sequence, alleles of each of the seven housekeeping genes searched for during mlst assignment were found. There were 37 unique ST assigned from the sequences **(Figure 5.4)**; 19 of these STs were associated with single isolates only, and 22 STs were carried by a single type of slaughterhouse worker only. Overall, the most common STs found among *E. coli* were ST10 (21.2%, n=25), ST617 (8.5%, n=10), ST361 (7.6%, n=9), ST48 and ST361 (6.8%, n=8 each). STs were distributed across all three groups of slaughterhouse worker, and this is evident particularly across the more common STs. Where workers from different types of slaughterhouse shared an ST, there was no significant difference (Mann-Whitney U, $p>0.05$) between the prevalence of that ST. The largest number of unique STs were isolated from *E. coli* from pig-only slaughterhouse workers (n=20). In total, eight isolates were found to belong to ST131; the highest proportion of these were also from pig-only slaughterhouse workers, though there was no significant difference between the proportion carried between different workers working at different types of slaughterhouse. Several distinct clades with low genetic variability (<500SNPs) can be seen, associated with ST131, ST361, ST10, ST43 and ST617.

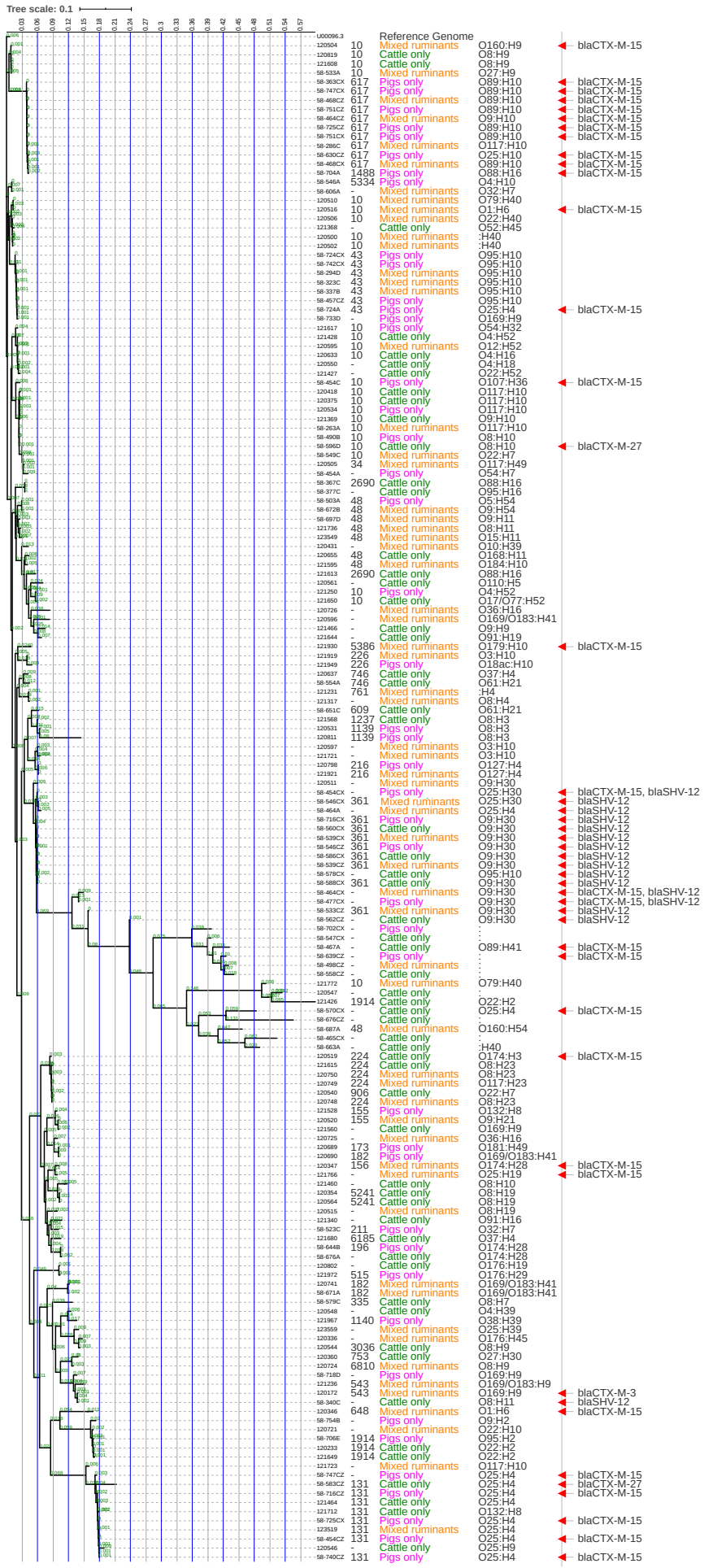


Figure 5.3 Maximum likelihood phylogenetic tree (using 832038 SNPs) of *E. coli* (n=187) isolated from humans working in three different types of slaughterhouse. Key: From left to right, 1st number is isolate identifier, MLST, type of slaughterhouse and serogroup. Red triangles indicate ESBL-producing *E. coli* and the genes which confer the ESBL phenotype are adjacent. All isolates are MDR (to at least 3 classes of antimicrobial) and/or carry an ESBL resistance gene. 5 clades with very low genetic variety (<0.0008, <650 SNPs) are visible on the tree. Green text on branches is branch length (rounded to 3dp).

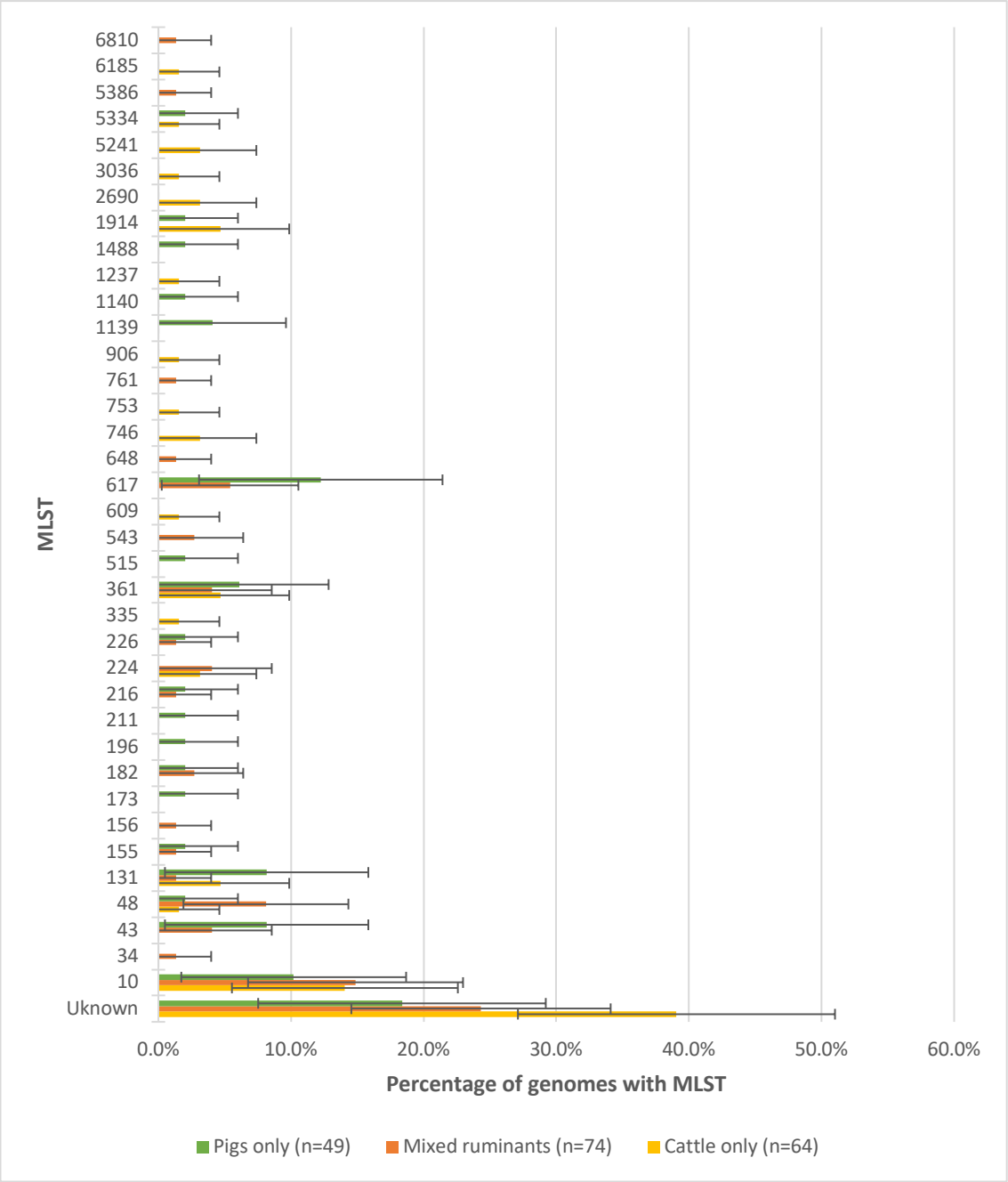


Figure 5.4 Distribution of multilocus sequence types, differentiated by the type of slaughterhouse.

All isolates were serotyped *in silico* to assign lipopolysaccharide (O) and flagellar (H) surface antigen groups. Of these, 8 were not assigned either an O or H group, 4 were only assigned an H group and 1 isolate was only assigned an O group. Overall, there were 86 unique O:H serotype assigned. Most of these groups were assigned to only one or two isolates. The two most common serotypes were O9:H30 (7.1%) and O25:H4 (6.5%). Individually, the most common O groups were O8 (13.7%), O9 (11.3%) and O25 (10.7%). Individually, the most common H groups were H10 (20.1%), H4 (10.7%) and H30 (8.9%).

When divided according to slaughterhouse type, the most common serogroups shared overlap between isolates from workers in cattle-only and cattle and shoat slaughterhouses, but not to isolates from pig-only slaughterhouse workers. In isolates from cattle and shoat workers, the most common serotypes were O95:H10 (7.7%), O25:H4 (7.7%) and O9:H30 (6.4%). For isolates from cattle only workers, the most common serotypes were O9:H30 (8.5%), O89:H10 (7.0%) and O25:H4 (7.0%). Isolates from pig only workers had no clear serotype association with each isolate had a different serotype. Those O25:H4 isolates were especially significant as these were mainly associated with ST131.

Finally, all isolates were phylotyped *in silico*. Thirty-eight isolates could not be phylotyped due to incomplete coverage of one or more of the genes (**Figure 5.5**). The largest proportion of the isolates were phylogroup A (38.5%), followed by B1 (13.9%) and E (13.4%). Phylogroups A and B1 (generally considered to be commensal, non-pathogenic strains) were approximately evenly distributed across all three types of slaughterhouse worker. Phylogroups B2 and D (generally considered to be more pathogenic) appeared to be most prevalent amongst pigs-only and cattle-only workers, respectively. Phylogroup E was least prevalent in pig-only workers. Finally, a single phylogroup F strain was isolated from a mixed ruminant slaughterhouse. There was a significant association between the type of slaughterhouse workers and phylogroup ($\chi^2 = 9.2$, $p<0.001$, Chi-squared test), but no significant difference between the proportions of *E. coli* with specific phylogroups between types of slaughterhouse (Kruskal-Wallis, $p>0.05$).

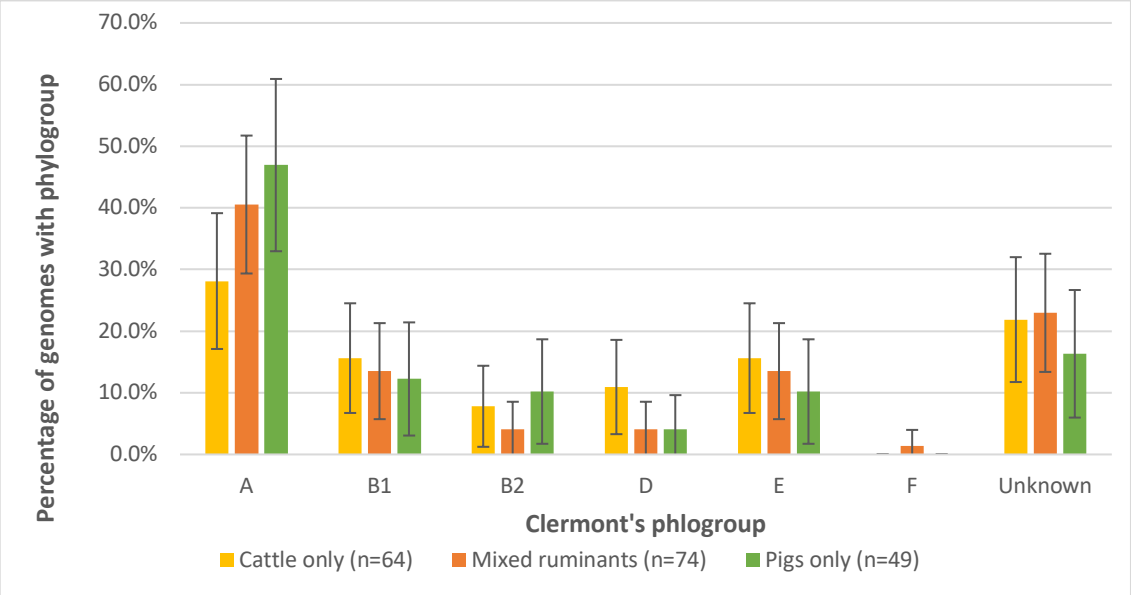


Figure 5.5 Distribution of phylogroups between slaughterhouse workers. Error bars are 95% CI.

5.3.2.2 Resistance Genes

Using the same ResFinder database as in Chapter 4 (October 2018), all detected resistance genes were tabulated, and the prevalence of each gene determined according to slaughterhouse type (mixed ruminants, cattle-only and pig-only). The most commonly identified resistance genes which encompassed two or more of the groups were examined for similarities (**Figure 5.6**).

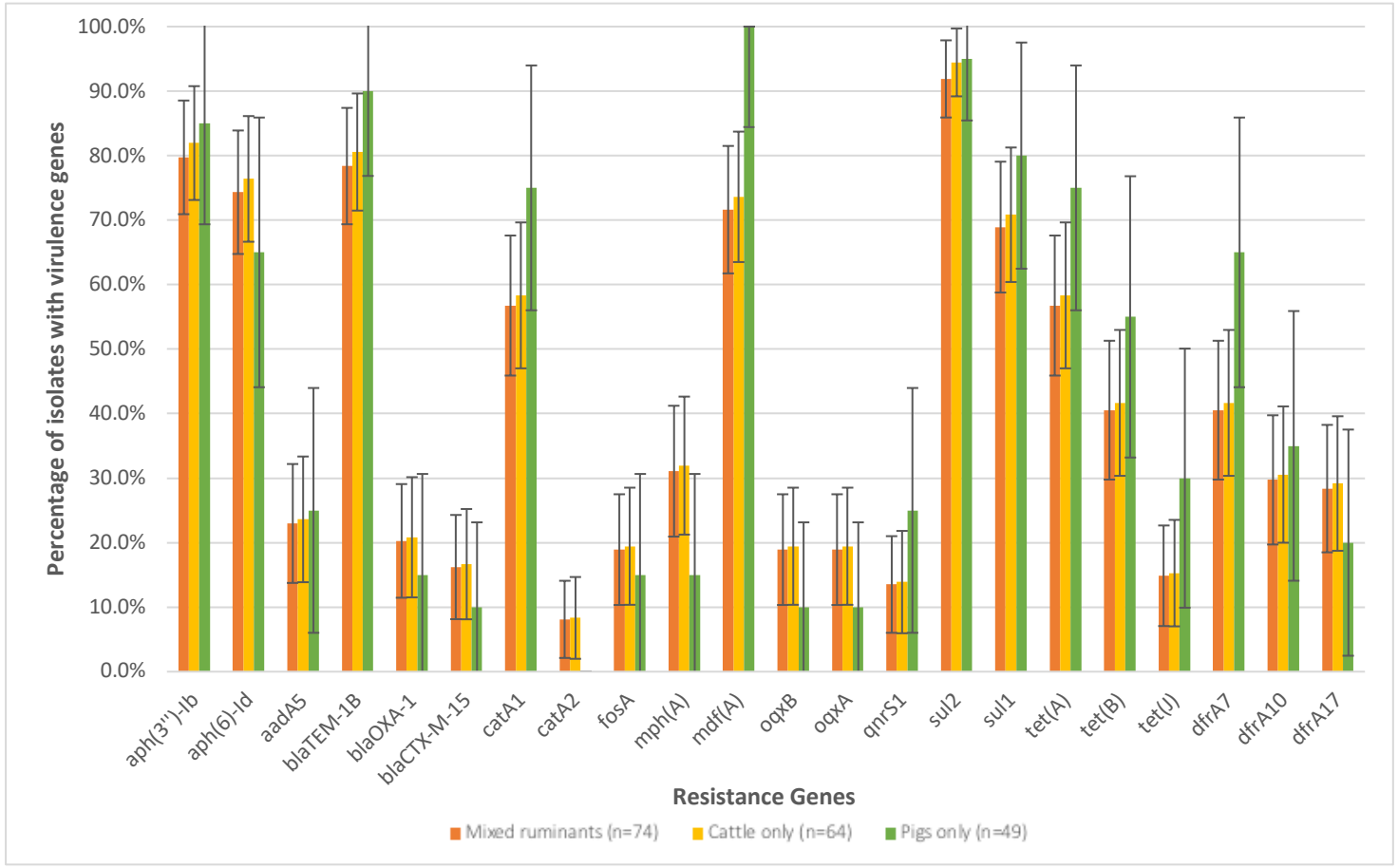
A total of 53 unique resistance genes were detected in 187 of the sequenced genomes. Using the previously defined thresholds for detection of genes (≥90% identity and ≥60% coverage), one genome was found to have no resistance genes. As such, analysis of resistance genes was rerun with a more modest threshold of ≥75% identity and ≥60% coverage. With these thresholds, all genomes were found to carry more than one resistance gene.

The most commonly identified resistance genes included to sulfonamides, aminoglycosides, the $bla_{\text{TEM-1B}}$ beta-lactamase (ampicillin), multi-drug transporters (macrolides-lincosamides-streptogramin) and chloramphenicol. There was no statistically significant difference between the carriage of 20 out of 22 of the most prevalent resistance genes i.e. those carried by human isolates from all three types of slaughterhouse. Carriage of chloramphenicol resistance gene, *catA2*, was not identified in humans working in pig-only slaughterhouses. There was a high prevalence of the multi-drug exporter gene *mdf(A).* In pig-only workers, the carriage of *mdf(A)* was 100%, and significantly (Mann-Whitney, p=0.003) more prevalent amongst pig-only workers than in cattle-only and mixed ruminant workers. There was a high carriage of sulfonamide resistance genes, particularly *sul2,* in all groups, with an average carriage rate of >90%. There was similarly high carriage of *tet* genes, specifically *tet(A), tet(B)* and *tet(J)* amongst all groups.

Two common aminoglycoside resistance genes were prevalent in all three groups: *aph(3")-1b* (also called *strA)* and *aph(6)-1d* (also called *strB).* A third aminoglycoside resistance gene (*aadA5)* was common to all three groups but significantly less prevalent (Mann-Whitney, p<0.001) than the other two.

Three common β-lactamase resistance gene were identified in the sequenced isolates. The most prevalent was $bla_{\text{TEM-1B}}$ which confers ampicillin resistance in *E. coli*. The second most prevalent was $bla_{\text{OXA-1}}$ which confer resistance to ampicillin, cephalothin, oxacillin and cloxacillin. The final

Figure 5.6 The proportion of isolates carrying a selection of 22 of the most common resistance genes, divided according to slaughterhouse type. Error bars indicate 95%CI. Resistance genes with less than 5% prevalence or found in single groups/species only, were excluded from this figure.



Antimicrobial Class	Gene
aminoglycoside	*aph(3'')-Ib*
aminoglycoside	*aph(6)-Id*
aminoglycoside	*aadA5*
β-lactamase	bla_{TEM-1B}
β-lactamase	bla_{OXA-1}
β-lactamase	$bla_{CTX-M-15}$
phenicol	*catA1*
phenicol	*catA2*
fosfomycin	*fosA*
macrolide	*mph(A)*
multi-drug transporter	*mdf(A)*
quinolone	*oqxB*
quinolone	*oqxA*
quinolone	*qnrS1*
sulfonamide	*sul2*
sulfonamide	*sul1*
tetracycline	*tet(A)*
tetracycline	*tet(B)*
tetracycline	*tet(J)*
trimethoprim	*dfrA7*
trimethoprim	*dfrA10*
trimethoprim	*dfrA17*

common gene was *bla*$_{CTX\text{-}M\text{-}15}$ which confers an ESBL phenotype; isolates carrying this gene also frequently co-carried quinolone (*qnr)* and aminoglycoside (*aph*) resistance genes. All the isolates carrying the *bla*$_{CTX\text{-}M}$ gene had an MDR genotype and phenotype. Whilst *bla*$_{CTX\text{-}M\text{-}15}$ was the most prevalent *bla*$_{CTX}$ gene carried, three other ESBL resistance genes were also carried amongst *E. coli*, including *bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{CTX\text{-}M\text{-}27}$ and *bla*$_{SHV\text{-}12}$.

There was also a high proportion of slaughterhouse workers harbouring *E. coli* with point mutations conferring resistance to both nalidixic acid and ciprofloxacin. The most common of these was in *gyrA* (D87N, 22.5%, n=42) and *parC* (S80I, 18.7%, n=35). In addition to these, there were also numerous point mutations found in the 16S and 23S ribosomal RNA (rRNA) genes; in 16S there were several mutations in both rrsB and rrsC. In 3.2% (n=1) genome each, there was r.1192A>G, r.1192T>G, r.1066C>T and r.1192T>A which confer resistance to spectinomycin and also 3.2% (n=1) with r.1058T>C, which confers resistance to tetracycline, doxycycline, minocycline and tigecycline. In rrsC, 6.4% (n=2) genomes contained the r.1519C>G mutation, conferring resistance to kasugamycin. In the 23S rRNA gene, 3.2% (n=1) genomes each also had the r.754T>A mutations conferring resistance to erythromycin and telithromycin and r.2032G>A, conferring resistance to linezolid. There was no significant difference (p>0.005, Kruskal Walis) between the presence of point mutations and the type of slaughterhouse which the workers worked at.

There was a low prevalence of resistance genes for fosfomycin and macrolides (*fosA* and *mph(A)),* though rates of carriage were similar in all three groups.

There was a low prevalence of quinolone and fluoroquinolone resistance genes in all slaughterhouse workers groups; three plasmid-mediated resistance genes, *oqxA*, *oqxB* and *qnrS1* were carried by isolates from all three groups. Both *oqxA* and *oqxB* are part of the *oqxAB* gene complex which encode for the OqxAB pump which confers low-level resistance to ciprofloxacin, and cross-resistance to trimethoprim. The *qnrS1* gene is also plasmid-mediated and confers low-level resistance to quinolones such as nalidixic acid.

5.3.2.3 Virulence Genes

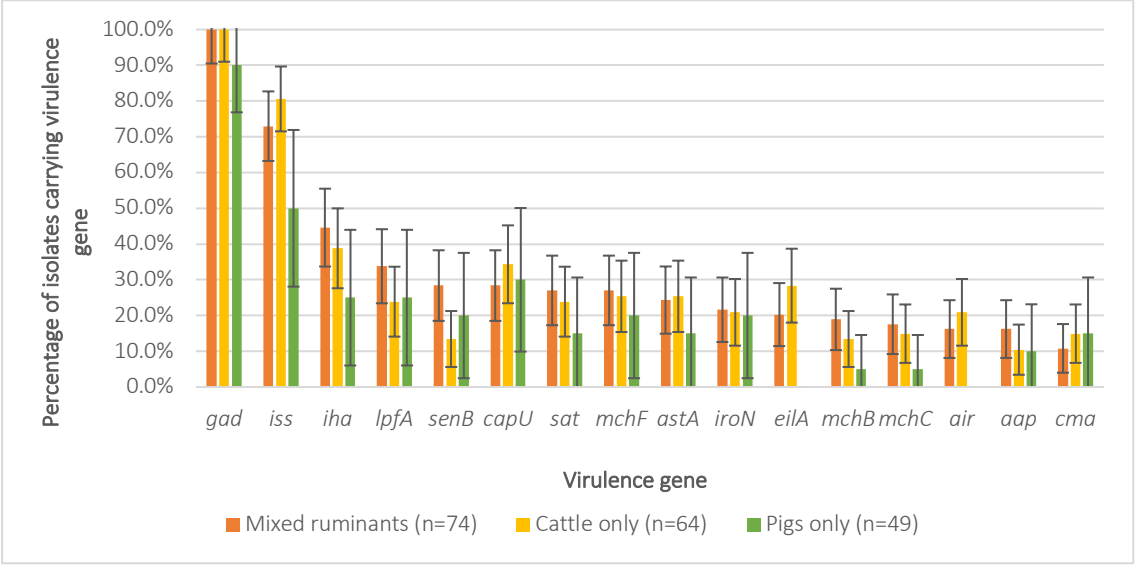
A total of 36 unique virulence genes were identified in all sequenced genomes. Six isolates carried no detectable virulence genes. Most isolates carried more than 3 unique virulence genes, with several isolates carrying more than 10 genes. Diversity of carried genes was similar in all three groups, 13 of the 15 most common genes were carried by isolates from all three

groups. A brief description of the most commonly identified virulence genes is described below **(Table 5.2).** Additional genes were also described in Chapter 4 and Chapter 1. As with the resistance genes, the most commonly identified virulence genes which spanned at least two groups were assessed for comparison of prevalence **(Figure 5.7)**.

Table 5.2 Brief description of the function of the most commonly carried virulence genes from a population of 187 human-origin *E. coli* from workers slaughtering only pigs, only cattle, and cattle and sheep and goats. (References in Chapter 1, Table 1.1)

Virulence Gene	Role	Function
gad	Glutamate decarboxylase	Converts glutamate to GABA; maintains intracellular pH when cells when traversing stomach acid – aids in colonisation.
iss	Protectin	Associated with exPEC strains; increased serum survival associated with serum resistance.
iha	Adhesin	Associated with StxEAEC and UPEC, homologue of *IrgA* adhesin; confers adherence to nonadherent strains.
lpfA	Adhesin	Encodes for chaperone-usher fimbriae used in adhering to gut wall.
senB	Toxin	Toxin active against Enterobacteriaceae, increased retention of water and associated with watery diarrhoea.
sat	Toxin	Associated with UPEC and exPEC strains, a secreted autotransporter toxin, triggers destruction of the cell cytoskeleton, followed by autophagy.
astA	Toxin	Produces EAST1 toxin resulting in diarrhoea in host organisms.
iroN	Siderophore	Scavenges iron from mineral phases of soluble iron complexes for growth and maintenance.
mchF	Antibacterial Peptide	Produces bacteriocin peptide, microcin. Compete with enteric pathogens by mimicking siderophores

Figure 5.7 The most prevalent virulence genes carried by *E. coli* from three groups of slaughterhouse worker. Error bars indicate 95%CI. The 16 most prevalent virulence genes were tabulated and those which covered at least two groups were plotted in this figure. A short explanation of virulence genes can be seen in (Table 1.1 and Table 5.2).



The most commonly identified virulence genes in all groups were *gad*, *iss*, *iha* and *capU*. There were two virulence genes which were not carried in pig-only sequences, including *eilA* and *air.* The *gad* virulence gene was carried almost universally by isolates from cattle and cattle and shoat workers, and in almost all isolates from pig-only workers. Besides these differences, the majority of other virulence genes identified in the genomes had similar patterns of prevalence.

There was low-level carriage of several genes relating to EAEC – the pathotype associated with diarrhoeal disease. These included *aap* (a dispersin, enhancing colonisation of the gut) and four different toxin-encoding genes: *senB, sat*, *astA* and *cma.* There was no difference between the carriage of these genes between any of the three groups, but they are important to note as they were associated with MDR and/or ESBL-producing isolates.

There was no identifiable pattern between the carriage of resistance genes and the carriage of virulence genes. For example, isolates from pig-only workers carried a large number of resistance genes (between 7-21), but commonly only 4-6 virulence genes. Almost all the isolates belonging to the ST131 group carried a wide variety of resistance genes, as well as toxin-producing virulence genes (*sat* and *senB*) but also only 3-4 other virulence genes. This suggests that whilst they have high AMR potential, they are not necessarily highly virulent.

5.3.2.4 Plasmids

Mobile genetic elements such as plasmids are known to carry both resistance and virulence genes and facilitate the spread of AMR genes between bacterial species via horizontal transfer, conferring antimicrobial resistance to previously antimicrobial-sensitive bacteria.

As with the farm dataset (**Chapter 4**), there was a high prevalence of MDR E. *coli* found amongst slaughterhouse workers (**Chapter 5**) working in Busia county. This could indicate that numerous AMR genes are being transferred between bacteria on (multiple) plasmids.

18 different plasmid replicon types were detected amongst humans working at three different types of slaughterhouse in Busia county (**Figure 5.8**). The most common plasmid replicon types found in *E. coli* genomes were IncFII (76.6%, n=141), IncFIB (65.1%, n=122), IncQ and IncQ1 (65.0%, n=121, and IncFIA (28.8%, n=51). A moderate proportion of the sequenced genomes had co-carriage of IncFI, IncFII, IncQ and IncQ1 (42.7%, n=80), though there was no significant difference between the type of slaughterhouse and carriage of those plasmids (Kruskal Wallis,

p>0.05). More than one replicon type was found in 94.7% (n=177) isolates, and in 60.9% of these isolates the most common plasmid replicon co-occurrence was IncFIB and IncFII (p<0.001). For plasmid replicons which were carried by workers from each of the three types of slaughterhouse, there was no significant difference between the proportion of the plasmids carried, and the type of slaughterhouse (p>0.05). This suggests that the type of slaughterhouse does not make a large difference to the diversity of plasmids acquired by *E. coli* of slaughterhouse workers.

Due to the high prevalence of AMR genes carried within this population, an examination of the number and type of plasmids found in the isolates with the largest number of AMR genes was made. Of note, was a significant association between the carriage of ESBL genes (specifically $bla_{\text{CTX-M-15}}$) and the presence of any IncF plasmid (IncFIA, IncFIB or IncFII). The combination of $bla_{\text{CTX-M-15}}$ and IncFIA was found in 26 isolates (78.7%) (p<0.001, Fisher's Exact Test). The combination of $bla_{\text{CTX-M-15}}$ and IncFIB was found in 28 isolates (72.7%) (p=0.01, Fisher's Exact Test). The combination of $bla_{\text{CTX-M-15}}$ and IncFII was found in 24 isolates (81.8%) (p=0.01, Fisher's Exact Test). The occurrence of $bla_{\text{CTX-M-15}}$ with all three of those plasmids was 45.4% (n=15) (p=0.04, Fisher's Exact Test). The high association of $bla_{\text{CTX-M-15}}$ with IncF plasmids, suggests that they have a significant role in the dispersal of ESBL genes amongst slaughterhouse workers in Busia county.

Conversely, the other major ESBL genotype (attributed to $bla_{\text{SHV-12}}$) was more likely to be associated with carriage of IncQ1 and IncX3 plasmids (p=0.02, Fisher's Exact Test), opposed to IncF plasmids.

Isolates which carried the same replicon types were likely to carry the majority of similar, but not identical resistance and virulence genes. Isolates carrying only IncFII and IncFIB were tabulated and the carriage of both resistance and virulence genes was noted (**Table 5.3**). Minor differences in the detected resistance and virulence genes may suggest that other methods of gene acquisition other than on mobile genetic elements may be occurring in this population.

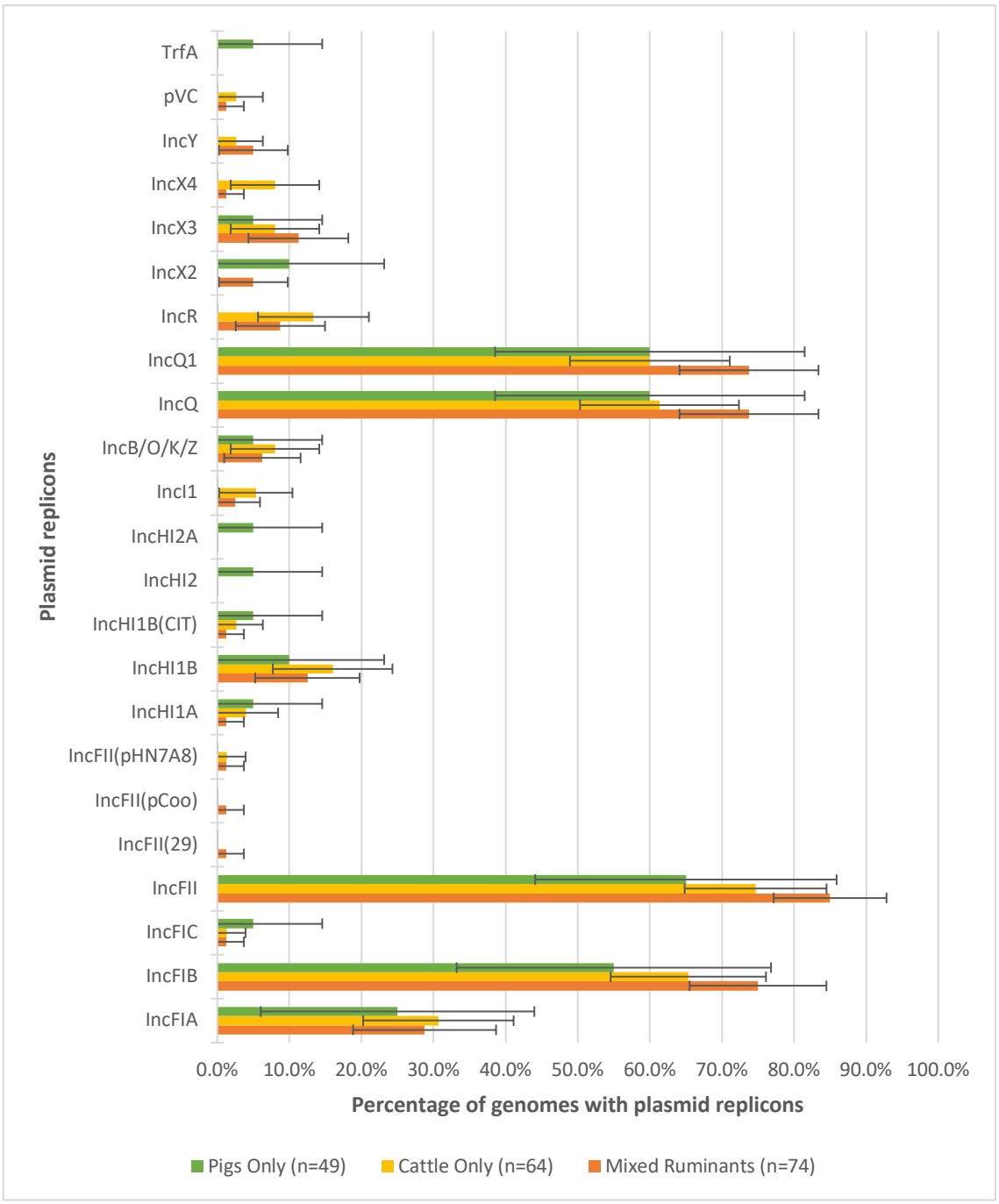


Figure 5.8 Proportions of each of the plasmids carried within *E. coli* genomes, isolated from farmers working at three different types of slaughterhouse. Error bars are 95% CI.

Table 5.3 Heat-map showing the virulence and resistance genes carried by a group of isolates carrying the same two plasmid replicon types, IncFII and IncFIB to highlight variability in strains with the same plasmids. One random isolate was selected from 9 different STs to ensure adequate diversity was compared.

Isolate Number	Slaughterhouse Type	MLST	Resistance Genes												Virulence Genes											
			A			B	C	D			E		F														
			aadA1	*strA*	*strB*	*blaTEM-1B*	*catA1*	*dfrA1*	*dfrA7*	*dfrA14*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(J)*	*aap*	*astA*	*capU*	*cma*	*eilA*	*gad*	*iha*	*iroN*	*iss*	*lpfA*	*mchF*	*senB*
121650	C	10	■	■	■	■	■	■	■		■	■	■		■		▒		▒	▒	▒	▒	▒	▒	▒	▒	
120520	C+S	155	■		■	■		■	■		■	■	■					▒			▒	▒	▒	▒	▒		▒
58-523C	P	211	■	■	■	■	■	■	■		■	■	■	■							▒				▒		
121919b	C+S	216	■	■	■	■		■			■	■	■				▒	▒			▒			▒	▒		
120748	C+S	224	■	■	■	■		■			■	■	■								▒			▒			
121919a	C+S	226	■	■	■	■		■			■	■	■								▒				▒		
121736	C+S	48	■	■	■	■		■			■	■	■								▒			▒			
121930a	C+S	5386		■	■	■				■		■	■								▒				▒		
121236	C+S	543		■	■	■	■		■		■	■	■		■	■	▒				▒			▒			

Key: **Slaughterhouse Type**: C = cattle only, C+S = Cattle + sheep and goats, P = Pigs only; **Resistance genes**: A = aminoglycosides, B = β-lactamases , C = chloramphenicol, D = Trimethoprim, E = Sulfonamides, F = Tetracyclines

5.3.2.5 Extended-Spectrum Beta Lactamases

Several clusters of ESBL-producing *E. coli* could be identified on the phylogenetic tree (**Figure 5.3**). *E. coli* from the ST617 clade carried a $bla_{CTX\text{-}M\text{-}15}$ resistance gene and were detected in pig-only and cattle and mixed ruminant slaughterhouse workers. Isolates from the ST361 clade carried a $bla_{SHV\text{-}12}$ resistance gene and were detected in all three types of slaughterhouse worker. All but one of the isolates from the ST131 clade also all carried a $bla_{CTX\text{-}M\text{-}15}$ resistance gene; one ST131 isolate carried a $bla_{CTX\text{-}M\text{-}27}$. ST131 isolates were also dispersed amongst all three types of slaughterhouse workers. 5 isolates belonging to the ST131 clade (which carried the $bla_{CTX\text{-}M\text{-}15}$ resistance gene) belonged to serogroup O25:H4. Interestingly, two of the ST131 isolates which were a part of the ST131 clade, carried numerous AMR genes, including to aminoglycosides, sulfonamides, tetracyclines and chloramphenicol, but they did not appear to carry an ESBL resistance gene, despite also being serogroup O25:H4. To confirm if this was accurate, resistance gene analysis was re-run using a lower threshold of detection (coverage remained at ≥90%, but identity dropped to ≥75%) and the same results were found.

One genome (58-596D) was found to carry a $bla_{CMY\text{-}135}$ resistance gene, conferring resistance to a wide array of antimicrobials. This was co-carried alongside $bla_{CTX\text{-}M\text{-}27}$ and belonged to the ST10 cluster (isolated from one cattle-only slaughterhouse worker).

The majority of isolates clustering within ST361 group belonged to serogroup O9:H30 (80.%, n=8); of these ST361:O9:H30 isolates, all carried the $bla_{SHV\text{-}12}$ ESBL resistance gene, and two of those also carried co-carried $bla_{CTX\text{-}M\text{-}15}$. Isolates were from geographically distinct areas and dispersed amongst each of the three types of slaughterhouse (mixed ruminants, cattle only and pigs only). A clade of 8 ST131 isolates was also dispersed amongst all three types of slaughterhouse worker. Of those, 6 isolates belong to serotype O25:H4, 4 carried the $bla_{CTX\text{-}M\text{-}15}$ ESBL resistance gene and 1 carried the $bla_{CTX\text{-}M\text{-}27}$ gene

The most commonly carried β-lactamase conferring the ESBL phenotype was $bla_{CTX\text{-}M\text{-}15}$. The $bla_{SHV\text{-}12}$ ESBL resistance gene was less common but was carried by several different *E. coli* from all three slaughterhouse types. There were three instances of co-carriage of $bla_{SHV\text{-}12}$, with $bla_{CTX\text{-}M\text{-}15}$, in pig-only isolates (n=2) and mixed ruminant isolates (n=1). Isolates carrying the $bla_{CTX\text{-}M}$ resistance genes were more associated with IncFIB, IncFIA and IncFII plasmids ($p<0.001$, Fisher's Exact Test), whereas those carrying a bla_{SHV} resistance gene were more commonly associated with IncQ1 and IncX3 plasmids ($p<0.001$, Fisher's Exact Test). However, several $bla_{SHV\text{-}12}$ carrying

isolates also carried IncF plasmids which proves exception to this rule and suggests that ESBL genes may be carried on different plasmids, or in combinations.

All isolates carried numerous resistance genes in association with different combinations of plasmids **(Table 5.4).** Despite the high degree of genetic similarity (<500 SNPs difference) shown on the phylogenetic tree, each group of STs contained isolates carrying non-identical plasmids and resistance genes. This is expected as there are numerous different plasmid replicons circulating in this population. This is true of ST131, four isolates carry identical resistance genes, but isolates **58-583CZ** carries almost entirely different resistance genes, as well as a different $bla_{CTX\text{-}M}$ resistance gene (27, instead of 15). Similarly, with ST617 isolates, six are identical, and **58-468CZ** is almost identical, except for the additional carriage of a fosfomycin resistance gene (*fosA)*.

Some similarities within serogroups were evident **(Table 5.5)** - similar virulence genes were associated with isolates belonging to the same groups. There was a large number of toxin-producing genes in serotypes within the O8 and O25 group, particularly of *sat* and *senB;* many of these isolates were found in cattle-only or cattle and mixed ruminant slaughterhouses. This could be of importance, as isolates belonging to phylogroup B2 have been associated with exPEC disease.

Table 5.4 A heatmap showing the details of *E. coli* isolates carrying *bla*$_{CTX-M}$ or *bla*$_{SHV}$ ESBL resistance genes. Results are sorted by the ESBL genes carried by the bacterium, then by ST and then plasmid replicon type.

Sample ID	Slaughterhouse No.	Slaughterhouse Type	ST	Resistance Phenotype	Plasmid Replicon Types	Resistance Genes																																		ESBL Resistance Genes
						aac(3)-IIa	*aac(3)-IId*	*aac(6')Ib-*	*aadA1*	*aadA2*	*aadA5*	*blaACT-*	*blaCMY-*	*blaOXA-1*	*blaTEM-*	*catA1*	*catB3*	*dfrA1*	*dfrA7*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA15*	*dfrA17*	*fosA*	*mph(A)*	*oqxA*	*oqxB*	*qepA*	*QnrS1*	*sph*	*strA*	*strB*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(c)*	*tet(j)*	
120516	119	C+S	10	SULFA TET AMP TRIM	IncFIA IncFII IncFIB IncQ1																																			*bla*$_{CTX-M-15}$
120504	108	C+S	10	SULFA TET AMP TRIM	IncFIB IncFII IncB/O/K/Z IncQ1		■	■	■		■			■	■		■	■						■		■					■	■	■	■	■	■			*bla*$_{CTX-M-15}$
58-454C	150	P	10	SULFA TET AMP TRIM	-				■	■					■	■		■	■		■										■	■	■	■	■	■			*bla*$_{CTX-M-15}$
58-724A	5	P	43	CIP SULFA TET AMP GENT TRIM	IncFIB IncFII IncFIA IncQ1				■		■	■	■		■	■		■	■				■		■	■					■	■	■	■	■	■		■	*bla*$_{CTX-M-15}$
120347	124	C+S	101	CIP SULFA CHLOR TET AMP TRIM	IncB/O/K/Z IncFII	■			■		■			■	■	■		■						■		■						■	■	■	■	■	■			*bla*$_{CTX-M-15}$
58-454CZ	150	P	131	CIP SULFA CHLOR TET AMP GENT TRIM	IncFIB IncFIA	■		■			■			■			■							■		■						■	■	■	■					*bla*$_{CTX-M-15}$
58-716CZ	5	P	131	SULFA TET AMP TRIM	IncFIB IncFIA	■		■			■			■			■							■		■						■	■	■	■					*bla*$_{CTX-M-15}$
58-725CX	37	P	131	CIP SULFA TET AMP TRIM	IncFIB IncFIA	■		■			■			■			■							■		■						■	■	■	■					*bla*$_{CTX-M-15}$
58-740CZ	4	P	131	SULFA TET AMP TRIM	IncFIB IncFIA	■		■			■			■			■							■		■						■	■	■	■					*bla*$_{CTX-M-15}$
120519	22	C	224	SULFA TET AMP TRIM	IncFII IncFIB IncFII IncR IncQ1				■	■					■	■		■	■		■	■											■	■	■	■		■	*bla*$_{CTX-M-15}$

Sample ID	Slaughterhouse No.	Slaughterhouse Type	ST	Resistance Phenotype	Plasmid Replicon Types	Resistance Genes																																		ESBL Resistance Genes
						aac(3)-IIa	*aac(3)-IId*	*aac(6')Ib-*	*aadA1*	*aadA2*	*aadA5*	*blaACT-*	*blaCMY-*	*blaOXA-1*	*blaTEM-*	*catA1*	*catB3*	*dfrA1*	*dfrA7*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA15*	*dfrA17*	*fosA*	*mph(A)*	*oqxA*	*oqxB*	*qepA*	*QnrS1*	*sph*	*strA*	*strB*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(c)*	*tet(j)*	
58-704A	40	P	617	SULFA TET AMP TRIM	IncFIA IncHI1B IncFIB IncFII						■	■												■		■						■	■	■	■					*bla*$_{CTX-M-15}$
58-363CX	6	P	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII					■					■	■					■							■					■			■				*bla*$_{CTX-M-15}$
58-464CZ	119	C+S	617	CIP SULFA TET AMP GENT TRIM	IncFIB IncFIA IncFII				■	■					■	■		■	■		■	■									■	■	■	■	■			■	*bla*$_{CTX-M-15}$
58-468CX	119	C+S	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
58-468CZ	119	C+S	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■	■				■		■		■						*bla*$_{CTX-M-15}$
58-725CZ	37	P	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
58-747CX	38	P	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
58-751CZ	38	P	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
58-751CX	38	P	617	SULFA TET AMP TRIM	IncFIB IncFIA IncFII										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
120346	124	C+S	648	CIP SULFA TET AMP TRIM	IncFIA IncFII IncFIB IncQ1										■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
121930	1	C+S	5386	SULFA TET AMP TRIM	IncFIB IncFIA IncFII IncQ1						■				■	■					■	■			■					■		■		■						*bla*$_{CTX-M-15}$
58-570CX	9	C	-	SULFA TET AMP TRIM	IncFIB IncFIA IncFII			■			■			■			■							■		■						■	■	■						*bla*$_{CTX-M-15}$
58-747CZ	38	P	-	SULFA TET AMP TRIM	IncFIB IncFIA IncFII			■			■			■			■							■		■						■	■	■						*bla*$_{CTX-M-15}$
58-639CZ	34	P	-	SULFA TET AMP TRIM	IncFIB IncFII	■		■			■			■			■							■		■						■	■	■						*bla*$_{CTX-M-15}$

Sample ID	Slaughterhouse No.	Slaughterhouse Type	ST	Resistance Phenotype	Plasmid Replicon Types	Resistance Genes																																	ESBL Resistance Genes
						aac(3)-IIa	*aac(3)-IId*	*aac(6')Ib-*	*aadA1*	*aadA2*	*aadA5*	*blaACT-*	*blaCMY-*	*blaOXA-1*	*blaTEM-*	*catA1*	*catB3*	*dfrA1*	*dfrA7*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA15*	*dfrA17*	*fosA*	*mph(A)*	*oqxA*	*oqxB*	*qepA*	*QnrS1*	*sph*	*strA*	*strB*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(c)*	*tet(j)*	
58-467A	22	C	-	CIP SULFA TET AMP TRIM	IncFIB IncFII IncFIA IncQ1	■		■			■			■			■							■		■						■	■	■		■				*bla*$_{CTX-M-15}$
121766	79	C+S	-	SULFA TET AMP TRIM	IncFII IncFIA IncR IncQ1			■			■			■			■							■		■						■	■	■		■				*bla*$_{CTX-M-15}$
58-630CZ	153	P	-	SULFA TET AMP TRIM	-			■			■			■			■							■		■						■	■	■		■				*bla*$_{CTX-M-15}$
58-464CX	119	C+S	-	CIP SULFA TET AMP GENT TRIM	IncFIA IncFIB IncFII IncX3 IncQ1			■			■			■			■							■		■						■	■	■		■				*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$
58-454CX	150	P	-	CIP SULFA CHLOR TET AMP GENT TRIM	IncFIB IncFIA IncX3 IncQ1			■			■			■			■							■		■						■	■	■		■				*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$
58-477CX	169	P	-	SULFA TET AMP TRIM	IncFII IncHI2A IncHI2 IncFIB IncFIA IncX3 IncQ1	■		■			■			■	■		■			■				■		■						■	■	■		■				*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$
58-596D	9	C	10	SULFA TET AMP TRIM	IncFIB IncFII IncFIA IncFIB IncQ1		■	■			■			■			■							■		■						■	■			■				*bla*$_{CTX-M-27}$
58-583CZ	9	C	131	SULFA TET AMP TRIM	IncFIA IncFII IncFIB	■					■			■			■							■		■			■			■	■	■		■				*bla*$_{CTX-M-27}$
120172	124	C+S	-	SULFA TET AMP TRIM	IncFII IncFIC IncI1 IncFIB IncQ1		■	■			■			■			■					■		■		■						■	■	■		■				*bla*$_{CTX-M-3}$
58-533CZ	26	C+S	361	SULFA TET AMP TRIM	IncFII IncFIB IncX3 IncQ1																			■							■		■							*bla*$_{SHV-12}$

Sample ID	Slaughterhouse No.	Slaughterhouse Type	ST	Resistance Phenotype	Plasmid Replicon Types	Resistance Genes																																	ESBL Resistance Genes
						aac(3)-IIa	*aac(3)-IId*	*aac(6')Ib-*	*aadA1*	*aadA2*	*aadA5*	*blaACT-*	*blaCMY-*	*blaOXA-1*	*blaTEM-*	*catA1*	*catB3*	*dfrA1*	*dfrA7*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA15*	*dfrA17*	*fosA*	*mph(A)*	*oqxA*	*oqxB*	*qepA*	*QnrS1*	*sph*	*strA*	*strB*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(c)*	*tet(j)*	
58-539CX	26	C+S	361	SULFA TET AMP TRIM	IncQ1 IncX3			■	■		■			■	■		■	■		■		■		■							■	■	■	■	■				bla_{SHV-12}
58-539CZ	26	C+S	361	SULFA TET AMP TRIM	IncQ1 IncX3	■		■			■			■			■						■		■							■	■	■	■	■				bla_{SHV-12}
58-560CX	3	C	361	SULFA TET AMP TRIM	IncQ1 IncX3	■		■	■		■			■		■	■	■						■		■							■	■	■	■	■				bla_{SHV-12}
58-586CX	3	C	361	SULFA TET AMP TRIM	IncQ1 IncX3	■		■			■	■		■			■							■		■							■		■						bla_{SHV-12}
58-588CX	3	C	361	SULFA TET AMP TRIM	IncQ1 IncX3					■					■			■															■	■	■	■			■	bla_{SHV-12}
58-716CX	5	P	361	SULFA TET AMP TRIM	IncQ1 IncX3	■		■	■		■			■	■	■	■	■	■						■	■							■	■	■	■			■	bla_{SHV-12}
58-546CX	110	C+S	361	CIP SULFA CHLOR TET AMP TRIM	-	■		■			■			■	■	■	■				■		■		■				■			■	■	■	■	■				bla_{SHV-12}
58-546CZ	110	C+S	361	CIP SULFA CHLOR TET AMP TRIM	-	■		■			■			■	■	■	■				■		■	■	■				■			■	■	■		■				bla_{SHV-12}
58-464A	119	C+S	-	CIP SULFA TET AMP GENT TRIM	IncFII IncFIB IncFIA IncX3 IncQ1	■		■			■			■	■	■	■				■		■	■	■				■			■	■	■		■				bla_{SHV-12}
58-562CZ	9	C	-	SULFA TET AMP TRIM	IncFII IncFIB IncX3 IncQ1						■				■	■					■		■		■				■			■	■	■	■	■				bla_{SHV-12}
58-340C	148	C	-	SULFA TET AMP TRIM	IncFII IncHI1B IncFIB IncR IncX3 IncQ1				■						■			■	■	■												■	■	■	■	■				bla_{SHV-12}
58-578CX	3	C	-	CIP SULFA TET AMP TRIM	IncX3 IncFII IncQ1						■				■	■			■			■	■		■				■			■	■	■	■	■				bla_{SHV-12}
120516	119	C+S	10	SULFA TET AMP TRIM	IncFIA IncFII IncFIB IncQ1										■	■						■		■	■				■			■	■		■	■				$bla_{CTX-M-15}$

Sample ID	Slaughterhouse No.	Slaughterhouse Type	ST	Resistance Phenotype	Plasmid Replicon Types	Resistance Genes																														ESBL Resistance Genes
						aac(3)-IIa	*aac(3)-IId*	*aac(6')Ib-*	*aadA1*	*aadA2*	*aadA5*	*blaACT-*	*blaCMY-*	*blaOXA-1*	*blaTEM-*	*catA1*	*catB3*	*dfrA1*	*dfrA7*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA15*	*dfrA17*	*fosA*	*mph(A)*	*oqxA*	*oqxB*	*qepA*	*QnrS1*	*sph*	*strA*	*strB*	*sul1*	*sul2*	*tet(A)*	*tet(B)*	*tet(c)*	*tet(j)*	
120504	108	C+S	10	SULFA TET AMP TRIM	IncFIB IncFII IncB/O/K/Z IncQ1				■					■	■	■		■				■			■				■		■	■		■		■			$bla_{CTX\text{-}M\text{-}15}$
58-454C	150	P	10	SULFA TET AMP TRIM	-				■						■	■		■	■			■			■	■	■	■		■	■	■	■	■	■	■	■		$bla_{CTX\text{-}M\text{-}15}$

Table 5.5 Serogroups, phylogroups and virulence genes carried by 46 isolates of ESBL-producing *E. coli.* The four detected toxin-producing virulence genes are highlighted in four respective colours (*astA* = red, *cma* = blue, *sat* = yellow and *senB* = green). Data is sorted according to O group, then H group, then phylogroup. Slaughterhouse type key: **C+S** = cattle and sheep and goats, **C** = cattle-only, **P** = pig-only.

Isolate	Slaughterhouse Type	Phylogroup	O group	H group	Virulence Genes																							ESBL Genes
					air	*aap*	*aatA*	*astA*	*capU*	*cma*	*eilA*	*espA*	*gad*	*iss*	*lpfA*	*iha*	*ireA*	*ironN*	*iss*	*mchB*	*mchC*	*mchF*	*nfaE*	*sat*	*sigA*	*senB*	*sta1*	
120516	C+S	E	1	6	■					■	■		■	■	■													*bla*$_{CTX-M-15}$
120346	C+S	F	1	6	■					■	■		■		■													*bla*$_{CTX-M-15}$
58-596D	C	-	8	10				■	■	■			■			■		■	■			■				■		*bla*$_{CTX-M-27}$
121930	C+S	D	8	10	■	■		■			■		■	■	■													*bla*$_{CTX-M-15}$
58-340C	C	E	8	11	■			■	■		■		■			■	■		■						■			*bla*$_{SHV-12}$
58-464CZ	C+S	A	9	10					■				■	■														*bla*$_{CTX-M-15}$
58-464CX	C+S	-	9	30					■					■									■					*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$
58-562CZ	C	-	9	30									■	■														*bla*$_{SHV-12}$
58-477CX	P	A	9	30					■				■	■									■					*bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$
58-533CZ	C+S	A	9	30									■	■														*bla*$_{SHV-12}$
58-539CX	C+S	A	9	30									■	■														*bla*$_{SHV-12}$
58-539CZ	C+S	A	9	30									■	■														*bla*$_{SHV-12}$
58-546CZ	C+S	A	9	30									■	■														*bla*$_{SHV-12}$
58-560CX	C	A	9	30									■	■														*bla*$_{SHV-12}$
58-586CX	C	A	9	30									■	■														*bla*$_{SHV-12}$
58-588CX	C	A	9	30									■	■														*bla*$_{SHV-12}$
58-716CX	P	A	9	30									■	■														*bla*$_{SHV-12}$

Isolate	Slaughterhouse Type	Phylogroup	O group	H group	Virulence Genes																							ESBL Genes
					air	*aap*	*aatA*	*astA*	*capU*	*cma*	*eilA*	*espA*	*gad*	*iss*	*lpfA*	*iha*	*ireA*	*iroN*	*iss*	*mchB*	*mchC*	*mchF*	*nfaE*	*sat*	*sigA*	*senB*	*sta1*	
120172	C+S	D	22	2		■	■	■	■		■		■			■	■		■					■				*bla*CTX-M-3
58-724A	P	-	25	4	■	■	■	■	■		■		■				■		■		■	■	■	■	■			$bla_{CTX-M-15}$
58-747CZ	P	-	25	4		■	■						■			■			■		■	■	■	■				$bla_{CTX-M-15}$
58-454CZ	P	B2	25	4									■			■			■				■					$bla_{CTX-M-15}$
58-464A	C+S	B2	25	4					■				■			■		■	■									bla_{SHV-12}
58-570CX	C	B2	25	4				■					■	■						■								$bla_{CTX-M-15}$
58-583CZ	C	B2	25	4				■					■			■			■					■				$bla_{CTX-M-27}$
58-716CZ	P	B2	25	4									■			■			■				■					$bla_{CTX-M-15}$
58-725CX	P	B2	25	4									■			■			■				■					$bla_{CTX-M-15}$
58-740CZ	P	B2	25	4									■			■			■				■					$bla_{CTX-M-15}$
58-630CZ	P	-	25	10					■				■			■			■				■					$bla_{CTX-M-15}$
121766	C+S	-	25	19				■				■	■	■									■					$bla_{CTX-M-15}$
58-454CX	P	-	25	30									■			■			■				■					$bla_{CTX-M-15}$, bla_{SHV-12}
58-546CX	C+S	A	25	30									■			■			■					■				bla_{SHV-12}
58-704A	P	A	88	16					■				■	■									■					$bla_{CTX-M-15}$
58-363CX	P	A	89	10					■				■	■														$bla_{CTX-M-15}$
58-468CX	C+S	A	89	10					■				■	■									■					$bla_{CTX-M-15}$
58-468CZ	C+S	A	89	10					■				■	■									■					$bla_{CTX-M-15}$
58-725CZ	P	A	89	10					■				■	■														$bla_{CTX-M-15}$

Isolate	Slaughterhouse Type	Phylogroup	O group	H group	Virulence Genes																								ESBL Genes
					air	*aap*	*aatA*	*astA*	*capU*	*cma*	*eilA*	*espA*	*gad*	*iss*	*lpfA*	*iha*	*ireA*	*iroN*	*iss*	*mchB*	*mchC*	*mchF*	*nfaE*	*sat*	*sigA*	*senB*	*sta1*	
58-747CX	P	A	89	10					■				■	■															*bla*$_{CTX-M-15}$
58-751CX	P	A	89	10					■				■	■															*bla*$_{CTX-M-15}$
58-751CZ	P	A	89	10					■				■	■															*bla*$_{CTX-M-15}$
58-467A	C	E	89	41	■			■	■		■					■			■				■	■		■	■		*bla*$_{CTX-M-15}$
58-578CX	C	-	95	10		■	■						■			■			■	■	■	■		■					*bla*$_{SHV-12}$
58-454C	P	A	107	36									■	■															*bla*$_{CTX-M-15}$
120504	C+S	-	160	9						■			■		■			■	■			■							*bla*$_{CTX-M-15}$
120519	C	B1	174	3									■	■	■														*bla*$_{CTX-M-15}$
120347	C+S	B1	174	28									■	■	■														*bla*$_{CTX-M-15}$
58-639CZ	P	B2	-	-																									*bla*$_{CTX-M-15}$

5.4 Discussion

This study examined the carriage and prevalence of AMR *E. coli* in the faeces of slaughterhouse workers, working in cattle only, mixed ruminant, and pig-only slaughterhouses across Busia county, in Western Kenya. Slaughterhouse workers are at high risk of coming into contact with livestock-associated *E. coli* during the slaughter process. Numerous shortcomings in the slaughter process may contribute to the increased risk for the transmission of AMR bacteria between livestock and humans in sub-Saharan Africa slaughterhouse environments however, in this region, there are no data investigating this. The results of this study indicate a high prevalence of AMR, MDR and ESBL-producing *E. coli* carriage amongst slaughterhouse workers, as well as a high level of diversity in the *E. coli* isolated. This is the first study investigating the prevalence of faecal carriage of AMR *E. coli* in slaughterhouse workers in western Kenya.

There was a high prevalence of AMR *E. coli* isolated from the faeces of slaughterhouse workers across the four sampled western Kenyan counties. The majority of isolates (95.6%) were resistant to at least one antimicrobial class, with approximately half being MDR in all slaughterhouse types. There are relatively few studies assessing rates of MDR *E. coli* in humans outside of clinical context and a lack of data in African countries, thus this study provides the first data in this respect.

There was a moderately high carriage of ESBL-*E. coli* amongst slaughterhouse workers. ESBL-producing *E. coli* was isolated from 6.5% (95%CI 2.8 - 10.2%) of cattle-only slaughterhouse workers, 11.8% (95%CI 6.6 – 17.1%) of mixed ruminant slaughterhouse workers, and 13.6% (95%CI 7.8 – 19.5%) of pig-only slaughterhouse workers. This is in-line with a previous study investigating the ESBL carriage in Dutch pig slaughterhouse workers, who reported an overall prevalence of 4.9% (95%CI 1.57 - 12.62%) (Dohmen et al., 2017). However, it is impossible to conclude from this study if ESBL-producing *E. coli* was acquired from livestock in the slaughterhouse or from elsewhere. Indeed, a number of these isolates (e.g. those belonging to the ST131, ST361 and other clades) are also associated with clinical infection amongst humans, all over the world (Ahmed et al., 2015; Manges et al., 2019). It is difficult to speculate on the increased rates of carriage for ruminant-slaughterhouses, though this could be related to AMU. It has been described that ESBL carriage is considerably higher in slaughterhouse workers who eviscerate pig carcasses (specifically the lungs, heart, liver, tongue) and remove the heads and spinal cords of these animals (Lowe et al., 2011; Lăpușan et al., 2012) and our findings mirror this, as the highest proportion of ESBL-*E.coli* was found in pig slaughterhouse workers.

Furthermore, evisceration may be a risk factor for people slaughtering other animals including cattle, particularly as the majority of Kenyan workers participate in several aspects of animal slaughter (Cook et al., 2017). There is poor hygiene in slaughterhouses in this region (Cook et al., 2017), thus there is potential transmission of *E. coli* from animal carcasses to workers, and then from worker-to-worker. Phenotypic resistance to antimicrobials commonly used in animals was apparent in all human *E. coli* isolates, similar to those noted in humans and animals in our farm study (**Chapter 4**). As there is significant overlap in the antibiotics used in humans and animals in this study site (oxytetracyclines, penicillin-streptomycin etc.), the same selective pressures may be placed on *E. coli* to develop AMR in humans, as in animals.

Tetracycline resistance was the most common amongst all workers (86.2%). This corresponds to the most commonly used antimicrobials in cattle and other farm animals, which is oxytetracycline (results from **Chapter 3**; (Founou et al., 2018). The most common phenotypes were similar in all groups, with no statistically significant differences found. More than half (61.3%) of the 31 detected phenotypic patterns were MDR. There was frequent co-carriage of resistance genes conferring resistance to sulfonamides, tetracycline, trimethoprim, and ampicillin. The high proportion of MDR *E. coli* isolated from slaughterhouse workers in this cohort, as well as the presence of numerous plasmid replicons found in *E. coli* genomes suggests that AMR genes are being mobilised between slaughterhouse workers on multiple mobile genetic elements, such as plasmids. This theory is consistent with the WGS results (**Chapter 5**) which showed a direct correlation between carriage of IncF plasmids and the presence of MDR (including *tet, dfr* and *sul* resistance genes) and ESBL-producing *E. coli*. Furthermore, the high degree of tetracycline resistance is fitting with the notion that humans have access to a wide variety of antimicrobials which can be purchased without a prescription (Muloi et al., 2019b). This finding is also consistent with commonly purchased antimicrobials according to the farm study (**Chapter 4**). Cook et al showed that 18% of slaughterhouse workers reported being unwell in the 3 months prior to sampling and that 18% of slaughterhouse workers also knowingly slaughtered sick animals (Cook et al., 2017). There is a significant risk of contracting AMR strains of *E. coli* during the slaughter process, particularly if workers have skin lacerations. As a number of these slaughterhouses did not also have ready access to running water and soap, and workers have to purchase their own PPE (Cook et al., 2017), there is adequate opportunity for colonisation of *E. coli* from animal carcasses to occur. As PPE was worn by only 27% of slaughterhouse workers and hand-washing facilities were only present at 20% of slaughterhouses (Cook et al., 2017), there is an opportunity for bacterial acquisition from animal

carcasses to humans via open wounds. Workers may contaminate their clothes with viscera and then spread bacteria to their homes and other people.

In silico MLST analyses and serotyping also revealed a variety of STs and serotypes amongst E. coli isolated from slaughterhouse workers. The most prevalent STs were ST10, ST617 and ST48. ST10 strains are usually generalists and are frequently found as intestinal commensals particularly in pigs (Lugsomya et al., 2018), but also in many other animals (Abraham et al., 2015), and also in humans (Fischer et al., 2017). The ST10 clonal complex (ST10 and closely related STs) have also been associated with human infections and ESBL production, being isolated from human clinical specimens, meat products, and food animals (Oteo et al., 2009; Cortes et al., 2010; Cohen Stuart et al., 2012; Peirano et al., 2012). ST10 *E. coli* was previously discovered in the farm study (**Chapter 4**) though none of these were found in human samples on farms. The second most common ST, ST617 is also part of the ST10 clonal complex and has recently been identified as a potential evolutionary descendant of ST10 and associated with carriage of ESBL genes (Zong et al., 2018). A study in rural farming communities in Mwanza, Tanzania showed that *E. coli* belonging to ST617 and carrying $bla_{\text{CTX-M-15}}$ was isolated from various animals (Seni et al., 2016a). It is possible that the crossover of *E. coli* ST617 from animals to humans has occurred via clonal spread. *E. coli* belonging to ST48 (also part of the ST10 clonal complex) appears to be a common type in human and animal isolate origins, according to the PubMLST database. In Tunisia, ST48 *E. coli* has been associated with the production of ESBLs in healthy human volunteers (Ben Sallem et al., 2012). Despite being relatively common, no ST48 isolates were reported to carry an ESBL gene in this study population.

From the phylogenetic tree, three major clades of *E. coli* with a high degree of genetic similarity (<500 SNPs difference) were detected, belonging to ST361, ST617 and ST131. The presence of ST131 *E. coli* is particularly relevant, as four of these isolates were associated with serotype O25:H4. ST131 has been identified as a pandemic, MDR, community- and hospital-associated strain (Rogers et al., 2010) and has severe implication for public health, owing to its difficulty in treatment. Four isolates from pig-only slaughterhouse workers were identified as having a high degree of similarity (fewer than 150 SNPs difference), all belonging to ST131, and all carrying the $bla_{\text{CTX-M-15}}$ resistance gene. Fimtyping showed that these all had the same *Fim* switch – H30. ST131:FimH30 have been described globally, and recently in Sweden (Ny et al., 2019). These four isolates do indeed belong to the ST131:O25/*Fim*H30 pandemic strain (Pitout and DeVinney, 2017; Kondratyeva et al., 2020). As discussed in **Chapter 4**, livestock may represent a major

reservoir of ESBL-producing *E. coli* and it is entirely possible that during the slaughter processes, there can be transmission from animal carcasses to humans which is then spread between workers. However, this strain could also be circulating specifically amongst slaughterhouse workers and spread from human-to-human due to poor hygiene practices, such as eating in the slaughterhouse or not having access to running water and soap (Cook et al., 2017)

The most commonly occurring serotypes were O9:H30 and O25:H4. Serotype O25:H4 is of particular importance, as it associated with the intercontinental emergence of ST131-O25b *E. coli* (Nicolas-Chanoine et al., 2008; Rogers et al., 2010). Typically, this lineage carries numerous virulence genes and is associated with extraintestinal infections. Indeed, all the O25:H4 isolates (8 isolates) carried 5 or more virulence genes, and all of them also carried a combination of toxin-associated virulence factors (*astA, sat* and *senB)* as well as other genes encoding for adhesins and siderophores. The number and combination of virulence genes found here strongly imply a robust virulence capability (Johnson et al., 2002). Characteristically, human *E. coli* derived from phylogroup B2 are associated with the ExPEC pathotype, whilst phylogroups A and B1 are associated with commensal carriage (Micenkova et al., 2016). Half of the O25:H4 isolates did belong to phylogroup B2 and two other isolates (which could not be phylotyped due to mismatches in the ClermontTyping database) each carried 16 virulence genes, alongside toxin-producing genes *sat* and *senB*. Whilst there is no certainty that these are also phylogroup B2 isolates, the virulence profile, in concordance with the serogrouping and carriage of $bla_{\text{CTX-M-15}}$ strongly indicates that these isolates carry a much more extensive array of virulence genes than a typical ESBL-producing B2 (Johnson et al., 2003). Other B2 isolates did not appear to carry specialised virulence factors to aid in colonisation (such as adhesins and siderophores) and thus, did not satisfy the criteria for ExPEC.

The emergence and dissemination of the pandemic clone is particularly concerning. As these isolates were first collected in 2012, it is possible that there has been additional spread since then. It is important to determine the origins, reservoirs, and transmission pathways of these ST131 isolates, so that appropriate interventions can be implemented. Previous transmission-based studies have been inconclusive in determining if bacteria is transmitted to or from livestock or indeed if bacterial populations are self-contained (Muloi et al., 2018), however specifically from these results it is possible that the ST131 pandemic strain may have been transferred to slaughterhouse workers from livestock, during the slaughter process. As the prevalence of ESBL-producing and MDR *E. coli* has not been previously investigated in western

Kenya, this finding highlights the need to increase AMR surveillance and better hygiene practices, in order to limit the spread or incidence of *E. coli* to/from carcasses intended for consumption. A better definition of the extent of this problem is needed, to clarify how great a public health threat these strains actually pose, so that resources can be allocated accordingly if necessary. Furthermore, it may be important to assess the carriage in other members of the community as this (whilst unlikely) may reflect normal carriage patterns. The other most common serogroup found in this population was O9:H30-ST361. These have been infrequently reported in the literature, though it has been reported that the ST131 pandemic evolved from an H30-ST131Rx subclone (Price et al., 2013; Nicolas-Chanoine et al., 2014). The ST131 has previously been reported in the faecal *E. coli* of malnourished children in Kenya (Mwangi, 2016) and so, by detecting it in another group of people, it is even more important to set up appropriate surveillance systems so that this can be tracked and prevented from spreading further.

There was a statistically significant difference in the carriage of the *mdf(a)* gene between isolates from different slaughterhouse workers; in isolates taken from workers in pig-only slaughterhouses, the prevalence was 100%. The clinical relevance of *mdf(a)* has been addressed in some studies and examined in Chapter 4; particularly in MDR *E. coli* there appears to be high carriage of *mdf(a)*, associated with IncX plasmids, especially in clinical patients (Wang et al., 2013; Wang et al., 2014; Yardeni et al., 2018). There was also a similarly high prevalence of *mdf(A)* carriage amongst farmers in Busia (**Chapter 4**). As all the sequenced isolates were MDR and/or ESBL-producers, it makes sense that prevalence is relatively high, however, there is no explanation for why it is more prevalent in pig-only isolates.

There was moderate carriage rate of a number of resistance genes, including *mph(a), fosA, oqxA/B* and *qnrS1* genes, these act on macrolides, fosfomycin and fluoroquinolones, respectively. In Chapter 3, farmers indicated extremely low use of macrolides, fosfomycin and fluoroquinolones in animals and in Chapter 4, the supporting questionnaire given to human participants also suggested that there was low fluoroquinolone (16.1%), erythromycin (3.2%) and cephalosporin (3.2%) use in farmers (though data was only collected in relation to antimicrobial therapies for gastrointestinal issues). Human isolates from farmers in Chapter 4 also showed a low-moderate prevalence of carriage of these resistance genes which was consistent with the low reported use. Patterns of carriage in livestock were also similar to both farmers and slaughterhouse workers. The high carriage of aminoglycoside resistance genes,

specifically *aph(3'')-Ib* and *aph(6)-Id* are both highly prevalent in companion and domestic farm animals in neighbouring Tanzania and typically associated (or co-carried) on plasmids alongside $bla_{CTX\text{-}M\text{-}15}$ (Seni et al., 2016a). This was also consistent with our findings amongst farmers (Chapter 4) and in slaughterhouse workers. As previously discussed in Chapter 3 and 4, there is particularly high usage of sulfonamides, tetracyclines and trimethoprim in farm animals, but despite there being little reported use in humans (results from questionnaires **Chapters 3 and 4**), the prevalence of resistance to these antimicrobials was similar in livestock, farmers and slaughterhouse workers. These similarities in the prevalence of AMR genes could be reflective of overlapping usage patterns in both humans and livestock, or it could suggest that having close contact with animals may increase the risk of humans acquiring AMR *E. coli* from animals, or it could reflect other indirect transmission patterns e.g. via the shared environment. To better answer this question, additional work in the form of constructing logistic regression models with animal contact and/or working in a slaughterhouse as a factor could be constructed (though this is currently being undertaken by Dr Annie Cook, hence it was omitted from this thesis). Additionally, instead of testing only slaughterhouse workers for the presence of AMR *E. coli,* it would have been beneficial to also collect samples from animal carcasses.

Despite being from different studies, with different selection criteria, there was an interesting difference between farmers and livestock, and slaughterhouse workers, which was the difference in the carriage of chloramphenicol resistance genes. *E. coli* from livestock and farmers had an extremely low carriage of *catA1,* whereas slaughterhouse workers had a high prevalence of *catA1* and *catA2.* Despite chloramphenicol use for livestock production being banned in Kenya (Global Antibiotic Resistance Partnership, 2011), there was still significant resistance detected amongst animals. This could be explained by the use of florfenicol, a fluorinated derivative of chloramphenicol, which shows some cross-resistance with chloramphenicol (White et al., 2000). The higher prevalence of resistance genes in slaughterhouse workers may be due to of unreported self-use of chloramphenicol or derivatives amongst human workers, which is being compounded by interactions with animal faeces during the slaughter process. It is important to note that this could be due to the selection bias introduced when choosing samples for WGS, but it is interesting to note, nonetheless. Indeed, the amplification and persistence of AMR determinants in faeces has been described and this could be the pathway by which these determinants are disseminated to slaughterhouse workers (Pornsukarom and Thakur, 2017). There is further evidence in the literature to suggest that there is sharing of bacteria between animal carcases and humans (Muloi et al., 2018); the questionnaire

accompanying the original collection of these isolates (Cook et al., 2017) stated that on average, only 32% (as volunteered by interviewees) of workers (27%, observed by interviewers) wore some form of personal protective equipment (PPE). This highlights that through close contact with animal carcasses (meat, viscera, faeces), resistance genes may be acquired by human *E. coli* on MGEs or AMR *E. coli* colonising the slaughterhouse workers through skin lacerations or via the faecal-oral route.

There was little significant difference in the carriage of the majority of virulence genes between the types of slaughterhouse workers. Two of the 16 most common virulence genes were not carried by *E. coli* isolated from humans working in pig-only slaughterhouses – *eilA* and *air*. As pig-only isolates made up a smaller proportion of the sequenced isolates compared to the other two groups, this may be reflective of the sample size. A number of toxin-producing virulence genes were found in isolates from each of the three groups. Toxin-producing genes carried by ESBL isolates associated with serogroup O25:H4 were almost identical, strongly suggesting clonal transfer within humans working at slaughterhouses slaughtering cattle only and cattle and small ruminants, in distinct locations, particularly within Busia sub-counties (approximately 40km apart). The most commonly carried toxin-producing virulence gene was *senB*, encoding ShET2 – this is commonly associated with EIEC/STEC diarrhoeal disease. As the investigation focused on enteric bacteria, this is an interesting finding as we previously observed extremely infrequent toxin gene carriage amongst livestock and human isolates in the farm study (Chapter 4). Toxin-carrying *E. coli* strains are being more commonly associated with food-borne diseases worldwide (European Food Safety Authority and Control, 2018) and this study has shown that on average, those isolates carrying toxin-producing genes, also carried more virulence and resistance genes than non-toxin producing counterparts. The high levels of resistance to tetracyclines and sulfonamides, in association with toxin-production genes is also supported by the literature (Ojo et al., 2010; Carrie-Ann et al., 2017; Sethulekshmi et al., 2018). As toxigenic *E. coli* was not found amongst farm animals, this also suggests that slaughterhouse workers may be a reservoir of toxigenic *E. coli* and these are not necessarily being acquired from livestock.

Four major plasmid replicons were carried by a high proportion of isolates from all three groups: IncFII, IncFIB, IncQ and IncQ1. The IncF plasmid is commonly associated with ESBL-producing *Enterobacteriaceae* (mostly $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$) in the literature (Novais et al., 2007; Carattoli, 2011; Irrgang et al., 2017) and was also found to be significantly correlated with ESBL-producing *E. coli* in our findings, specifically with the presence of $bla_{\text{CTX-M-15}}$. Three isolates

carrying *bla*$_{CTX\text{-}M\text{-}15}$ also carried the *aac(6')-Ib-cr* variant gene which simultaneously induces resistance to aminoglycosides and ciprofloxacin. IncFII plasmids carrying *bla*$_{CTX\text{-}M\text{-}15}$ are known to be highly transferable (Carattoli, 2009) and the dispersal of both IncF plasmids and *bla*$_{CTX\text{-}M\text{-}15}$ amongst a variety of slaughterhouse workers, working at different types and location of slaughterhouses in Busia, supports this. This presents a public health issue as there is evidence to suggest that IncF and IncQ plasmids carrying multiple AMR and ESBL genes are circulating amongst slaughterhouse workers in Busia. As such, there is potential for the spread of such genes along the food chain via human-contaminated meat products. The other major plasmid replicon type, IncFIB was found to be commonly carried in our human, animal, and environmental study (**Chapter 4**). IncFIB plasmids are reported in the literature and are thought to have resulted from recombinational events between other IncFII plasmids (Coque et al., 2008b; Partridge et al., 2011). The majority of isolates carrying an IncFIB plasmid were genotypically drug-resistant, carrying between 6-15 different AMR genes. However, these isolates also carried other replicon types, which may indicate that multiple resistance genes were being carried on multiple plasmids. Indeed, approximately one in five isolates harbouring the IncFIB plasmids also carried the IncFIA plasmid (21.3%) – these two plasmids have been reported in the literature as being part of a complex, occurring together and carrying *bla*$_{CTX\text{-}M}$ genes (and *bla*$_{CMY}$ in USA studies) (Freitag et al., 2018; Touzain et al., 2018).

There were limitations to this study; the collection of human faecal samples was conducted by Cook et al in 2012 and analysed retrospectively. Only samples from Busia county were located and analysed, thus a number of background samples from neighbouring sub-counties which could have been included in phylogenetic reconstruction have been missed. As multiple workers from each slaughterhouse were sampled and slaughterhouses were visited in the same sub-county, there may have been a clustering effect which was not considered in this analysis. As with **Chapter 4,** raw reads were subject to QC and then mapped to a reference *E. coli* strain (U00096.3), this would have had some effect on calling SNPs for phylogenetic analysis. In future studies, using *de novo* assemblies and filtering of contaminating reads with custom Kraken databases may yield additional information; this is particularly relevant with the thresholds used for detection of resistance genes – it is possible that many genes could have crossed multiple reads and were therefore not detected. Patterns of resistance detected in *E. coli* in this cohort are interesting to compare to our more recent farm study, but due to the large time delay in collecting both sets of isolates, and the different subsampling criteria for WGS, comparisons should be interpreted with caution. This study did not assess the clinical history of patients with infections potentially caused by *E. coli* or any compounding effects of immunocompetence due

to HIV or other conditions, although basic information on health was available as part of the questionnaire given at the time of the study. Akin to the various other studies examining the transmission of AMR bacteria between humans and animals, this study only focused on one bacterium. Realistically, there is rapid dissemination of AMR determinants between bacterial species, which makes it difficult to track infective sources (Sheppard et al., 2016). Furthermore, this study only considered *E. coli* isolated from slaughterhouse workers, and crucially, not from livestock or the slaughter environment.

A number of sequences could not be assigned an MLST even though alleles of each of the seven housekeeping genes were detected. Those ST assigned as unknown were due to the particular combination of alleles not being present in the EnteroBase database. Furthermore, a number of interesting findings related to plasmid carriage within these genomes was found, however shortcomings in plasmid reconstruction meant that no deeper analysis could be performed. It may be beneficial to use alternate, next-generation sequencing platforms such as Oxford Nanopore (Jain et al., 2016). As Illumina fragments are approximately 150-250bp long, reconstruction into whole genomes can often lead to errors in genome construction. Using a method such as Nanopore may allow for the construction of DNA fragments which are kilobases long, which could reduce the errors in genome construction and allow better insight into plasmids and the genes they carry.

It is difficult to distinguish if there has been molecular transmission of AMR *E. coli* between humans and animal carcasses, or if AMR has arisen from selection pressures due to antibiotic use (Muloi et al., 2018). There is a clear overlap in the farming and slaughterhouse environments, slaughterhouse workers, livestock carcasses and food supply chains in Busia, and all of these sectors are interconnected (Alarcon et al., 2017b). It can therefore be difficult to determine the relative contribution to AMR that each of these sectors play. Further studies investigating transmission of AMR determinants between humans and livestock need to consider indirect transmission, particularly from the wider environment (e.g. soil, hospital, and farm effluents), to complement direct animal to human and/or human to animal transmission.

In conclusion, a high prevalence of MDR *E. coli* was isolated from faeces of slaughterhouse workers in Western Kenya. Three major clonal groups of ESBL-*E. coli* were detected in sequenced isolates found in geographically distinct areas and from different types of slaughterhouses which may indicate there is transmission occurring, but there is insufficient

evidence to infer in which direction this is spreading. One group of O25:H4 isolates were all associated with MDR, ESBL production and a high number of toxin-producing virulence genes and shown to belong to the ST131 global pandemic clone, which is a public health concern affecting both food safety and human health. It is difficult to conclude if there is a definitive transmission of resistance determinants from humans to animals or vice-versa, as further work including longitudinal sampling of both slaughterhouse workers, livestock carcasses and the slaughter environment would be required to reconstruct accurate transmission patterns.

This work suggests that there is urgent need to monitor AMR amongst slaughterhouse workers and animal carcasses as well as better regulation of slaughterhouses, alongside targeted measures to improve biosecurity and hygiene to reduce the spread of AMR. These issues are likely to be addressed as part of the new Kenya NAP, which will place focus on increasing education of slaughterhouse workers, and meat inspectors and increasing hygiene practices within slaughterhouses.

Chapter 6

Concluding Discussion

6.1 General Discussion

The increasing levels of AMR in human and veterinary medicine represents one of the largest global public health threats (WHO, 2019a). The role that complex ecological niches (such as those at the animal-human-environment interface) play in the dissemination of AMR, is the subject of much scrutiny and speculation. There is significant overlap in the *E. coli* populations and transmission pathways between all members of this niche, including in humans, livestock, and their shared environment. This interplay is particularly evident in LMICs, where there is a significant overlap of humans and livestock, particularly as they exist in close proximity and have shared environments, which both receive human and animal waste. This means that human, livestock and environmental populations may act as distinct reservoirs of AMR bacteria, meaning that there is an opportunity for resistance determinants to be transmitted in various directions (Woolhouse et al., 2015). Recently, research has focused on AMR gene dissemination between natural ecosystems and humans or livestock (Nesme et al., 2014; Guo et al., 2017); whilst some studies have tried to suggest evidence that transmission of AMR from animals to humans may occur, no robust conclusions on the directionality of that transmission have been drawn, due to limitations in study methodologies (Muloi et al., 2018).

Recent research investigating transmission of AMR bacteria and/or AMR determinants has relied on low-resolution typing tools and the majority of studies in the last 10 years have been based on opportunistic sampling with little spatiotemporal overlap between humans, livestock and their shared environment. In this thesis, I have used high-resolution analysis of bacterial genomes obtained from farmers, livestock, their shared environment and slaughterhouse workers and used a combination of phylogenetic and ecological methods to try and determine AMR transmission between humans, livestock, slaughterhouse workers, and their shared environment. I used *E. coli* as a sentinel organism to investigate the prevalence of AMR, MDR and ESBL-producing *E. coli* in a potentially high-risk rural interface for AMR transmission between humans and livestock in Busia, Kenya. The overarching hypothesis of this thesis is that there is an epidemiologically significant crossover of AMR bacteria and AMR determinants between livestock, humans and their shared environments.

This is the first ever study of this nature to be conducted in western Kenya, and it highlights the major AMR phenotypes amongst humans, rural livestock, and the farming/living environment. Overall there was a high carriage of AMR *E. coli* found in all sectors.

6.1.1 Knowledge and Attitudes towards Antimicrobials and AMR

In **Chapter 3** this study collected data regarding knowledge and attitudes of antimicrobial users and prescribers as well as characterising patterns of AMR *E. coli* amongst farmers, livestock and farming environments. Collecting these data simultaneously in both animals and humans has provided novel insight which could help determine the drivers for the development and maintenance of AMR and is consistent with the One Health approach advocated by global authorities concerned with combatting AMR. With respect to the Kenyan National AMR action plan (NAP) (Fleming Fund, 2019), specific aims of this plan included strengthening the knowledge base and evidence of AMR, improving monitoring and regulation of antimicrobials and optimising the use of antimicrobials in human and veterinary medicine. The work presented in this study is directly relevant to informing the implementation of that plan, in order to combat the spread of AMR. It does this primarily by reducing the paucity of data regarding AMR amongst rural human and livestock populations in this region.

As a result of the questionnaire-based interviews, it was shown that knowledge and understanding of antimicrobials and the dangers they pose in terms of the development of AMR was superficial. Questionnaire-based interviews were used to investigate the patterns of antibiotic purchase, as a proxy for antibiotic usage in farm animals. Broad-spectrum β-lactams, sulphonamides (penicillin-streptomycin) and oxytetracyclines were the most commonly purchased for animal treatments. Colistin – a drug considered as being of last resort in human medicine, was also used by one farmer (1.4%) to treat his animals. Analysis of attitudes and practices of agrovet staff and community animal healthcare workers shows a high level of knowledge amongst AHAs, but knowledge of good prescription practices and appropriate levels of qualifications for dispensing antibiotics is insufficient amongst approximately 50% of agrovet staff. Similarly, knowledge of AMR was found to be lacking amongst both AHAs and agrovet staff. Finally, the practice of selling antimicrobials without prescription was common to this study site and is also common elsewhere in Kenya and other sub-Saharan African countries, which also describe unrestricted access to veterinary antimicrobials in informal shops (Higham et al., 2016; Chem et al., 2018; Basulira et al., 2019; Muloi et al., 2019a).

All antimicrobial users in the veterinary sector access antimicrobials through agrovet shops suggesting that agroveterinary suppliers are key nodes of antimicrobial distribution to end users. Both human and veterinary pharmacists play an important role in enhancing antimicrobial stewardship initiatives, not just by highlighting issues surrounding AMU and AMR,

but by influencing crucial prescribing decisions (Sakeena et al., 2018; Haddadin et al., 2019). However, there is the constant tension between priorities as a business owner and priorities which advocate public health. Pharmacists, agrovet staff, and AHAs can only provide accurate information regarding AMU and AMR, if sufficiently trained, and in line with economic drivers and pressures placed on them. The NAP can reduce the sale of inappropriate antimicrobials by ensuring that antimicrobial providers are appropriately trained. Furthermore, if a clear antimicrobial supply chain is set up, this can help to reduce the number of informal outlets selling antimicrobials, whilst allowing for better enforcement of legislation regarding prudent use of antimicrobials, as well as ensuring that all people have access to essential antimicrobials at all levels.

6.1.2 Carriage of AMR bacteria amongst farmers, livestock and their shared environment

In **Chapter 4**, by using a combination of AMR phenotyping and genotyping, I explored variation in the carriage of AMR determinants (such as virulence and resistance genes) of AMR *E. coli* between human, livestock and environmental populations. 596 livestock and 130 human *E. coli* isolates were tested for susceptibility to a panel of 7 different antimicrobials (chosen based on their commonality from the questionnaire-based study, **Chapter 3**). There was a high prevalence of resistance to tetracycline, sulfonamides, trimethoprim and β-lactams, but extremely low prevalence of resistance to fluoroquinolones, chloramphenicol and aminoglycosides. More than half of the isolates (53.9%) were MDR (to >3 classes of antibiotic). There is significant overlap in the patterns of AMR between farmers, livestock, slaughterhouse workers. Evidence of a common co-occurring phenotype (conferring resistance to tetracycline, sulfathiazole, ampicillin and trimethoprim antibiotic classes) was found amongst livestock, farmers and environmental isolates, suggesting that a conjugative plasmid was disseminating MDR genes within the *E. coli* populations in each group, and that there are similar selection pressures and co-selection of resistance determinants in all hosts. Phylogenetic analysis of a number of isolates showed a highly diverse population of *E. coli* which were dispersed amongst each of the three groups; this strongly suggests that the general *E. coli* population within the study site is largely shared and that there are numerous overlapping transmission pathways which are not necessarily differentiated by the human, livestock or the environment compartments sampled in this study.

Using WGS I characterised the carriage of and diversity of AMR genes within commensal *E. coli* isolated from farmers, livestock and the environment. A total of 60 unique acquired resistance genes were found amongst human, livestock and environmental *E. coli*, and 6 point mutations

were found amongst human and livestock *E. coli*. *sul2, bla$_{TEM-1B}$, mdf(A), tet(A), aph(3")-Ib and aph(6)-Id* conferring resistance to sulfonamides, β-lactams, tetracycline and aminoglycosides, respectively were the most common AMR genes amongst all three groups of isolates. There was no significant difference between the carriage of any of the genes except for *sul2*, which was significantly more prevalent than *sul1* amongst all groups (except for pigs). According to maximum-likelihood phylogenetics, two large groups of isolates with low genetic variation and common STs and serogroups were found to be circulating within the study populations. These were not isolated from the same farms, rather, they often circulated in the same sub-counties (region). Interestingly, there was a particularly high prevalence of ESBL-*E. coli* in water samples, which may indicate that shared human-animal water sources (such as boreholes, rivers and piping leading to taps) are reservoirs of ESBL-*E. coli.* The production of ESBL enzymes is particularly important for AMR in the general population, as ESBL genes were co-carried alongside other AMR genes (such as aminoglycoside and fluoroquinolone resistance) which can make them more difficult to treat. This can place undue strain on healthcare facilities and potentially increase AMU in efforts to treat resistant bacteria in the absence of effective diagnostics.

The high prevalence of AMR *E. coli* in this population indicates that there is potential for further dissemination of AMR strains and their resistance determinants within the wider community (such as between livestock, when they are brought to farmers' markets). I suggest that bacterial sharing is more dynamic than previously hypothesised; as part of the farm study (**Chapter 4**), I suggested that close proximity between livestock and animals may facilitate the transmission of bacteria. However, it may be more likely that there is a high proportion of indirect sharing of bacteria, via the shared environment, which encompasses both animals and livestock. There is a growing body of evidence which suggests that livestock play a minimal role in acquisition and infection of AMR bacteria in humans (Gouliouris et al., 2018; Ludden et al., 2019) and more attention needs to be directed towards the environment.

The NAP suggests that the general population has a high risk of exposure to AMR bacteria. By constructing a baseline of carriage of AMR *E. coli* amongst this population, this study has reduced that paucity of data. Furthermore, the high rates of carriage of AMR *E. coli* suggest the potential for greater levels of resistance amongst bacterial populations, should other drugs (including HPCIAs) become more commonly used, in this setting. It should therefore be a priority for the NAP to also secure the long-term viability of 2nd and 3rd line drugs to ensure their

continued effectiveness. From this data, it is difficult to link specific patterns of AMR to specific hosts and this indicates a generalised risk of emergence of new strains and reaffirms the need to investigate AMR using a One Health approach. Expanded efforts on characterising all compartments of this complex ecosystem may allow for the interpretation of complex transmission pathways between all groups.

6.1.4 Risk factor analysis of AMR outcomes amongst farmers, livestock and the environment

In **Chapter 4**, risk factor analysis provided insight into AMR patterns amongst livestock, farmers, and the environment. Few risk factors were identified, indicating that resistance patterns were generalised across humans, livestock and the environment. This suggests that there may be other factors involved in the variability identified between strains found within these populations. Multivariable models showed that there was a low variance between sub-counties, indicative of local transmission of bacteria. There was greater variance attributed to farm level AMR, especially with respect to tetracycline resistance and ESBL-*E. coli*. A dairy farm in Madagascar noted that "livestock size" i.e. larger farms with >25 cattle and "disinfection" were associated with decreased risk of ESBL production (Gay et al., 2018). This suggests that by improving biosecurity measures and overall hygiene on farms, the risk of maintaining faecal carriage of ESBL-*E. coli* can be decreased. As such, improving hygiene practices on farms may help to reduce transmission of AMR.

There were no significant risk factors associated with the ESBL-producing *E. coli*, despite a high proportion of tested water samples in this study being both MDR and ESBL-producing. As few water samples were collected, this could preclude further conclusions being drawn. MDR and ESBL-*E. coli* have previously been associated with irrigation water (Gekenidis et al., 2018), which lends credibility to the theory that common water sources are reservoirs of ESBL-producing *E. coli*, having become contaminated with both human and animal wastes. Additionally, there was risk associated with recent antimicrobial treatment (use of penicillin-streptomycin in the 3 months prior to the study) for the MDR and ampicillin outcomes. This finding is consistent with AMU providing selection pressures for the development of AMR (Oz et al., 2014; Caudell et al., 2017), though there are also additional environmental factors which must be important in indirect AMR transmission.

Another point of the NAP is to reduce AMR by adopting preventative measures. Vaccination is an important strategy for reducing AMR as it aims to prevent disease outbreaks, reducing

reliance on antimicrobials. However, vaccination of animals was found to be a significant risk factor for tetracycline resistance. This was due to potential confusion between vaccinations and injectable treatments. As AHAs and agrovets are the main providers of vaccinations and antimicrobials, by adequately informing farmers and targeting educational programmes to antimicrobial and vaccine providers, it is possible to reduce the risk of a small proportion of AMR attributed to misuse.

6.1.3 Carriage of AMR bacteria amongst slaughterhouse workers

In **Chapter 5**, again, by using a combination of AMR phenotyping and genotyping, I explored variation in the carriage of AMR determinants of *E. coli* isolated from slaughterhouse workers, working at three different types of slaughterhouse. The three types included slaughterhouses which slaughtered i) mixed ruminants, ii) cattle only, and iii) pigs-only. 447 human faecal samples were tested for susceptibility to the same panel as for the farm study (**Chapter 4**). There was a high prevalence of resistance to the same antibiotics as in farm study, specifically to tetracycline, trimethoprim, sulfathiazole and (to a lesser degree) ampicillin. There was a low prevalence of resistance to fluoroquinolones, chloramphenicol and aminoglycosides. Almost two-thirds of isolates (61.5%) were MDR (to >3 classes of antibiotic). There was no major difference in the overlap of AMR patterns amongst slaughterhouse workers suggesting that commonly co-occurring phenotypes (conferring resistance to tetracycline, sulfathiazole, ampicillin and trimethoprim antibiotic classes) were common to all types of slaughterhouse workers. This points to similar selection pressures and co-selection of resistance determinants in all slaughterhouse workers.

A total of 53 unique acquired resistance genes were found amongst slaughterhouse workers, and 8 point mutations were found amongst *E. coli* isolated from slaughterhouse workers. *sul2, $bla_{TEM-1B,}$ mdf(A), tet(A), catA1, aph(3")-Ib and aph(6)-Id* conferring resistance to sulfonamides, β-lactams, tetracycline, chloramphenicol and aminoglycosides, respectively were the most common AMR genes amongst all three groups of slaughterhouse worker. A higher proportion of point mutations were found amongst slaughterhouse workers than in farmers (Chapter 4), though the difference may be due to differences in subsampling strategies. There was no significant difference between the carriage of any AMR genes between groups, except in pig-only slaughterhouse workers, where carriage of *mdf(A)* was significantly higher than in cattle only and mixed ruminant slaughterhouses. Phylogenetic analysis of a number of isolates showed a highly diverse population of *E. coli* which were dispersed amongst all slaughterhouse workers.

Numerous clades association with ST131, ST361, ST10, ST43 and ST617 were found with low genetic variation indicating that there is a high degree of bacterial sharing between slaughterhouse workers in this population.

There was high carriage of toxin-producing virulence genes (*astA*, *cma*, *sat* and *senB*) amongst *E. coli* isolated from slaughterhouse workers, many of which were co-carried with ESBL genes (specifically $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-12)}}$. Toxin-producing strains of *E. coli* are particularly relevant for food production, and toxin-producing *E. coli* are increasingly becoming attributed to carriage amongst farm animals, food-borne infections, and subsequently diarrhoeal disease, all over the world (Veilleux and Dubreuil, 2006; Ochi et al., 2017; Dubreuil, 2019). The danger and relevance of these toxin-producing *E. coli* strains circulating amongst slaughterhouse workers, is the potential for transmission from the workers, to animal products and then wider distribution to consumers. These strains can cause diarrhoeal disease and may be difficult to treat if they persist, particularly in children, immunocompromised, pregnant and older people (GBD 2016 Diarrhoeal Disease Collaborators, 2018). Contaminating meat products during the slaughter process is therefore a public health risk.

A startling finding was the presence of the ST131 global pandemic strain – four isolates were found to be ST131:O25b:Fim*H30.* These isolates were MDR and highly virulent; further spread of these clones could be important for transmission prevention-based control strategies, which are to be implemented as part of the NAP, due to their resistance to effective antibiotics.

Slaughterhouse workers were an important group to consider in assessing carriage of AMR *E. coli*, as they are responsible for slaughtering animals and preparing food-products destined for sale and consumption. As part of the slaughter, evisceration and butchering process, workers are exposed to a higher density of bacteria from animals, which may put them at high risk of colonisation with new bacterial strains from animal carcases. Due to their regular exposure to animals, slaughterhouse workers may be acting as a sink - acquiring diverse bacterial populations through occupational exposure, as well as their normal lives. Slaughterhouse workers could therefore be key populations of AMR *E. coli* emergence.

There is a need to urgently address shortcoming during the slaughter process (such as inadequate hygiene practices and addressing biosecurity) to ensure that there is limited spread of highly successful AMR, MDR, ESBL- and toxin-producing strains amongst slaughterhouse workers and potentially to food-products. The importance of good hygiene practices in

slaughterhouses, access to running water and soap (to clean utensils, carcasses and hands), and use of PPE such as gloves and aprons (to prevent bacteria from colonising open wounds) are paramount to ensure that AMR bacteria does not spread to humans through contact with animals, to animals products through cross-contamination, and to the environment via disposed of carcasses. The ZooLiNK project has undertaken several such activities, providing workers with new slaughter equipment, PPE, and training. More needs to be done to continue to raise the standard in rural slaughterhouses in Kenya.

6.1.5 Implications of this study for surveillance and public health

Numerous initiatives have been taken by regulatory agencies and governments to combat AMR, but many of these have only been implemented in developed countries (Government of the United Kingdom, 2013; WHO, 2015). In Kenya, a GARP working group is established, but the lack of resources means that policies and the means to penalise those who do not follow policy, is limited (Global Antibiotic Resistance Partnership, 2011). With further support from the government, informed by data collected in this study, there are clear targets for optimising supply chain of antimicrobials and education of antimicrobial suppliers.

The high prevalence of clinically relevant AMR phenotypes and genotypes described amongst farmers, livestock, the shared environment and slaughterhouse workers in this thesis support previous findings (Woolhouse et al., 2016; Caudell et al., 2018; Ingle et al., 2018) that AMR is becoming increasingly common in LMICs. Data collected as part of this thesis regarding the carriage of AMR *E. coli* in key populations in Western Kenya is a significant contribution to the paucity of data regarding AMR. These results reiterate the need for integrated surveillance of AMR, and an urgent need for public health policy to adopt effective strategies which will aim to reduce the emergence and spread of AMR determinants.

The use of WGS in this thesis in a low-income setting is important, given the recent efforts to implement WGS in public health settings as the cost of sequencing and infrastructure becomes more accessible. In public health settings, genomics can help in characterising and tracking the spread of bacterial strains, as well as providing rich surveillance data regarding genomic mechanisms of resistance. Indeed, with the advent of NGS platforms such as Oxford Nanopore, sequencers are no larger than a matchbox and ‘real-time’ genomics can be performed in the field.

AMU use is likely one of the most important drivers of AMR in both humans and livestock (Holmes et al., 2016; Robinson et al., 2016). In LMICs, where resistance is beginning to emerge, there is a short window of opportunity to limit the spread of AMR, by encouraging the switch to sustainable farming practices and reducing the reliance on antimicrobials. This can be achieved through policy recommendations aimed at selectively reducing inappropriate use of antibiotics, increasing appropriate use of antibiotics to treat and prevent disease, and reducing the need for antibiotics (Van Boeckel et al., 2019). As described, the results of this study are directly useful in informing policies to be rolled out as part of the NAP. To summarise, the KAP study **Chapter 3** highlighted gaps in the knowledge of antimicrobial users and providers – these could be the targets for public communication campaigns, particularly the providers. Furthermore, practices such as over-the-counter sales of veterinary and human antimicrobials by informal sources, as well as unlicensed agroveterinary business may be targeted for government intervention, which can attempt to set up clearer antimicrobial supply chains, enforce prudent use of antimicrobials and allow the wider population to access essential antimicrobials. In **Chapter 4** and **5**, the high carriage of AMR, MDR and ESBL *E. coli* amongst all sampled populations highlights shortcomings in infectious diseases control practices – notably the lack of promotion of vaccination programmes as an alternative to prophylactic AMU, while better food and water safety may assist in preventing transmission of AMR bacteria. In order to track and report AMR, and AMU, a national database needs to be established and used, and the use of mobile phone-based technology to do this may be worth investigating. Clearly, the issues raised are complex and will require significant cross-sectorial collaboration but addressing these will be an important part of protecting human and animal health in Kenya in the future.

6.2 Future Work

In **Chapter 3** and **4** I designed and implemented questionnaire-based interviews which were administered to antimicrobial users and providers. This showed superficial knowledge of the risks of AMR but also highlighted issues with translating questionnaires into the local dialect. In future studies, adequately briefing all staff on the perceived outcomes of the study will reduce the incidence of leading questions or over-explaining of questions to compensate for differences in language. This will allow for consistent data which are fully comparable between questionnaires.

Whilst the largest number of locatable AHAs and agrovet shops in the county were interviewed, there are a number of informal sources of antimicrobial sale throughout the region which may

not have been interviewed. Similarly, only a relatively small subset of 70 farms were visited across the region, by expanding the number of participants and future studies to a longitudinal survey of AMR use, additional data could be captured, giving a more accurate portrayal of the drivers of AMR. As no weighting was given to questionnaire results, it is possible that results from the captured sample are not fully representative of the entire population. As the sample size was relatively small, by using post-analysis weighting, this may increase the representativeness of the sample.

The basis of good surveillance systems for AMR and AMU, is accurate record-keeping of purchased and dispensed antimicrobials. A standardised reporting system can feed into a national surveillance system to provide targeted interventions and combat AMR. Whilst Kenya is an LMIC, approximately 93% of Kenyans (across the whole country) use mPesa, the largest mobile wallet in Kenya. This highlights the fact that when people are empowered, they can use digital technology to innovate. The possibility of using mobile phones and technology for such surveillance purposes is certainly realistic, and through education, people could be taught why surveillance to reduce AMR is of importance for their future. The NAP seeks to implement a national database in which antimicrobial use and AMR reporting is compulsory, and this may be done using existing technology. A surveillance system, using data directly from antimicrobial prescribers and users could be designed and implemented using the same software as was used for the KAP study (Chapter 3) which can be rolled out to users cheaply and using existing mobile phone capacity.

In **Chapters 4** and **5**, I demonstrated an overlap of *E. coli* between and within human, livestock and environmental populations. However, a number of isolates were not sequenced due to a cost-based trade-off. Also, a subset of *E. coli* isolates from each population was selected for sequencing using different selection criteria. In the farm study, only isolates from farms where *E. coli* was isolated from human, animal, and environmental samples were sequenced, and thus, a number of farms were not studied further. However, as a broad range of farms from across all sub-counties were included in the analysis, despite some selection bias, the results should be representative of the study site. In the slaughterhouse study, only MDR and ESBL-producing isolates were sequenced. This introduced selection bias, as also sequencing antimicrobial-sensitive isolates would have provided an opportunity to also assess carriage of non-screened resistance phenotypes and resistance determinants. Regarding the phenotypic analysis of isolates, numerous samples were collected from animals and humans on each farm, but as

deposited faeces were collected in a non-random way, selection bias may have skewed the interpretation of the results, and samples may have belonged to the same animal which may have reduced the captured diversity (or conversely, increased it, as additional bacteria spread from walking across the ground could have been sampled). In future studies, use of additional methods such as rectal swabbing and additional sequencing of a wider variety of randomly selected isolates may yield different results relating to the gut microbiome and prevalence of AMR determinants amongst these populations.

Despite characterising a number of plasmids circulating amongst AMR *E. coli*, shortcomings in plasmid reconstruction and other mobile genetic elements from short-read data meant that this study was unable to pinpoint an exact mechanism by which AMR determinants are acquired and dispersed. Additional studies may look to incorporating long-read sequencing techniques to accurately reconstruct plasmids to better determine if carriage of AMR genes is on single or multiple plasmids (Rozwandowicz et al., 2018).

Risk factor analysis could not be completed for a number of low-prevalence outcomes and was not estimated as part of the slaughterhouse study. By increasing the sample size, additional data can be captured, allowing for more rigorous risk factor analyses for a larger number of outcomes to be determined. As the role that food-producing animals and the environment play in the transmission and maintenance of AMR bacteria is still being actively investigated, a longitudinal study which can highlight antimicrobial residues passing through the food chain may provide further insights.

6.3 Concluding Remarks

The findings presented in this study suggest that there are gaps in the knowledge of both antimicrobial users and prescribers, particularly in areas such as the risk of antimicrobial resistance and withdrawal periods. This has far-reaching implications and may lead to a situation where there is significantly reduced efficacy of antimicrobials in both humans and animals. This thesis has demonstrated how fine-scale analysis of bacterial genomes, which, if embedded in an epidemiologically structured sampling framework can be used to i) inform surveillance of the prevalence, emergence and spread of AMR, ii) identify risk factors for carriage of AMR strains and AMR determinants, and iii) track bacterial overlap in a rural LMIC setting. I have provided evidence of carriage and resistance to a number of antimicrobials including tetracyclines, trimethoprim, sulfonamides and ampicillin amongst farmers, animals, slaughterhouse workers

and the environment *E. coli* populations. At the SNP level, there was evidence of bacterial sharing between humans, livestock and the environment which indicates that transmission between groups is occurring. As there is a clear overlap between humans, animals, and the environment in rural settings, it is important to consider not only direct routes of transmission of bacteria between humans and animals, and humans and humans, but also indirect transmission via their shared environments. The use of WGS as a tool to investigate AMR determinants has been particularly useful in elucidating the molecular epidemiology of resistance amongst commensal *E. coli,* and risk factor analysis has highlighted the complex interactions involved in AMR. Overall, this study has highlighted the usefulness of an integrated One Health approach, as well as the need for surveillance systems to collect AMR data across countries, to allow for targeted interventions where required.

Chapter 7

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Chapter 8

Appendices

Appendix I

Material concerning Chapter 3

Figure I-i Participant Information Sheet for KAP Study

Ref ☐☐☐☐

UNIVERSITY OF LIVERPOOL

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

Antibiotic use and patterns of antibiotic resistance in bacteria which can be transmitted from animals to humans in small farms in Western Kenya: **Information Sheet**

Dear Participant,

Thank you for taking the time to read this.

We are currently undertaking a study looking at resistance to antibiotics among bacteria which are found in animals and are transmissible to humans. We would be very grateful if you could answer the questions of this questionnaire, or consent to us taking an audio recording of you as you answer the questions. The questions are mainly about antibiotic purchases and themes around their use in your animals.

Please read the following information carefully. Your participation is voluntary, and if you decide to NOT take part in this study this will have no negative consequences on you.

Why are we asking about antibiotic purchases?
All animals carry bacteria in their gastrointestinal tracts (digestive system), *most* of which are not harmful to the animals, though some of these bacteria can cause diarrhoea or other symptoms in animals. When animals are treated with antibiotics, some of the bacteria which are not killed by them develop resistance – these antibiotic resistant bacteria can also be passed to humans through close interactions with the animals. We are trying to determine how much antibiotic resistance there is in the normal bacteria carried in the digestive tract of cattle and other animals and eventually, how much of this has spread to humans. We want to know where you purchase your antibiotics from so that we can determine the origin of the antibiotics (if they are imported or produced locally etc.) and other factors such as if the seller has had appropriate training and is able to give guidance when selling antibiotics to you. A mixture of questions will allow us to best determine how people access antibiotics, which will eventually allow us to assess correlation between antibiotic administration and resistance patterns. Ultimately, this will allow us to better educate farmers and animal healthcare workers about the appropriate administration of antibiotics, possibly reducing the amounts required for animals.

What information are we collecting?
For this portion of the study we are simply collecting information from animal healthcare workers and farmers – no samples are required directly from the animals. Questions revolve around where your antibiotics are purchased from and how you administer them to your animals.

What does this involve?
We will give you a questionnaire to fill in either as a paper copy or as an electronic copy on an Android tablet. If you would prefer to give us answers verbally, we will write your answers directly onto the questionnaire. If you are not comfortable speaking in English, we will take an audio recording of you answering the questions in Kiswahili as you are asked them by a translator. We will then be able to have this translated and analyse your responses at a later date.

Ref

What will we do with the information we collect?
Once this data has been collected, we will analyse it and eventually be able to give advice on how to better treat animals with antibiotics and give information on how to prevent resistance from occurring.

Further information
Information obtained from the questionnaire may be retained for up to ten years and all data will remain anonymous. If you decide you want to withdraw from the study you may do so without explanation, and any information you have given can be destroyed. Results from the study may be published in academic journals, but no-one will be identifiable from any published work and we will not provide individual results, but can provide you with the overall findings of this study at the end of the study if you wish.

What next?
If you are happy to become involved, then please read and sign the consent form. Please note, unfortunately due to the nature of this study, we will not be able to give you back any individual results, but we are happy to provide you with a copy of the general findings from the study. This will be disseminated through the district veterinary office of your sub-county.

Many thanks,

Steven Kemp

Steven Kemp
(Supervised by Professor Eric Fevre)

If you have any cause for concern or wish to withdraw from the study at any time, please contact us:

Project Manager
Dr Victoria Kyallo
International Livestock Research Institute
Old Naivasha Road
PO Box 30709-00100,
Nairobi
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0204223252
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If you have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer at the University of Liverpool, UK on +44151 794 8290 or email ethics@liv.ac.uk. Alternatively, you can also contact the Research Compliance Office at ILRI, Nairobi on 0204223000/3375/3887 or email EOHSKenyaHelpdesk@cgiar.org.

Information sheet Jan 16 v0.2

Figure I-ii Informed consent form given to all participants before collecting data during the KAP Study

Ref ☐☐☐☐

UNIVERSITY OF LIVERPOOL

Antibiotic use and patterns of antibiotic resistance in bacteria which can be transmitted from animals to humans in small farms in Western Kenya: **Informed Consent Form**

Please read the following information carefully. You may also request a copy for yourself.

Researcher: Steven Kemp, supervised by Professor Eric Fevre

Please initial box

1. I confirm that I have read and have understood the information sheet dated January 2016 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐
3. I understand that I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. I understand that I may refuse to answer particular questions and individual privacy will be maintained in all published and written data from the study. ☐

Name of Person giving consent	Date	Signature
Researcher	Date	Signature

The contact details of the Project Manager are:

Dr Victoria Kyallo
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Old Naivasha Road
PO Box 30709-00100,
Nairobi
Kenya
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Informed consent Form Jan 16 v1

Figure I-iii Questionnaire framework for KAP Study

Veterinary antibiotic dispensing practices in Busia County, Western Kenya.

As part of our research into the way that antibiotics are accessed and administered to farm animals, we are conducting a survey of farmers, animal healthcare workers, veterinary surgeons and agrovets. A separate information sheet detailing why we undertaking this research is provided and we ask all participants to read this carefully before signing an informed consent form.

The following questions are investigating themes around antibiotic acquisition, antibiotic prescription and antibiotic administration to farm animals.

Your participation in this study will help us to better understand antibiotic dispensing practices in farmed animals in Busia county, Western Kenya.

All responses will remain anonymous and are completely confidential.

The information that you provide will be maintained and analysed at the University of Liverpool and will not be made available to other parties. Participation in this study is entirely voluntary.

About Yourself

1. **GeoLocation**

2. **Shop Name/Location**

3. **Gender**
   Mark only one oval.
   - ◯ Male
   - ◯ Female

4. **Age Group**
   Mark only one oval.
   - ◯ 18-24
   - ◯ 25-44
   - ◯ 45-64
   - ◯ 65 +

5. **Current Occupation**

6. **Do you have more than one job?**
Mark only one oval.

- ◯ Yes
- ◯ No

7. **What else do you do?**

_____

8. **What is the highest degree or level of school you have completed?**
If currently enrolled, highest degree received.
Mark only one oval.

- ◯ No Formal Education
- ◯ Primary Education
- ◯ Secondary Education (KCSE or other)
- ◯ College (Certificate/Diploma/Higher Diploma)
- ◯ University

9. **What sort of establishment is this?**
Mark only one oval.

- ◯ Agrovet *Skip to question 69.*
- ◯ Farm *Skip to question 171.*
- ◯ Veterinary Practice *Skip to question 10.*
- ◯ Community Animal Healthcare Practice *Skip to question 10.*
- ◯ Market Stall *Skip to question 122.*

Animal Healthcare Workers and Veterinarians

10. **What is your job at this outlet?**
Mark only one oval.

- ◯ Veterinary Surgeon
- ◯ Animal Healthcare Worker
- ◯ Pharmacist
- ◯ Shop Attendant
- ◯ Owner
- ◯ Laboratory Staff / Technician
- ◯ Other: _____

11. **How long have you worked here for?**
Mark only one oval.

- ◯ Less than 1 year
- ◯ 1-2 Years
- ◯ Longer than 3 Years

12. **How many other staff work here and what are their roles?**
Please write down a job title and the number of staff e.g. 3 x Veterinary Surgeons, 1 x Laboratory Staff, using separate lines for each.

13. **How many pharmacies or agrovets are there nearby (within 3km)?**
Please give name and address. Use a separate line for each place.

14. **Does the Owner work at this outlet?**
Mark only one oval.

- Yes
- No

15. **What qualification(s) do they hold?**

16. **Do you or the owner hold a current license to sell antibiotics for animal use?**
Mark only one oval.

- Yes
- No
- Not Sure

17. **Do you have a veterinary degree?**
Mark only one oval.

- Yes
- No

18. **Have you had specific training in Livestock Health and/or Diseases?**
Mark only one oval.

- Yes
- No

19. **Have you ever received training or are you still training to dispense antibiotics for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

20. **Please describe the nature of your training.**
Please write down the name of the course, the location and when it was undertaken. Use a separate line for each course.

_____

_____

_____

_____

_____

21. **Which of the following products do you sell here?**
Tick all that apply.

- ☐ Veterinary Antibiotics
- ☐ Vaccines
- ☐ Animal Feeds (with Antibiotics)
- ☐ Animal Feeds (without Antibiotics)
- ☐ Animals (Chickens/Cattle etc.)
- ☐ Farm Equipment
- ☐ Pet care products
- ☐ Crop inputs (seeds, fertilisers)
- ☐ Other

22. **What other products do you sell?**

_____

_____

_____

_____

_____

23. **Which of the following services do you provide here?**
Tick all that apply.

- ☐ Clinical Animal Health services
- ☐ Diagnostic Testing
- ☐ Farmer Training
- ☐ Government Surveillance or disease reporting
- ☐ Artificial Insemination
- ☐ Write prescriptions for animal drugs
- ☐ Other

24. **What other services do you provide?**

_____

_____

_____

_____

_____

25. **If you provide diagnostic testing, what specifically do you do?**
Tick all that apply.

- ☐ Bacterial Cultures to determine species
- ☐ Antibiotic Susceptibility tests
- ☐ Molecular Testing (PCR etc.)
- ☐ Blood Smears
- ☐ Milk Testing (For residues etc.)
- ☐ Skin Scrapes (Mites)
- ☐ Post-mortems
- ☐ Other

26. **What other diagnostic tests do you perform?**

_____

_____

_____

_____

_____

27. **Which of the following do you serve most frequently?**
Tick all that apply.

- ☐ Owners of Dairy Cattle
- ☐ Owners of Beef Cattle
- ☐ Owners of Sheep/Goats
- ☐ Owners of Poultry
- ☐ Mixed livestock farmers
- ☐ Crop farmers
- ☐ Clinicians (vets, animal healthcare workers etc.)

28. **Which of the following do you serve least frequently?**
Tick all that apply.

- [ ] Owners of Dairy Cattle
- [ ] Owners of Beef Cattle
- [ ] Owners of Sheep/Goats
- [ ] Owners of Poultry
- [ ] Mixed livestock farmers
- [ ] Crop farmers
- [ ] Clinicians (vets, animal healthcare workers etc)

29. **What are the main reasons for customers to come to this shop? To buy/get:**
Tick all that apply.

- [ ] Veterinary Antibiotics
- [ ] Vaccines
- [ ] Animal Feeds (with Antibiotics)
- [ ] Animal Feeds (without Antibiotics)
- [ ] Animals (Chicks, cattle etc.)
- [ ] Farming Equipment
- [ ] Crop Inputs (Fertiliser, seeds etc.)
- [ ] Animal Advice
- [ ] Other

30. **What other reasons do people visit the shop for?**

_____

_____

_____

_____

_____

31. **On average, how many customers do you serve...**
On separate lines, please indicate the number of people you serve on 1) Market Day, 2) Non-Market Days and 3) The whole week

_____

_____

_____

_____

_____

32. **Do you write prescriptions for antibiotics?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

33. **How often do you give advice to a customer before they purchase antibiotics?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

34. **When giving information to customers about withdrawal periods and antibiotic residues, what specific information do you give and where do you get that information from?**

_____

_____

_____

_____

_____

35. **When recommending/prescribing antibiotics, which of the following pieces of information do you give the customer?**
Tick all that apply.

- ☐ Directions for use
- ☐ Potential side effects
- ☐ Correct dosages
- ☐ Expiry date
- ☐ How to store
- ☐ Contra-indications with other antibiotics
- ☐ Withdrawal Period
- ☐ Residues
- ☐ Other

36. **What other information do you give to customers?**

_____

_____

_____

_____

_____

37. **What factors do you take into account when recommending antibiotics to a customer?**
Tick all that apply.

- ☐ Cost of drug to customer (profit)
- ☐ Customer Preference
- ☐ Availability of drugs
- ☐ Results of a diagnostic test
- ☐ How the drug is given to the animal
- ☐ Other

38. **What other factors do you take into account when recommending antibiotics to a customer?**

_____

_____

_____

_____

_____

39. **How often does your customer request a specific antibiotic?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

40. **Have you ever recommended or prescribed antibiotics to a farmer for animals without examining the animals first?**
Mark only one oval.

- ◯ Yes
- ◯ No

41. **Why did you make a recommendation without examining the animals?**

_____

_____

_____

_____

_____

42. **Once a person has obtained the antibiotics you have recommended or prescribed, how often do you administer them to the animals on their behalf?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

43. **Once treatment of the animals has begun/been completed, do you perform check-ups on the farm to determine the clinical outcome?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

44. **Why don't you perform check-ups?**

45. **Where do you acquire your antibiotics from?**

46. **Are there any types of antibiotics which you cannot get from your supplier?**

47. **How do you store your antibiotics?**
Tick all that apply.

- [ ] Fridge
- [ ] Shelves
- [ ] Cool Box
- [ ] Other

48. **How else do you store your antibiotics?**

49. **Do you keep a record of all the antibiotics you dispense/prescribe?**
Mark only one oval.

- ◯ Yes
- ◯ No

50. **Please can we see/take a copy?**
Mark only one oval.

- ◯ Yes
- ◯ No

Please read carefully before answering the following questions.

The next five questions are all linked and ask about the most commonly sold antibiotics. The first question will ask about the most commonly sold antibiotics. Please type the brand name and active Ingredient on a separate line - the one at the top being the most commonly sold antibiotic and the one at the bottom being the least sold antibiotic. The following questions should be typed in the same order e.g. if Tetracycline is the most commonly sold antibiotic, the answer on the top line will refer to Tetracycline. Please ask for clarification if you need help answering these questions.

51. **What are the five most commonly prescribed/sold antibiotics?**
Please list each on a different row.

52. **What/which condition was each antibiotic sold to treat?**
Please list each on a different row.

53. **What species was this each antibiotic for?**
Please list each on a different row.

54. **What is the typical dose, frequency and duration of treatment for each antibiotic?**
Please list each on a different row.

55. **What is the price for the customer and what is the price you paid to buy the antibiotics?**
Please list each on a different row.

56. **Are the antibiotics you sell primarily used for:**
Mark only one oval.

- ◯ Growth Promotion
- ◯ Treatment of Disease
- ◯ Disease Prevention

57. **Are there any antibiotics which you are not allowed to recommend/prescribe?**
Mark only one oval.

- ◯ Yes
- ◯ No

58. **Please list the name of each antibiotic and the reason you cannot prescribe them.**

_____

_____

_____

_____

_____

59. **Are you aware of any specific guidelines which should be consulted when recommending or prescribing antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Not Sure

60. **Please list any organisations and policies that you are aware of.**

_____

_____

_____

_____

_____

61. **Are you aware of any critical/important antibiotics which are of high importance to human medicine, but can also be prescribed for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

62. **Please give examples of critical/important antibiotics that you are aware of.**

_____

_____

_____

_____

_____

63. **Have you ever prescribed 3rd or 4th generation Cephalosporins or Fluoroquinolone antibiotics for animal use?**
Tick all that apply.

- [ ] 3rd or 4th Generation Cephalosporins (ceftiofur, cefaqui-nome etc.)
- [ ] Fluoroquinolones (enrofloxacin, marbofloxacin etc.)
- [ ] Neither

64. **Please give details of the clinical scenario in which they were prescribed.**

65. **If you recommend or prescribe an antibiotic and the farmer returns to you and complains that it hasn't treated the disease, what do you do?**

66. **Please indicate in the space below what you think/know about what antibiotic resistance is.**

67. **Have you had any evidence of antibiotic resistance when prescribing or treating animals with antibiotics?**

68. **What did you do to overcome the antibiotic resistance?**
Are there other available antibiotics or treatments? Please give as much detail as possible.

_____

_____

_____

_____

_____

Thank you for your responses!

Stop filling out this form.

Question for Agrovets

69. **What is your job at this shop?**
Mark only one oval.

- ◯ Shop Attendant
- ◯ Pharmacist
- ◯ Veterinary surgeon
- ◯ Owner
- ◯ Other: _____

70. **How long have you worked here for?**
Mark only one oval.

- ◯ Less than 1 year
- ◯ 1-2 Years
- ◯ Longer than 3 Years

71. **How many other staff work here and what are their roles?**
Please write down a job title and the number of staff e.g. 3 x Veterinary Surgeons, 1 x Laboratory Staff, using separate lines for each.

_____

_____

_____

_____

_____

72. **How many other pharmacies/agrovets are there close by (within 3km)?**
Please give names and addresses where possible. Use a separate line for each.

_____

_____

_____

_____

_____

73. **Does the owner work here?**
Mark only one oval.

- ◯ Yes
- ◯ No

74. **What qualification(s) do they hold?**

_____

75. **Do you or the owner hold a current license to sell antibiotics for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

76. **Have you had specific training in Livestock Health and/or Disease?**
Mark only one oval.

- ◯ Yes
- ◯ No

77. **Have you ever received any training in, or are you still training to dispense antibiotics for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

78. **What sort of training have you undertaken or are still undertaking?**
Please write down the name of the course, the location and when it was undertaken. Use a separate line for each course.

_____

_____

_____

_____

_____

79. **Do you think you need additional training to help you do your work?**
Mark only one oval.

- ◯ Yes
- ◯ No

80. **What additional training would be of most benefit to your work?**

_____

81. **What types of product do you sell in this shop?**
Tick all that apply.

- [ ] Veterinary Antibiotics
- [ ] Vaccines
- [ ] Animal Feeds (with antibiotics)
- [ ] Animal Feeds (without antibiotics)
- [ ] Animals (Chicks, cattle etc.)
- [ ] Farming Equipment
- [ ] Crop Inputs (Fertiliser, seeds etc.)
- [ ] Pet Care Products
- [ ] Other

82. **What other types of products do you sell?**

_____

_____

_____

_____

_____

83. **Do you provide any other services?**
Tick all that apply.

- [ ] Farmer Training
- [ ] Government Surveillance of animal diseases
- [ ] Farm visits
- [ ] Other

84. **What other services do you provide?**

_____

_____

_____

_____

_____

85. **Who are your most frequent customers?**
Tick all that apply.

- [ ] Owners of Dairy Cattle
- [ ] Owners of Beef Cattle
- [ ] Owners of Sheep/Goats
- [ ] Owners of Poultry
- [ ] Mixed livestock farmers
- [ ] Crop farmers
- [ ] Clinicians (Vets or animal healthcare workers)

86. **Who are your least frequent customers?**
Tick all that apply.

- [ ] Owners of Dairy Cattle
- [ ] Owners of Beef Cattle
- [ ] Owners of Sheep/Goats
- [ ] Owners of Poultry
- [ ] Mixed livestock farmers
- [ ] Crop farmers
- [ ] Clinicians (Vets or animal healthcare workers)

87. **What are the main reasons for customers to come to this shop? To buy/get:**
Tick all that apply.

- [ ] Veterinary Antibiotics
- [ ] Vaccines
- [ ] Animal Feeds (with antibiotics)
- [ ] Animal Feeds (without antibiotics)
- [ ] Animals (Chicks, cattle etc.)
- [ ] Farming Equipment
- [ ] Crop Inputs (Fertiliser, seeds etc.)
- [ ] Animal Advice
- [ ] Other

88. **What other reasons do people visit the shop for?**

_____

_____

_____

_____

_____

89. **On average, how many customers do you serve...**
On separate lines, please indicate the number of people you serve on 1) Market Day, 2) Non-market days and 3) The whole week

_____

_____

_____

_____

_____

90. **Do you fulfil prescriptions for antibiotics here?**
Mark only one oval.

- ◯ Yes
- ◯ No

91. **How often do you give a customer advice before they purchase antibiotics?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

92. **When giving information to customers about Withdrawal Periods and Residues, what specific information do you give and where do you get that information from?**

_____

_____

_____

_____

_____

93. **When you sell antibiotics to a customer, which of the following pieces of information do you give them?**
Tick all that apply.

- ☐ Directions for use
- ☐ Potential side effects
- ☐ Correct dosage
- ☐ Expiry Date
- ☐ Storage instructions
- ☐ Contra-indications with other antibiotics
- ☐ Withdrawal period
- ☐ Residues
- ☐ Other

94. **What other information do you give to customers?**

_____

_____

_____

_____

_____

95. **What factors do you take into account when recommending antibiotics to a customer?**
Tick all that apply.

- ☐ Cost of drug to customer (profit)
- ☐ Customer preference
- ☐ Availability of drugs
- ☐ How easy it is to give the drug to the animals
- ☐ Other

96. **What other factors do you take into account when recommending antibiotics to a customer?**

_____

_____

_____

_____

_____

97. **How often does your customer make a request a specific antibiotic?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

98. **If you are unsure of the best antibiotic treatment options for animals, do you refer the customer to a veterinary or other animal healthcare worker?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

99. **Where do you acquire your antibiotics from?**

_____

100. **Are there any types of antibiotics which you cannot get from your supplier?**

_____

_____

_____

_____

_____

101. **How do you store your antibiotics?**
Tick all that apply.

- ☐ Fridge
- ☐ Shelves
- ☐ Cool box
- ☐ Other

102. **How else do you store your antibiotics?**

_____

103. **Do you keep a record of all of the antibiotics you sell?**
Mark only one oval.

- ◯ Yes
- ◯ No

104. **Please can we see/have a copy of this?**
Mark only one oval.

- ◯ Yes
- ◯ No

Please read carefully before answering the following questions.

The next five questions are all linked and ask about the most commonly sold antibiotics. The first question will ask about the most commonly sold antibiotics. Please type the brand name and active Ingredient on a separate line - the one at the top being the most commonly sold antibiotic and the one at the bottom being the least sold antibiotic. The following questions should be typed in the same order e.g. if Tetracycline is the most commonly sold antibiotic, the answer on the top line will refer to Tetracycline. Please ask for clarification if you need help answering these questions.

105. **What are the five most commonly sold antibiotics?**
Please list each on a separate line, indicating the Brand name and active ingredient.

_____

_____

_____

_____

_____

106. **What/which condition was each antibiotic sold to treat?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

107. **What species was each antibiotic for?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

108. **What is the typical dose, frequency and duration of each treatment?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

109. **What is the price for the customer and how much did you pay for the antibiotic?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

110. **Are the antibiotics you sell primarily used for:**
Mark only one oval.

- ◯ Growth Promotion
- ◯ Treatment of Disease
- ◯ Disease Prevention

111. **Are you aware of any specific guidelines which should be consulted when recommending or selling antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Not Sure

112. **Please list any organisations and policies that you are aware of.**

_____

_____

_____

_____

_____

113. **Are you aware of any critical/important antibiotics which are of high importance to human medicine, but can also be used for animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

114. **Please give examples of critical/important antibiotics which you are aware of.**

115. **Do you stock 3rd or 4th generation Cephalosporins or Fluoroquinolones?**
Tick all that apply.

- [ ] 3rd or 4th Generation Cephalosporins (ceftiofur, cefaqui-nome etc.)
- [ ] Fluoroquinolones (enrofloxacin, marbofloxacin etc.)
- [ ] Neither

116. **Have you ever sold 3rd or 4th generation Cephalosporins or Fluoroquinolones?**
Tick all that apply.

- [ ] 3rd or 4th Generation Cephalosporins (ceftiofur, cefaqui-nome etc.)
- [ ] Fluoroquinolones (enrofloxacin, marbofloxacin etc.)
- [ ] Neither

117. **What did you sell and what was the clinical scenario in which they were recommended for?**

118. **If you recommend and sell an antibiotic to a farmer and the farmer returns and states that his animal has not been cured, what do you do?**

119. **Please indicate in the space below what you think/know about what antibiotic resistance is.**

120. **Have you had any evidence of antibiotic resistance when prescribing or treating animals with antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No

121. **What did you do to overcome the antibiotic resistance?**
e.g Are there other available antibiotics or treatments? Please give as much detail as possible.

_____

_____

_____

_____

_____

Thank you for your responses!

Stop filling out this form.

Questions for Market Stalls

122. **How long have you worked at this stall for?**
Mark only one oval.

- ◯ Less than 1 year
- ◯ 1-2 Years
- ◯ Longer than 3 Years

123. **Are you the owner/does the owner of the stall work here?**
Mark only one oval.

- ◯ Yes
- ◯ No

124. **What qualifications do you/the owner hold?**

_____

_____

_____

_____

_____

125. **Do you/the owner hold a current license to sell antibiotics for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

126. **Have you had specific training in Livestock Health and/or Disease?**
Mark only one oval.

- ◯ Yes
- ◯ No

127. **Have you ever received any training in, or are you still training to sell antibiotics for animal use?**
Mark only one oval.

- ◯ Yes
- ◯ No

128. **What sort of training have you undertaken or are still undertaking?**
Please write down the name of the course, the location and when it was undertaken. Use a separate line for each course.

_____

_____

_____

_____

_____

129. **Do you think you need additional training to help you do your work?**
Mark only one oval.

- ◯ Yes
- ◯ No

130. **What additional training would be of most benefit to your work?**

_____

_____

_____

_____

_____

131. **What types of product do you sell at this stall?**
Tick all that apply.

- ☐ Veterinary Antibiotics
- ☐ Vaccines
- ☐ Animal Feeds (with antibiotics)
- ☐ Animal Feeds (without antibiotics)
- ☐ Animals (Chicks, cattle etc.)
- ☐ Farming Equipment
- ☐ Crop Inputs (Fertiliser, seeds etc.)
- ☐ Pet care products
- ☐ Other

132. **What other types of product do you sell here?**

_____

_____

_____

_____

_____

133. **Do you provide any services to customers?**
Tick all that apply.

- ☐ Government surveillance of animal diseases
- ☐ Farm visits
- ☐ Farmer training
- ☐ Other

134. **What other services do you provide?**

_____

_____

_____

_____

_____

135. **Who are your most frequent customers?**
Tick all that apply.

- ☐ Owners of Dairy Cattle
- ☐ Owners of Beef Cattle
- ☐ OWners of Sheep/Goats
- ☐ Owners of Poultry
- ☐ Mixed Livestock farmers
- ☐ Crop farmers
- ☐ Clinicians (Vets or animal healthcare workers)

136. **Who are your least frequent customers?**
Tick all that apply.

- ☐ Owners of Dairy Cattle
- ☐ Owners of Beef Cattle
- ☐ OWners of Sheep/Goats
- ☐ Owners of Poultry
- ☐ Mixed Livestock farmers
- ☐ Crop farmers
- ☐ Clinicians (Vets or animal healthcare workers)

137. **What are the main reasons for customers to come to this stall? To buy/get:**
Tick all that apply.

- [ ] Veterinary Antibiotics
- [ ] Vaccines
- [ ] Animal Feeds (with antibiotics)
- [ ] Animal Feeds (without antibiotics)
- [ ] Animals (Chicks, cattle etc.)
- [ ] Farming Equipment
- [ ] Crop Inputs (Fertiliser, seeds etc.)
- [ ] Pet care products
- [ ] Other

138. **What other reasons do people visit this shop for?**

_____

_____

_____

_____

_____

139. **On average, how many customers do you serve...**
On separate lines, please indicate the number of people you serve on 1) Market Day, 2) Non-market days and 3) The whole week

_____

_____

_____

_____

_____

140. **How often do you give a customer advice before they purchase antibiotics?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

141. **When giving information to customers about Withdrawal Periods and Residues, what specific information do you give and where do you get that information from?**

_____

_____

_____

_____

_____

142. **When you sell antibiotics to a customer, which of the following pieces of information do you give them?**
Tick all that apply.

- [ ] Directions for use
- [ ] Potential side effects
- [ ] Correct dosage
- [ ] Expiry Date
- [ ] Storage Instructions
- [ ] Contra-indications with other antibiotics
- [ ] Withdrawal periods
- [ ] Residues
- [ ] Other

143. **What other information do you give to customers?**

_____

_____

_____

_____

_____

144. **What factors do you take into account when recommending antibiotics to a customer?**
Tick all that apply.

- [ ] Cost of drug to customer (profit)
- [ ] Customer preference
- [ ] Availability of antibiotics
- [ ] How easy it is to administer to the animals
- [ ] Other

145. **What other factors do you take into account when recommending antibiotics to a customer?**

_____

_____

_____

_____

_____

146. **How often does your customer make a request for a specific antibiotic?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

147. **If you are unsure of the best antibiotic treatment options for animals, do you refer the customer to a veterinary or other animal healthcare worker?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

148. **Where do you acquire your antibiotics from?**

_____

_____

_____

_____

_____

149. **Are there any types of antibiotics which you cannot get from your supplier?**

_____

_____

_____

_____

_____

150. **How do you store your antibiotics?**
Tick all that apply.

- ☐ Fridge
- ☐ Cool box
- ☐ Shelves
- ☐ Other

151. **How else do you store your antibiotics?**

_____

_____

_____

_____

_____

152. **Do you keep a record of all of the antibiotics you sell?**
Mark only one oval.

- ◯ Yes
- ◯ No

153. **Please can we see/have a copy of this?**
Mark only one oval.

- ◯ Yes
- ◯ No

Please read carefully before answering the following questions.

The next five questions are all linked and ask about the most commonly sold antibiotics. The first question will ask about the most commonly sold antibiotics. Please type the brand name and active Ingredient on a separate line the one at the top being the most commonly sold antibiotic and the one at the bottom being the least sold antibiotic. The following questions should be typed in the same order e.g. if Tetracycline is the most commonly sold antibiotic, the answer on the top line will refer to Tetracycline. Please ask for clarification if you need help answering these questions.

154. **What are the five most commonly sold antibiotics?**
Please list each on a separate line, indicating the Brand name and active ingredient.

_____

_____

_____

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_____

155. **What/which condition was each antibiotic sold to treat?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

156. **What species was each antibiotic for?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

157. **What is the typical dose, frequency and duration of each treatment?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

158. **What is the price for the customer and how much did you pay for the antibiotic?**
Please answer on a separate line for each.

_____

_____

_____

_____

_____

159. **Are the antibiotics you sell primarily used for:**
Mark only one oval.

- ◯ Growth Promotion
- ◯ Treatment of Disease
- ◯ Disease Prevention

160. **Are you aware of any specific guidelines which should be consulted when recommending or selling antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Not Sure

161. **Please list any organisations and policies that you are aware of.**

_____

_____

_____

_____

_____

162. **Are you aware of any critical/important antibiotics which are of high importance to human medicine, but can also be used for animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

163. **Please give examples of critical/important antibiotics which you are aware of.**

_____

_____

_____

_____

_____

164. **Do you stock 3rd or 4th generation Cephalosporins or Fluoroquinolones?**
Tick all that apply.

- [ ] 3rd or 4th Generation Cephalosporins (ceftiofur, cefaquinome etc.)
- [ ] Fluoroquinolones (enrofloxacin, marbofloxacin etc.)
- [ ] Neither

165. **Do you ever sold 3rd or 4th generation Cephalosporins or Fluoroquinolones?**
Tick all that apply.

- [ ] 3rd or 4th Generation Cephalosporins (ceftiofur, cefaquinome etc.)
- [ ] Fluoroquinolones (enrofloxacin, marbofloxacin etc.)
- [ ] Neither

166. **What did you sell and what was the clinical scenario in which they were recommended for?**

_____

_____

_____

_____

_____

167. **If you sell an antibiotic to a farmer and the farmer returns and states that his animal has not been cured, what do you do?**

_____

_____

_____

_____

_____

168. **Please indicate in the space below what you think/know about what antibiotic resistance is.**

_____

_____

_____

_____

_____

169. **Have you had any evidence of antibiotic resistance when selling antibiotics for animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

170. **What did you do to overcome the antibiotic resistance?**

Thank you for your responses!

Stop filling out this form.

Questions for Farmers

171. **Which types of animal do you keep on your farm?**
Tick all that apply.

- [ ] Cattle
- [ ] Sheep
- [ ] Goats
- [ ] Chickens
- [ ] Pigs
- [ ] Other

172. **What other animals do you keep here?**

173. **How many of each animal do you have?**

174. **What is the main purpose of your animals?**

175. **How many of your animals are used for breeding?**
Please write each species of animal on a separate line.

_____

_____

_____

_____

_____

176. **How many of your animals are used for animal products for sale (milk/meat/eggs etc.)**
Please write each species of animal on a separate line.

_____

_____

_____

_____

_____

177. **How many of your animals are used for animal products for your own consumption (milk/meat/eggs etc.)**
Please write each species of animal on a separate line.

_____

_____

_____

_____

_____

The following questions will ask about antibiotic use in your animals. If you cannot remember the name of the antibiotic or you are unsure if the treatment is an antibiotic, please let the interviewer know and we will request to see the packaging and may ask your permission to take a photograph of it.

178. **Do you treat your animals with antibiotics?**
Mark only one oval.

- ◯ Often
- ◯ Occasionally
- ◯ Rarely
- ◯ Never

179. **Can you estimate how often you treat your animals with antibiotics e.g. weekly, monthly, yearly?**

_____

_____

_____

_____

_____

180. **Which of the following reasons do you treat your animals with antibiotics for?**
Tick all that apply.

- [ ] Growth Promotion
- [ ] Treatment for Disease
- [ ] To prevent disease

181. **Have any of your animals required antibiotic treatment in the last year?**
Mark only one oval.

- ◯ Yes
- ◯ No

182. **Please list the illnesses and which types of antibiotics, you used to treat your animals with.**
If you used different antibiotics for each species, please list on separate lines.

_____

_____

_____

_____

_____

183. **Do you ever purchase commercial feeds containing...**
Tick all that apply.

- [ ] Antibiotics only
- [ ] Vitamins and Minerals only
- [ ] Vitamins, Mineral and Antibiotics
- [ ] Do not purchase feeds

184. **When purchasing commercial feeds containing antibiotics, what is it mainly used for?**
Tick all that apply.

- [ ] Growth Promotion
- [ ] Treating Disease
- [ ] Disease Prevention

185. **Do you keep a record of all of the antibiotics you treat your animals with?**
Mark only one oval.

- ◯ Yes
- ◯ No

186. **Can we have a copy?**
Mark only one oval.

- ◯ Yes
- ◯ No

187. **Do you seek the advice of a vet before purchasing antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

188. **If you do not seek advice from a vet before purchasing antibiotics, do you seek advice elsewhere?**
Tick all that apply.

- ☐ Agrovet
- ☐ Another farmer
- ☐ Animal Healthcare worker
- ☐ Internet
- ☐ Books
- ☐ Other

189. **Which other sources do you consult?**

_____

_____

_____

_____

_____

190. **How often do your request a specific antibiotic from an agrovet or pharmacy?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

191. **Why do you request specific antibiotics?**

_____

_____

_____

_____

_____

192. **How do you know to request that specific antibiotic?**

193. **Where do you purchase your antibiotics from?**
Please provide name of person/shop and/or contact details and an address or location if you know them.

194. **What is the reason for choosing this place?**

195. **How many pharmacies or agrovets are there nearby (within 3km)?**
Please give name and address. Use a separate line for each place.

196. **What are the main factors when purchasing antibiotics?**
Tick all that apply.

- [ ] Price
- [ ] Amount Needed (Dose)
- [ ] Availability in a specific shop
- [ ] Other

197. **What other factors do you take into consideration when purchasing antibiotics?**

198. **Do you have an issue with the amount of antibiotics you need to purchase e.g. you have two sheep, but you can only buy packs to treat ten sheep?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

199. **Do you use any extra antibiotics for any other animal species or do you store them?**
Tick all that apply.

- ☐ Store them for next time
- ☐ Use for another species
- ☐ A mixture of storage and use for other species

200. **How do you store any extra antibiotics which do you do not use?**
Tick all that apply.

- ☐ Fridge
- ☐ Cool box
- ☐ Shelf
- ☐ Other

201. **How else do you store any antibiotics which you do not use?**

202. **Once you have purchased the antibiotic(s), do you follow the recommended dosage instructions?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

203. **If you treat an animal with antibiotics and it appears to get better before the end of the full treatment, do you stop giving it antibiotics and save them for later?**
Mark only one oval.

- ◯ Occasionally
- ◯ Sometimes
- ◯ Rarely
- ◯ Never

204. **Can you estimate how often you treat your animals e.g. daily, weekly, monthly or yearly?**

_____

_____

_____

_____

_____

205. **How do you work out the correct dosage for each animal you need to treat with antibiotics?**
Tick all that apply.

- ☐ Weigh animals with scales
- ☐ Weigh animals with a weighing band
- ☐ Estimate
- ☐ Follow recommendation of Vet/Agrovet
- ☐ Other

206. **What other ways do you use to estimate the correct dose of antibiotics?**

_____

_____

_____

_____

_____

207. **How do you give the antibiotics to the pigs?**
Tick all that apply.

- ☐ Injection
- ☐ In Feed
- ☐ In water
- ☐ Other

208. **How do you give the antibiotics to the chickens?**
Tick all that apply.

- ☐ Injection
- ☐ In Feed
- ☐ In water
- ☐ Other

209. **How do you give the antibiotics to the cattle?**
Tick all that apply.

- [ ] Injection
- [ ] In Feed
- [ ] In water
- [ ] Other

210. **How do you give the antibiotics to the sheep?**
Tick all that apply.

- [ ] Injection
- [ ] In Feed
- [ ] In water
- [ ] Other

211. **How do you give the antibiotics to the goats?**
Tick all that apply.

- [ ] Injection
- [ ] In Feed
- [ ] In water
- [ ] Other

212. **If you have selected 'other' for any of the questions asking how you give the antibiotics to your animals, please write below the name of the animals and how else you give antibiotics to them.**
Please use a different row for each type of animal.

_____

_____

_____

_____

_____

213. **Do you always give the recommended dosage to your animals?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

214. **Why don't you always give the recommended dosage to your animals??**

_____

_____

_____

_____

_____

215. **Imagine the following clinical scenario: You have a 10 cattle, but you need antibiotics for just 1 of them. You check the dose instructions on the antibiotics packaging but it does not allow for just 1 animal to be treated. What do you do?**

216. **If you purchase one type of antibiotic to treat the animals with and it does not work, what do you do?**

217. **What do you know about antibiotic resistance?**

218. **Have you had any evidence of antibiotic resistance when giving antibiotics to your animals?**

Mark only one oval.

- ◯ Yes
- ◯ No

219. **What do you think causes antibiotic resistance?**

220. **What did you do to overcome the antibiotic resistance?**
Are there other antibiotics available to you? What did you use; please give as much detail as possible.

_____

_____

_____

_____

_____

221. **What do you know about antibiotic withdrawal periods?**
For each type of animal, please write on a separate row.

_____

_____

_____

_____

_____

222. **Do you sell any animal products (meat/cheese/milk/eggs) whilst the animals are on antibiotics or have recently finished treatment?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

223. **Do you sell consume any animal products (meat/cheese/milk/eggs) whilst the animals are on antibiotics or have recently finished treatment?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

224. **If you sell milk obtained from your animals, do you pasteurise or boil your milk before selling it?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

225. **If you consume milk obtained from your animals, do you pasteurise or boil your milk before consuming it?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

226. **Have you ever purchased antibiotics intended for human consumption from a pharmacy or doctor to give to your animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

227. **Please give details of what the antibiotic was and why it was used instead of a veterinary antibiotic.**

Appendix II

Material concerning Chapter 4

Figure II-i Information sheet given to all participants regarding the Farm study

Ref

UNIVERSITY OF LIVERPOOL

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

Antibiotic use and patterns of antibiotic resistance in bacteria which can be transmitted from animals to humans in small farms in Western Kenya: **Information Sheet**

Dear Participant,

We are currently undertaking a study looking at resistance to antibiotics among bacteria which are found in animals and are transmissible to humans. We would be very grateful if you could answer the questions of the attached questionnaire. The questions relate mainly to the animals you keep on your farm, their overall health, any recent antibiotic treatments they have. We would also like to collect faecal samples from the animals to test at our laboratory in Busia. In addition to collecting data and faecal samples, we will also ask some questions about how you look after the animals you keep on the farm and also request a faecal sample from yourself. Your participation is voluntary, and if you decide to NOT take part in this study this will have no negative consequences on you or your animals.

Why are we asking about you animals and antibiotic?
All animals carry bacteria in their digestive system, *most* of which are not harmful to the animals, though some of these bacteria can cause diarrhoea or other symptoms in animals. When animals are treated with antibiotics, some of the bacteria which are not killed by them develop resistance – these antibiotic resistant bacteria can also be passed to humans through close contact with the animals. We are trying to determine how much antibiotic resistance there is in the normal bacteria carried by cattle and other animals and eventually, how much of this has spread to humans. We want to know which types of diseases your animals commonly have and what sort of antibiotics you use to treat these diseases. A mixture of questions and collection of samples will allow us to best determine which types of antibiotics will have the best effect in treating diseases as we will be able to identify if there is any antibiotic resistance occurring. We will look specifically for a type of gut bacteria, called *E. coli* which can also cause diarrhoea in humans. Ultimately, this study will allow us to better educate farmers and animal healthcare workers and advise them on the types of antibiotics which are likely to be the most successful in treating animals and helping to reduce antibiotic resistance.

What are we collecting?
We will aim to collect two types of samples. We will collect a small amount of faeces from a number of different animals/poultry on your farm and take this back to our lab to determine the different types of antibiotic resistance found in your animals. We will also collect a small amount of faeces from a number of people on the farm (those who have direct interactions with the animals) for the same reason (we will give you a tube and a scoop with which to collect a small amount of stool). In some cases, persons under the age of 18 may spend large amounts of time in direct contact with any animals on your farm; in this case, we will request your permission to take a stool sample from a person under the age of 18, who, you are either the parent or legal guardian of. We are also investigating the amount of bacteria which can be found on the floor of your home (if you allow animals inside the house).

What does this involve?
A short questionnaire will be given to the farm manager or person(s) on each farm who spend significant amounts of time with each the animals/poultry. We will administer the questionnaire using a mobile phone or a tablet computer by asking you each of the questions in turn and writing down your answers. If you are not comfortable speaking in English, we will ask you the questions in Kiswahili, through a trained

Ref

field translator (Mr Cleophas Maseno). Faecal samples from animals will be collected from the floor and transported back to our lab for testing. Faeces will be collected from human participants differently – you will be provided with a faecal pot which has an attached spoon. Please defecate onto a sheet of newspaper and then collect on spoonful of faeces and place in the pot. This will also be transported back to our lab for testing. Samples will be collected from the floor of your house, using shoe covers – we will put these on and walk back and forth over a small area of your floor and transport these back to the lab to assess if any bacteria has been transferred from the outside of your house, to the inside via any animals.

What will we do with the information we collect?
Once this data has been collected, we will analyse it and perform laboratory tests on the faeces. We will not be able to disseminate individual results, but once written up, we will provide copies of the findings to the District Veterinary Office and they can be picked up from them. Eventually, this report will be able to provide advice on how to better treat animals with antibiotics and also give information on how to prevent resistance from occurring. We will also be able to give advice to humans on which antibiotics are the best to use and not to use if a gastrointestinal infection is present.

Further information
Information obtained from the questionnaire may be retained for up to ten years and all data will remain confidential. If you decide that you want to withdraw from the study you may do so without explanation, and any information you have given to us will be destroyed. Results from the study may be published in academic journals, but no-one will be identifiable from any published work.

What next?
If you are happy to become involved, then please read and sign the consent form. Before signing the consent form, it is your right to ask any questions and to have these answered satisfactorily before giving us any information/samples. It is your right to NOT answer any questions that may implicate you in any way or you are uncomfortable with answering. It is your right to refuse to allow us to take samples from your animals if you do not wish us to do so or to refuse to give us faecal samples from human participants.

Many thanks,

Steven Kemp

Steven Kemp
(Supervised by Professor Eric Févre)

If you have any cause for concern or wish to withdraw from the study at any time, please contact us:
Project Manager
Dr Christine Mosoti
International Livestock Research Institute
Old Naivasha Road
PO Box 30709-00100,
Nairobi
Kenya
c.mosoti@cgiar.org

If you have a complaint which you feel you cannot come to us with, then you should contact the Research Governance Officer at the University of Liverpool, UK on +44151 794 8290 or email ethics@liv.ac.uk. Alternatively, you can also contact the Research Compliance Office at ILRI, Nairobi on 0204223000/3375/3887 or email EOHSKenyaHelpdesk@cgiar.org.

Information sheet March 2017 v0.2

Figure II-ii Informed Consent Form completed by all participants prior to data and sample collection during the Farm Study

UNIVERSITY OF LIVERPOOL

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

Ref ☐☐☐

Antibiotic use and antibiotic resistance in bacteria from farmers and farm animals in Western Kenya: **Informed Consent Form**

Please read the following information carefully. You may also request a copy for yourself.

Researcher: Steven Kemp, supervised by Professor Eric Fevre

Please write your initials in EACH BOX e.g. Theodatyl West = TW

1. I confirm that I have read and have understood the information sheet dated July 2016 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is entirely voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐
3. I understand that I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. I understand that I may refuse to answer particular questions and individual privacy will be maintained in all published and written data from the study. ☐
4. I understand that a stool sample has been requested from a child, under the age of 18, that I am either the parent or legal guardian of, and I give my permission for this to be collected via the previously discussed method. ☐
5. I understand that a stool sample has been requested from one or more of my animals, and I give my permission for this to be collected via the previously discussed method. ☐

Name of Person giving consent	Date	Signature
Researcher	Date	Signature

The contact details of the Project Manager are:

Dr Christine Mosoti
International Livestock Research Institute
Old Naivasha Road
PO Box 30709-00100,
Nairobi Kenya
c.mosoti@cgiar.org

Informed Consent Form v0.2 - Mar 2017

Figure II-iii Questionnaire framework used in Farm Study

1. **Please indicate your gender:**
   Mark only one oval.
   - ◯ Male
   - ◯ Female

2. **Please indicate your age:**
   Mark only one oval.
   - ◯ <18
   - ◯ 18-24
   - ◯ 25-44
   - ◯ 45-64
   - ◯ >65

3. **Which type of farming is undertaken here:**
   Mark only one oval.
   - ◯ Intensive
   - ◯ Semi-intensive
   - ◯ Self-sufficiency

4. **Overthe last 12 months, how many animals have you kept on the farm? (Animal - Number)**

   _____

5. **In the last 12 months, how many animals/poultry have you sold?**

   _____

6. **Do you sell any of your animals/poultry?**
   Mark only one oval.
   - ◯ Yes
   - ◯ No
   - ◯ Sometimes

7. **Where do you usually sell any of your animals/poultry?**
Tick all that apply.

- [ ] Farmers Market
- [ ] Exported to other districts
- [ ] Exported within this district
- [ ] Other

8. **Where else do you sell any of your animals/poultry?**

_____

9. **In the last 12 months, have you slaughtered any animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

10. **Which species of animals did you slaughter?**
Tick all that apply.

- [ ] Dairy cows
- [ ] Beef cows
- [ ] Sheep/Goats
- [ ] Pigs
- [ ] Poultry
- [ ] Other

11. **Which other animals did you slaughter?**

_____

12. **Was any protective clothing worn during the slaughter?**
Mark only one oval.

- ◯ Yes
- ◯ No

13. **What was the main purpose of slaughtering animals?**
Mark only one oval.

- ◯ For home consumption
- ◯ For sale within district
- ◯ For sale outside of district
- ◯ Other

14. **What other purposes are there for slaughtering animals?**

_____

15. **In your own words, can you explain what you understand by "antibiotic withdrawal" period**

16. **How do you know the correct withdrawal period for each antibiotic that you use?**

17. **Do you always wait for the withdrawal period to end before selling any animals/poultry?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

18. **In your own words, can you explain what you understand by "antibiotic resistance"**

19. **Over the last 12 months, what are the three most common diseases that you, or an AHA/veterinary officer, has treated with antibiotics, for any animals/poultry?**

20. **Which diseases (or clinical signs) has killed the most animals on this farm?**

21. **How do you know that this was the correct disease?**
Tick all that apply.

- ☐ Agrovet has diagnosed
- ☐ AHA/veterinary officer has diagnosed
- ☐ Self-diagnosed
- ☐ Other

22. **How else did you know this this was the correct disease?**

_____

23. **Have any of the animals on the farm developed any signs of gastrointestinal disease or illnesses within the last 3 months? (diarrhea, upset stomach etc.)?**
Tick all that apply.

- [ ] Abdominal pain
- [ ] Diarrhea
- [ ] Bloody Diarrhea
- [ ] Other

24. **Which other gastrointestinal signs have you witnessed in any of the animals?**

_____

25. **When you buy new animals (or animals are born), do you vaccinate them (inject to protect against diseases)?**
Mark only one oval.

- ◯ Yes
- ◯ No

26. **How often/when do you vaccinate animals/poultry? (Animal - vaccination - Age - disease protected against)**

_____

_____

_____

_____

_____

27. **If one animal becomes ill, do you treat the entire group or just that animal?**
Mark only one oval.

- ◯ Whole group
- ◯ Single animal

28. **Do you separate the ill animal from the rest?**
Mark only one oval.

- ◯ Yes
- ◯ No

29. **Are you the person who is responsible for purchasing all antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No

30. **During the last 3 months, have you purchased any antibiotics to treat any animals/poultry?**
Mark only one oval.

- ◯ Yes
- ◯ No

31. **Where do any antibiotics usually come from?**
Tick all that apply.

- ☐ Agrovet
- ☐ Veterinary officer/AHA
- ☐ Another farmer
- ☐ Other

32. **Where else do antibiotics usually come from?**

_____

33. **Do you ever add antibiotics (not vitamins!) to animal feeds?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

34. **When mixing antibiotics with animal feeds and water, do you alter the antibiotic does to the size (weight) or age of the animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

35. **How do you estimate the size/weight?**

_____

36. **During the last 3 months, which are the 3 most common antibiotics you have used for any animals/poultry? (animal - antibiotic)**

_____

_____

_____

_____

_____

37. **When treating animals, do you use the same needle/syringe for all antibiotics?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Do not use a syringe

38. **If you do not use a syringe, how do you measure out the antibiotic?**

39. **Do you clean the needles/syringe between doses/animals?**
Mark only one oval.
- ◯ Yes
- ◯ No
- ◯ Sometimes

40. **How do you clean the needles/syringes between doses/animals?**

Water Questions

41. **What are the main source(s) of water on this farm?**
Tick all that apply.
- ☐ Taps
- ☐ Bore hole
- ☐ Well
- ☐ Local river
- ☐ Other

42. **What other sources of water are there?**

43. **What do you use the water for?**
Tick all that apply.
- ☐ Cooking
- ☐ Drinking
- ☐ Giving to animals/poultry
- ☐ Crop farming
- ☐ Cleaning tools (needles/syringes)
- ☐ Other

44. **What else do you use the water for?**

45. **Do you routinely boil the water before drinking it?**
Mark only one oval.
- ◯ Yes
- ◯ No
- ◯ Sometimes

46. **Is there a communal sources of water (river/stream/lake) used by the local population for personal use?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Don't know

47. **Is there a communal sources of water (river/stream/lake) used by the local population to graze animals?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Don't know

48. **Where is the nearest water source? *latlong***

_____

Gastrointestinal Pathogens

49. **What type of latrine do you have?**
Mark only one oval.

- ◯ Latrine completely closed
- ◯ Latrine partially closed
- ◯ Open pit
- ◯ Other

50. **What other type of latrine do you have?**

_____

51. **How often do you use the latrine when you need to defecate?**
Mark only one oval.

- ◯ Always
- ◯ Sometimes
- ◯ Never

52. ***For interviewer* Is the latrine in close proximity to any animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

53. **What do you do with waste/contaminated water (including what you use for the latrine/animals)?**

_____

54. **(How) do you treat/process any waste water before discarding it?**

55. **On a weekly basis, how much contact do you have with any of the animals on the farm?**
Mark only one oval.

- ◯ 1-2 days
- ◯ 3-5 days
- ◯ 6-7 days

56. **Do you wash you hands with soap and water after you have contact with animals/poultry?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

57. **Do you wash you hands with soap and water before cooking/eating?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

58. **Do you wash you hands with soap and water after using the latrine?**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Sometimes

59. **Have you/any of the people who have interaction with the animals developed any symptoms of gastrointestinal disease or illnesses within the last 3 months? (diarrhea, upset stomach etc.)?**
Tick all that apply.

- ☐ Abdominal pain
- ☐ Constipation
- ☐ Diarrhea
- ☐ GI bleeding (bloody stool)
- ☐ Nausea
- ☐ Other

60. **Which other symptoms have you had?**

61. **Did you use any antibiotics to treat the symptoms/disease?**
Mark only one oval.

- ◯ Yes
- ◯ No

62. **Which antibiotics did you use to treat the symptoms/disease?**

_____

63. **How long did you take it for?**

_____

64. **Do any of your animals/poultry have access to the inside of your house?**
Mark only one oval.

- ◯ Yes
- ◯ No

65. **Which animals have access to the inside of your house?**
Tick all that apply.

- ☐ Dairy cows
- ☐ Beef cows
- ☐ Sheep/Goats
- ☐ Pigs
- ☐ Poultry

66. **Are you willing to allow us to collect some samples from your floor using the aforementioned boot-socks?**
Mark only one oval.

- ◯ Yes
- ◯ No

67. **Are you willing to provide a faecal samples for us?**
Mark only one oval.

- ◯ Yes
- ◯ No

68. **Are you willing to allow us to take faecal samples fro, your animals?**
Mark only one oval.

- ◯ Yes
- ◯ No

69. ***For interviewer* Water sample collected?**
Mark only one oval.

- ◯ Yes
- ◯ No

Results of Univariable Analysis

Complete results of univariable analysis for all outcomes of interest are presented on pages 285 – 290.

Table II-i Results of univariable analysis for risk factors associated with multi-drug resistance and ESBL-production in faecal *E. coli* samples from humans, livestock, and the environment.

		MDR							ESBL						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
Species	Cattle	Ref							**Ref**						
	Human	0.704	0.24	2.941	0.003	1.265	3.234	2.023	0.076	0.359	0.212	0.832	0.534	2.18	1.079
	Poultry	0.179	0.207	0.862	0.389	0.796	1.794	1.195	-0.13	0.324	-0.4	0.689	0.465	1.658	0.878
	Pig	-0.098	0.316	-0.31	0.757	0.488	1.684	0.907	0.24	0.476	0.505	0.614	0.501	3.23	1.271
	Sheep/Goats	0.429	0.265	1.623	0.105	0.915	2.58	1.536	0.484	0.379	1.277	0.202	0.772	3.409	1.622
EnvironmentMDR	No	Ref							Ref						
	Yes	-0.408	0.283	-1.441	0.149	0.382	1.158	0.665	0.384	0.453	0.849	0.396	0.605	3.566	1.469
EnvironmentESBL	No	Ref							Ref						
	Yes	3.115	1.131	2.753	0.006	2.452	206.886	22.523	1.248	0.916	1.363	0.173	0.579	20.992	3.485
Gender	Female	Ref							Ref						
	Male	0.024	0.221	0.11	0.912	0.665	1.579	1.025	0.224	0.388	0.579	0.563	0.586	2.676	1.252
Age	18-24	Ref							Ref						
	25-44	-0.191	0.522	-0.367	0.714	0.297	2.298	0.826	0.88	1.122	0.784	0.433	0.268	21.711	2.41
	45-64	-0.078	0.554	-0.14	0.888	0.312	2.742	0.925	1.131	1.161	0.974	0.33	0.318	30.152	3.097
	<65	0.171	0.567	0.302	0.763	0.39	3.608	1.187	1.055	1.18	0.894	0.371	0.284	29.011	2.871
Farm Size	GrandMean	0.006	0.004	1.42	0.156	0.998	1.014	1.006	0.004	0.007	0.552	0.581	0.991	1.017	1.004
Knowledge of Abs	Don't Know	Ref							Ref						
	Good	0.376	0.298	1.264	0.206	0.813	2.61	1.457	0.315	0.446	0.706	0.48	0.572	3.282	1.37
	Bad	0.143	0.285	0.502	0.616	0.66	2.017	1.154	-0.786	0.464	-1.694	0.09	0.183	1.131	0.456
KnowledgeAbResistance	Don't Know	Ref							Ref						
	Good	0.109	0.311	0.349	0.727	0.606	2.05	1.115	0.637	0.493	1.293	0.196	0.72	4.972	1.892
KnowledgeAbWithdrawal	Don't Know	Ref							Ref						
	Some	0.178	0.261	0.681	0.496	0.716	1.995	1.195	-0.371	0.472	-0.785	0.432	0.274	1.742	0.69
	Good	0.557	0.527	1.057	0.291	0.621	4.906	1.746	1.892	0.708	2.672	0.008	1.656	26.585	6.635
	Actively Ignores	1.103	0.678	1.628	0.104	0.798	11.384	3.015	0.463	0.989	0.468	0.64	0.228	11.045	1.589
Mastitis Last 12 months?	No	Ref							Ref						

		MDR							ESBL						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
	Yes	0.484	0.233	2.073	0.038	1.027	2.563	1.622	0.233	0.417	0.559	0.576	0.558	2.858	1.263
Diarrhoea 12 months?	No	Ref							Ref						
	Yes	-0.067	0.261	-0.255	0.798	0.561	1.56	0.936	-0.23	0.473	-0.486	0.627	0.315	2.007	0.795
Diarrhoea 3 months?	No	Ref							Ref						
	Yes	0.024	0.265	0.091	0.928	0.61	1.721	1.024	-0.781	0.429	-1.819	0.069	0.198	1.062	0.458
Bloody diarrhoea 3 months?	No	Ref							Ref						
	Yes	-0.491	0.211	-2.33	0.02	0.405	0.925	0.612	-0.116	0.388	-0.3	0.764	0.416	1.904	0.89
Animals Vaccinated?	No	Ref							Ref						
	Yes	0.22	0.217	1.014	0.311	0.814	1.908	1.246	0.062	0.383	0.161	0.872	0.502	2.252	1.064
Treat whole group?	Single Animal	Ref							Ref						
	Whole Group	-0.266	0.213	-1.245	0.213	0.505	1.165	0.767	-0.298	0.381	-0.784	0.433	0.352	1.565	0.742
Separate Ill animals?	No	Ref							Ref						
	Yes	-0.225	0.215	-1.047	0.295	0.524	1.217	0.799	0.012	0.378	0.031	0.975	0.483	2.121	1.012
BoughtTetracyckine3Months?	No	Ref							Ref						
	Yes	-0.017	0.23	-0.074	0.941	0.626	1.544	0.983	0.261	0.388	0.672	0.502	0.607	2.776	1.298
BoughtPen-strep3months?	No	Ref							Ref						
	Yes	0.483	0.294	1.643	0.1	0.911	2.884	1.621	0.029	0.508	0.058	0.954	0.381	2.787	1.03
UsedTetracycline3months?	No	Ref							Ref						
	Yes	0.221	0.214	1.029	0.303	0.819	1.898	1.247	0.49	0.373	1.311	0.19	0.785	3.393	1.632
UsedPenstrep3months?	No	Ref							Ref						
	Yes	0.582	0.212	2.747	0.006	1.181	2.709	1.789	-0.035	0.394	-0.088	0.93	0.446	2.092	0.966
Sameneedlesyringe?	No	Ref							Ref						
	Yes	0.24	0.216	1.115	0.265	0.833	1.941	1.272	0.19	0.378	0.503	0.615	0.576	2.54	1.21
Mainwatersource	Taps	Ref							Ref						
	Borehole/Well	-0.321	0.353	-0.91	0.363	0.363	1.449	0.725	1.009	0.764	1.322	0.186	0.614	12.25	2.743
	River/Lake	-0.779	0.401	-1.943	0.052	0.209	1.007	0.459	1.087	0.833	1.305	0.192	0.579	15.162	2.964
	Rainwater	-0.679	0.467	-1.455	0.146	0.203	1.266	0.507	1.145	0.93	1.232	0.218	0.508	19.43	3.142

***P*-values** highlighted in green are statistically significant (p<0.25) values from the Wald Chi squared test; **OR** = Odds Ration; **SE** = Standard Error; **95%CI** = 95% Confidence Intervals.

Table II-ii Results of univariable analysis for risk factors associated with tetracycline and sulfathiazole resistance in faecal *E. coli* samples from humans, livestock, and the environment.

		Tetracycline							Sulfathiazole						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
Species	Cattle	**Ref**							**Ref**						
	Human	0.51	0.496	1.027	0.304	0.63	4.403	1.665	1.479	0.309	4.792	<0.001	2.396	8.033	4.387
	Poultry	-0.519	0.349	-1.487	0.137	0.3	1.18	0.595	0.334	0.225	1.488	0.137	0.899	2.169	1.397
	Pig	0.693	0.687	1.01	0.313	0.521	7.681	2	0.503	0.355	1.419	0.156	0.825	3.314	1.654
	Sheep/Goats	0.867	0.586	1.479	0.139	0.754	7.51	2.38	0.703	0.299	2.354	0.019	1.125	3.628	2.02
EnvironmentMDR	No	Ref							Ref						
	Yes	-0.957	0.61	-1.57	0.117	0.116	1.269	0.384	-0.069	0.353	-0.196	0.845	0.468	1.863	0.933
EnvironmentESBL	No	Ref							Ref						
	Yes	35.51	0.907	39.145	<0.001	4.5^{E+14}	1.5^{E+16}	2.6^{E+15}	36.243	0.481	75.324	<0.001	2.1399E+15	1.411E+16	5.495E+15
Gender	Female	Ref							Ref						
	Male	-0.019	0.408	-0.047	0.962	0.441	2.184	0.981	0.293	0.273	1.076	0.282	0.786	2.287	1.341
Age	18-24	Ref							Ref						
	25-44	-0.967	1.441	-0.671	0.502	0.023	6.414	0.38	0.268	0.564	0.474	0.635	0.432	3.951	1.307
	45-64	-0.128	1.513	-0.085	0.932	0.045	17.059	0.88	0.302	0.598	0.505	0.614	0.419	4.366	1.352
	<65	-0.94	1.522	-0.617	0.537	0.02	7.724	0.391	1.285	0.642	2.001	0.045	1.027	12.736	3.616
Farm Size	GrandMean	0.017	0.014	1.171	0.242	0.989	1.045	1.017	0.012	0.005	2.186	0.029	1.001	1.023	1.012
Knowledge of Abs	Don't Know	Ref							Ref						
	Good	0.382	0.712	0.536	0.592	0.363	5.917	1.465	0.311	0.357	0.871	0.384	0.678	2.749	1.365
	Bad	-0.521	0.611	-0.854	0.393	0.179	1.966	0.594	0.126	0.338	0.372	0.71	0.584	2.2	1.134
KnowledgeAbResistance	Don't Know	Ref							Ref						
	Good	0.306	0.73	0.419	0.675	0.325	5.676	1.357	0.389	0.385	1.01	0.313	0.693	3.141	1.476
KnowledgeAbWithdrawal	Don't Know	Ref							Ref						
	Some	Error in convergence							0.277	0.341	0.812	0.417	0.676	2.571	1.319
	Good								-0.026	0.601	-0.044	0.965	0.3	3.16	0.974
	Actively Ignores								1.286	1.016	1.266	0.206	0.494	26.5	3.618
Mastitis Last 12 months?	No	Ref							Ref						
	Yes	0.629	0.599	1.05	0.293	0.58	6.068	1.876	0.279	0.306	0.915	0.36	0.727	2.407	1.322
Diarrhoea 12 months?	No	Ref							Ref						
	Yes	0.056	0.614	0.091	0.927	0.318	3.523	1.058	-0.068	0.322	-0.211	0.833	0.497	1.756	0.934

		Tetracycline							Sulfathiazole						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
Diarrhoea 3 months?	No	Ref							Ref						
	Yes	0.019	0.619	0.03	0.976	0.303	3.43	1.019	0.135	0.326	0.414	0.679	0.604	2.17	1.145
Bloody diarrhoea 3 months?	No	Ref							Ref						
	Yes	-0.058	0.528	-0.11	0.912	0.335	2.658	0.943	-0.359	0.275	-1.306	0.192	0.407	1.197	0.698
Animals Vaccinated?	No	Ref							Ref						
	Yes	0.869	0.451	1.929	0.054	0.986	5.771	2.386	0.479	0.259	1.85	0.064	0.972	2.683	1.615
Treat whole group?	Single Animal	Ref							Ref						
	Whole Group	-0.711	0.477	-1.491	0.136	0.193	1.251	0.491	-0.105	0.262	-0.4	0.689	0.539	1.505	0.9
Separate Ill animals?	No	Ref							Ref						
	Yes	0.043	0.475	0.09	0.929	0.411	2.65	1.044	-0.133	0.255	-0.521	0.602	0.53	1.444	0.875
BoughtTetracyckine3Months?	No	Ref							Ref						
	Yes	0.062	0.552	0.112	0.911	0.361	3.137	1.064	-0.156	0.283	-0.55	0.582	0.491	1.491	0.856
BoughtPen-strep3months?	No	Ref							Ref						
	Yes	0.744	0.763	0.975	0.329	0.472	9.389	2.105	0.568	0.356	1.594	0.111	0.878	3.55	1.765
UsedTetracycline3months?	No	Ref							Ref						
	Yes	-0.07	0.479	-0.146	0.884	0.364	2.386	0.932	0.154	0.26	0.591	0.555	0.7	1.942	1.166
UsedPenstrep3months?	No	Ref							Ref						
	Yes	0.593	0.512	1.157	0.247	0.663	4.935	1.809	0.511	0.274	1.863	0.062	0.974	2.852	1.666
Sameneedlesyringe?	No	Ref							Ref						
	Yes	0.354	0.495	0.716	0.474	0.541	3.757	1.425	0.084	0.267	0.315	0.753	0.645	1.835	1.088
Mainwatersource	Taps	Ref							Ref						
	Borehole/Well	0.306	0.786	0.389	0.697	0.291	6.337	1.357	-0.295	0.445	-0.662	0.508	0.311	1.782	0.745
	River/Lake	0.019	0.869	0.021	0.983	0.185	5.598	1.019	-0.635	0.495	-1.283	0.199	0.201	1.398	0.53
	Rainwater	-0.328	1.002	-0.328	0.743	0.101	5.131	0.72	-0.526	0.574	-0.918	0.359	0.192	1.818	0.591

***P*-values** highlighted in green are statistically significant (p<0.25) values from the Wald Chi squared test; **OR** = Odds Ration; **SE** = Standard Error; **95%CI** = 95% Confidence Intervals.

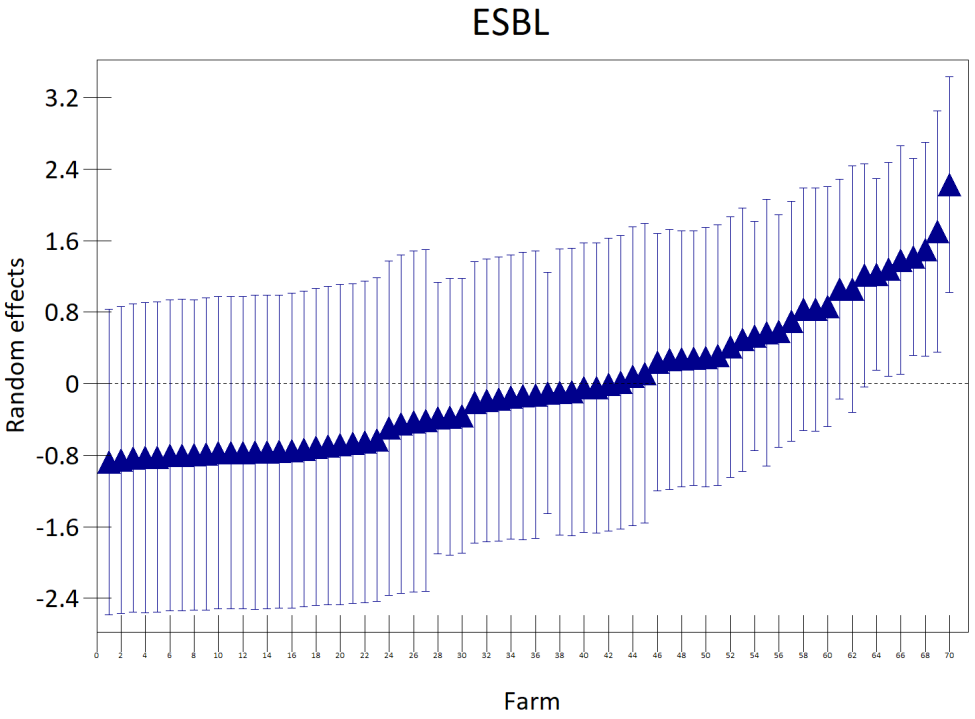
Table II-iii Results of univariable analysis for risk factors associated with ampicillin and trimethoprim resistance in faecal *E. coli* samples from humans, livestock, and the environment.

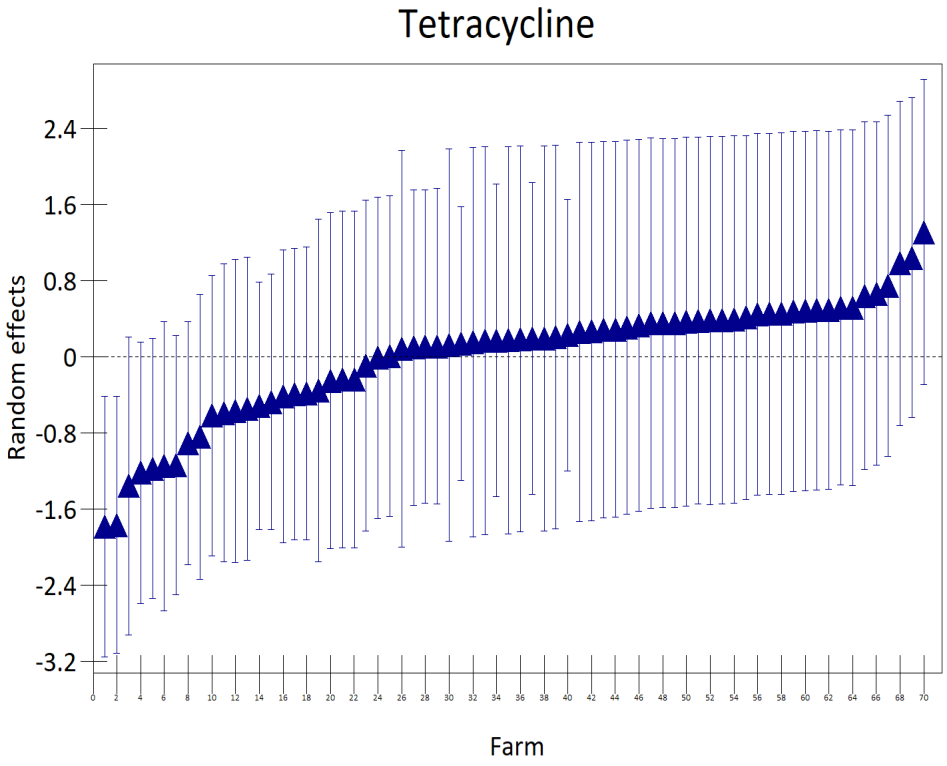
		Ampicillin							Trimethoprim						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
Species	Cattle	**Ref**							**Ref**						
	Human	0.343	0.239	1.437	0.151	0.883	2.25	1.409	1.185	0.301	3.938	0	1.813	5.899	3.27
	Poultry	0.042	0.207	0.203	0.839	0.695	1.565	1.043	0.318	0.229	1.389	0.165	0.878	2.151	1.374
	Pig	-0.057	0.311	-0.184	0.854	0.514	1.736	0.945	0.378	0.357	1.059	0.29	0.725	2.937	1.459
	Sheep/Goats	0.436	0.271	1.609	0.108	0.909	2.629	1.546	0.655	0.305	2.145	0.032	1.058	3.503	1.925
EnvironmentMDR	No	Ref							Ref						
	Yes	-0.231	0.263	-0.88	0.379	0.474	1.328	0.794	-0.23	0.369	-0.623	0.533	0.386	1.638	0.795
EnvironmentESBL	No	Ref							Ref						
	Yes	2.79	1.088	2.563	0.01	1.928	137.434	16.277	35.734	0.523	68.372	0	1.1863E+15	9.2031E+15	3.3041E+15
Gender	Female	Ref							Ref						
	Male	0.101	0.206	0.489	0.625	0.738	1.658	1.106	0.408	0.286	1.427	0.154	0.859	2.634	1.504
Age	18-24	Ref							Ref						
	25-44	-0.16	0.494	-0.324	0.746	0.324	2.243	0.852	-0.052	0.607	-0.086	0.932	0.289	3.117	0.949
	45-64	-0.046	0.525	-0.088	0.93	0.341	2.671	0.955	0.034	0.641	0.053	0.958	0.294	3.637	1.035
	<65	0.025	0.536	0.046	0.963	0.358	2.934	1.025	1.194	0.695	1.717	0.086	0.844	12.897	3.3
Farm Size	GrandMean	0.003	0.004	0.751	0.453	0.995	1.011	1.003	0.015	0.006	2.509	0.012	1.003	1.027	1.015
Knowledge of Abs	Don't Know	Ref							Ref						
	Good	0.316	0.278	1.133	0.257	0.794	2.366	1.371	0.423	0.379	1.117	0.264	0.727	3.206	1.526
	Bad	0.039	0.265	0.148	0.883	0.619	1.748	1.04	0.136	0.356	0.381	0.703	0.57	2.3	1.145
KnowledgeAbResistance	Don't Know	Ref							Ref						
	Good	0.158	0.293	0.538	0.59	0.659	2.078	1.171	0.793	0.428	1.854	0.064	0.956	5.116	2.211
KnowledgeAbWithdrawal	Don't Know	Ref							Ref						
	Some	0.271	0.243	1.118	0.264	0.815	2.112	1.312	0.602	0.361	1.668	0.095	0.9	3.701	1.825
	Good	1.019	0.545	1.868	0.062	0.951	8.064	2.77	-0.121	0.621	-0.195	0.845	0.262	2.992	0.886
	Actively Ignores	0.802	0.622	1.289	0.197	0.659	7.552	2.23	2.006	1.314	1.527	0.127	0.566	97.727	7.436
Mastitis Last 12 months?	No	Ref							Ref						
	Yes	0.53	0.22	2.405	0.016	1.103	2.617	1.699	0.376	0.325	1.159	0.246	0.771	2.753	1.457

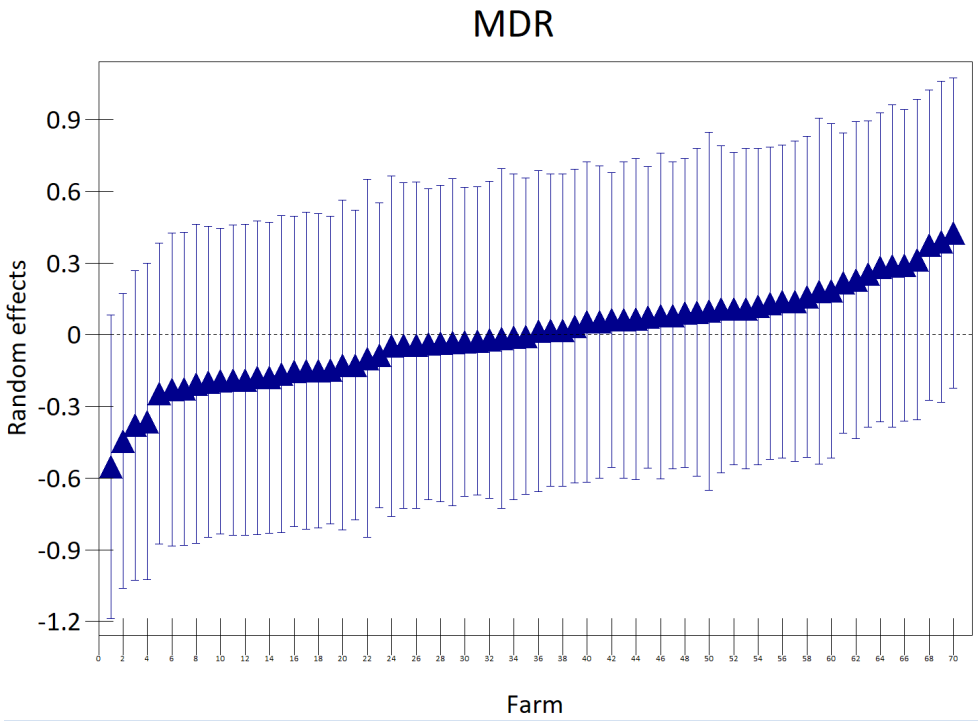
		Ampicillin							Trimethoprim						
Variable	**Category**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**	**β**	**SE**	**z-ratio**	**p-value**	**L95%CI**	**U95%CI**	**odds ratio**
Diarrhoea 12 months?	No	Ref							Ref						
	Yes	0.108	0.245	0.441	0.659	0.689	1.8	1.114	0.193	0.347	0.557	0.578	0.615	2.394	1.213
Diarrhoea 3 months?	No	Ref							Ref						
	Yes	0.08	0.246	0.324	0.746	0.669	1.754	1.083	0.233	0.345	0.674	0.5	0.641	2.484	1.262
Bloody diarrhoea 3 months?	No	Ref							Ref						
	Yes	-0.421	0.198	-2.129	0.033	0.446	0.967	0.657	-0.429	0.288	-1.492	0.136	0.37	1.144	0.651
Animals Vaccinated?	No	Ref							Ref						
	Yes	0.072	0.204	0.353	0.724	0.721	1.603	1.075	0.429	0.275	1.556	0.12	0.895	2.635	1.535
Treat whole group?	Single Animal	Ref							Ref						
	Whole Group	-0.187	0.201	-0.931	0.352	0.559	1.23	0.829	0.008	0.276	0.028	0.978	0.587	1.731	1.008
Separate Ill animals?	No	Ref							Ref						
	Yes	-0.146	0.202	-0.724	0.469	0.581	1.284	0.864	-0.176	0.27	-0.65	0.515	0.494	1.424	0.839
BoughtTetracyckine3Months?	No	Ref							Ref						
	Yes	0.092	0.217	0.427	0.67	0.717	1.677	1.097	0.303	0.301	1.009	0.313	0.751	2.442	1.355
BoughtPen-strep3months?	No	Ref							Ref						
	Yes	0.436	0.28	1.559	0.119	0.894	2.677	1.547	0.567	0.381	1.488	0.137	0.835	3.717	1.762
UsedTetracycline3months?	No	Ref							Ref						
	Yes	0.28	0.199	1.411	0.158	0.897	1.954	1.324	0.271	0.273	0.993	0.321	0.768	2.241	1.312
UsedPenstrep3months?	No	Ref							Ref						
	Yes	0.529	0.204	2.597	0.009	1.139	2.529	1.697	0.52	0.29	1.79	0.073	0.952	2.972	1.682
Sameneedlesyringe?	No	Ref							Ref						
	Yes	0.307	0.201	1.528	0.127	0.917	2.014	1.359	0.269	0.28	0.961	0.337	0.756	2.265	1.309
Mainwatersource	Taps	Ref							Ref						
	Borehole/Well	-0.145	0.335	-0.432	0.666	0.449	1.669	0.865	0.199	0.199	1	0.318	0.826	1.804	1.221
	River/Lake	-0.561	0.379	-1.483	0.138	0.272	1.198	0.57	0.071	0.227	0.315	0.753	0.689	1.674	1.074
	Rainwater	-0.392	0.439	-0.893	0.372	0.285	1.598	0.675	0.071	0.272	0.262	0.793	0.63	1.831	1.074

***P*-values** highlighted in green are statistically significant (p<0.25) values from the Wald Chi squared test; **OR** = Odds Ration; **SE** = Standard Error; **95%CI** = 95% Confidence Intervals.

Figure II-iii. Farm level residuals plotted against the overall mean for each mixed effect model (n=70 farms). X-axis is farm number in order from 1 at the left, to 70 at the right. Error bars are 95% CI.



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0.3
0
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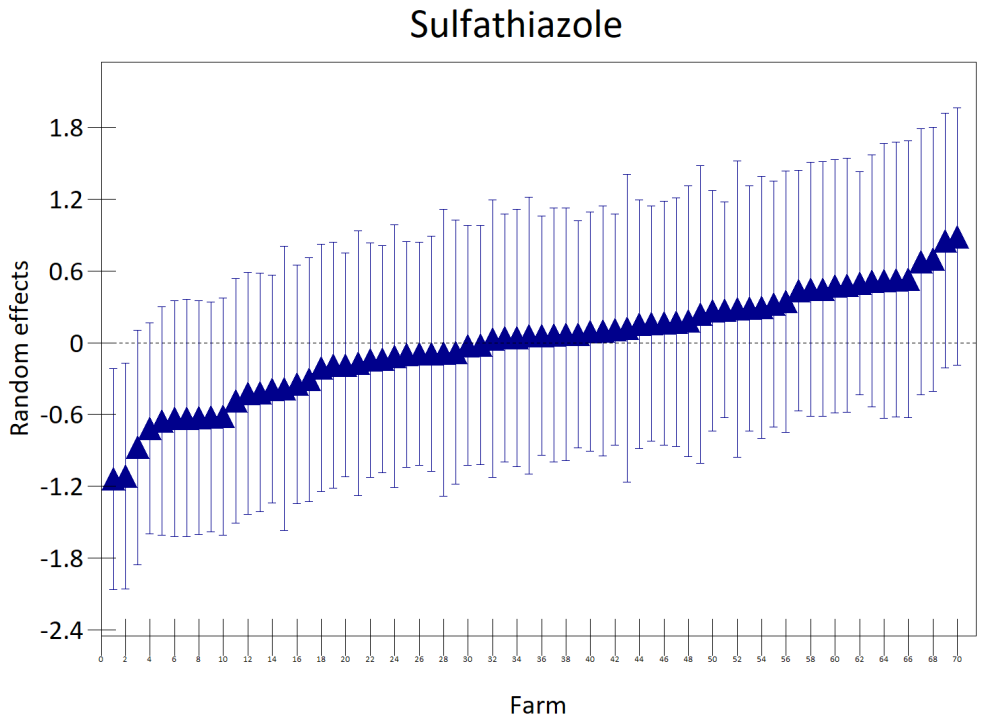
-0.6
-0.9
-1.2
Farm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Random effects
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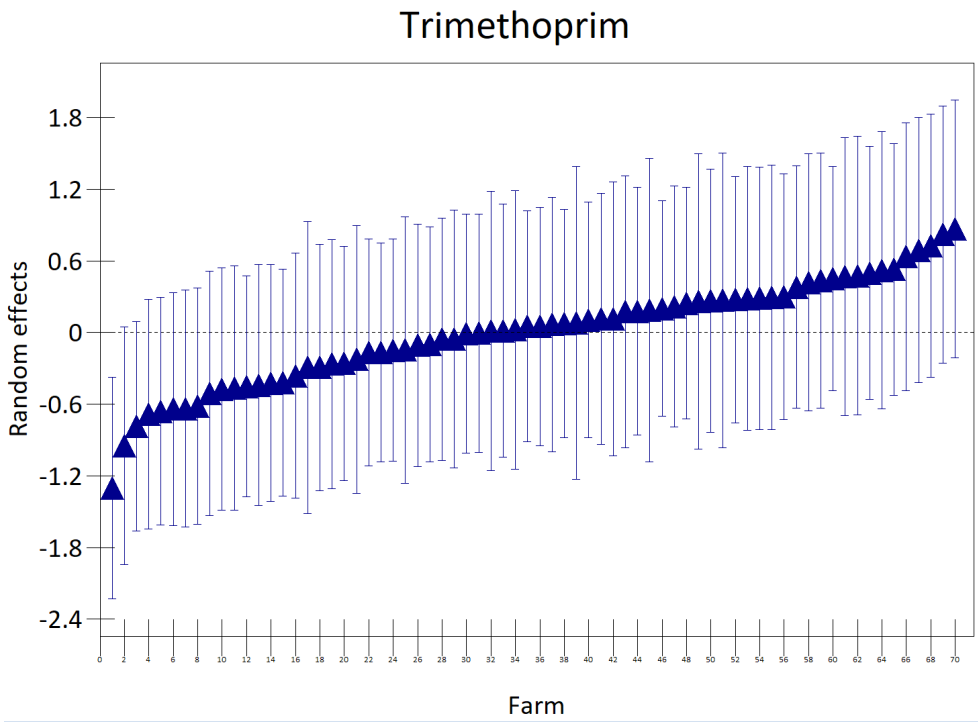
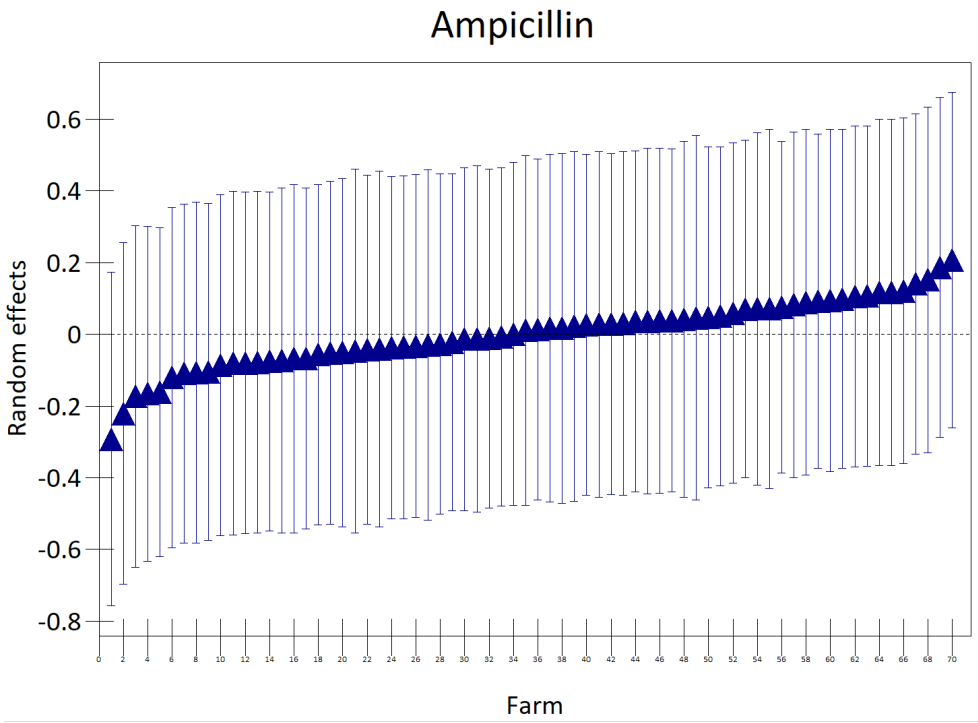
1.2
0.6
0
-0.6
-1.2
-1.8
-2.4
Farm


Trimethoprim
Random effects
1.8
1.2
0.6
0
-0.6
-1.2
-1.8
-2.4
Farm


Ampicillin
Random effects
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Farm

Whole Genome Sequencing

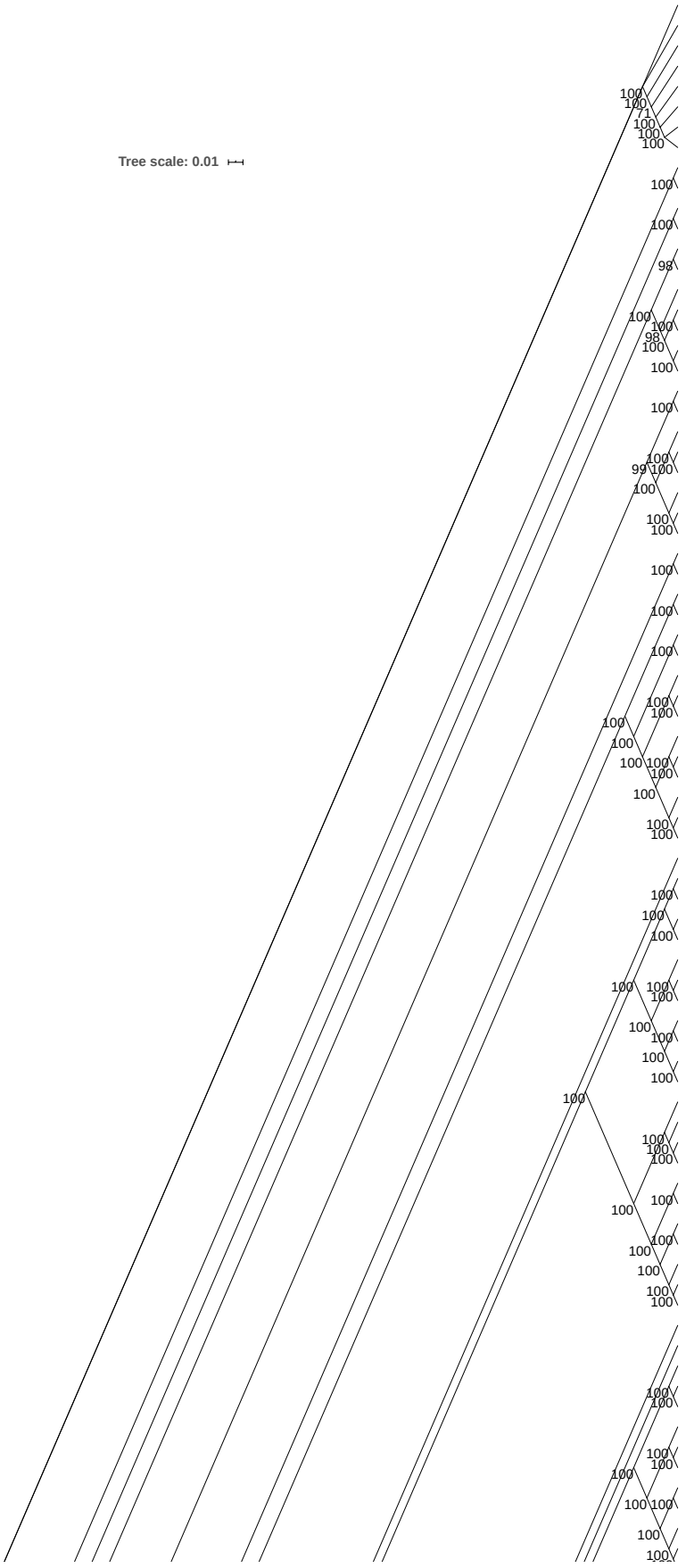
Table II-iv Standard metrics of 150 sequences obtained from whole genome sequencing of livestock, humans, and environmental isolates. Raw reads were QC and then mapped to reference genome K12 *E. coli*

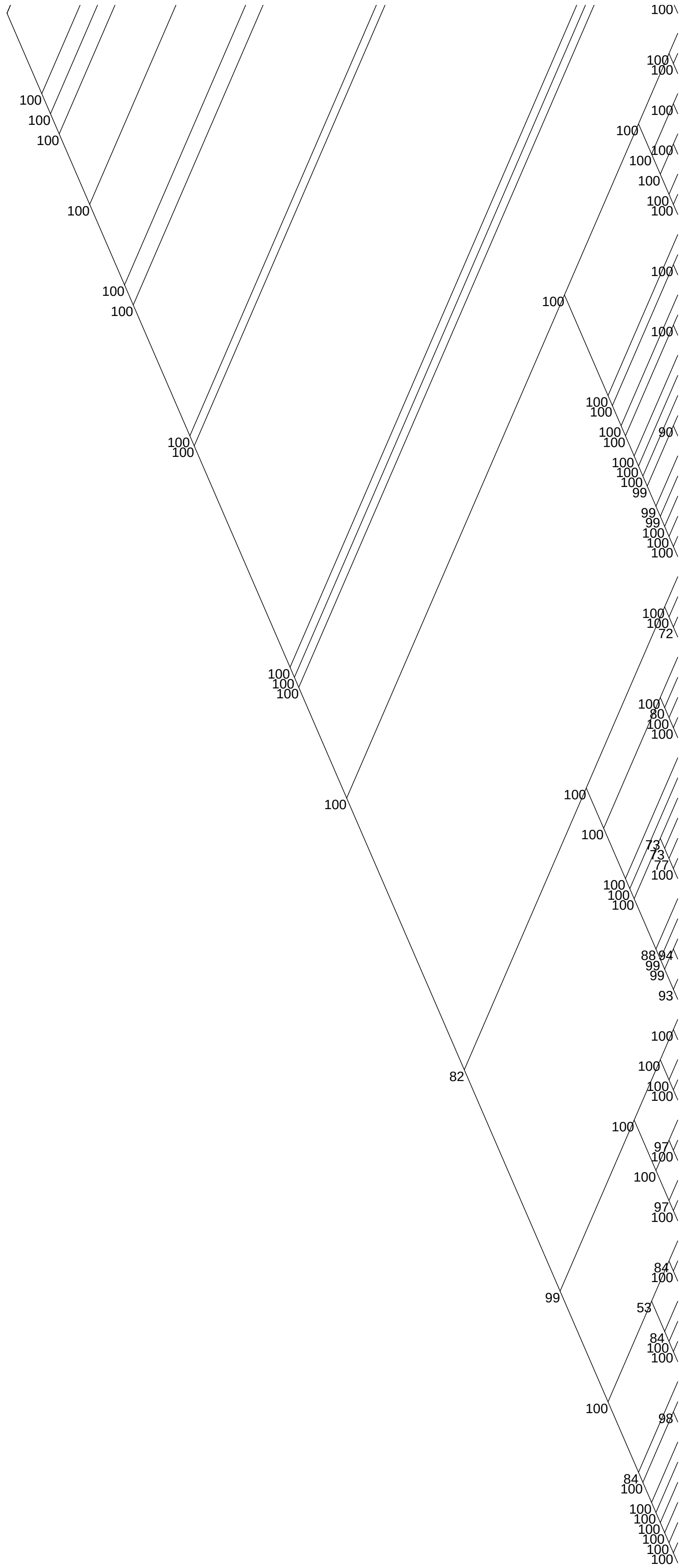
Isolate	Bacterial Host	Number of sequences	Genome Size	Mean Coverage
F4H2C	Human	7594049	5010573	48
F6C3B	Cattle	7409363	4669865	48
F6C3C	Cattle	6915014	4664779	43
F9C1A	Cattle	6349380	4721225	117
F9C3A	Cattle	6349380	4701158	86
F9C4CX	Cattle	6207820	4825261	86
F9C5A	Cattle	6207820	4695529	127
F9C5B	Cattle	6206940	4657781	128
F9H2A	Human	6051696	4665384	165
F9WATER	Environment	6029597	4709000	75
F11C3CZ	Cattle	6029597	4709006	97
F11C4CZ	Cattle	5967560	4709006	144
F11C4E	Cattle	5776919	4651216	144
F11H3A	Human	5699804	4651921	127
F11H3CZ	Human	5699804	4708245	127
F11P3E	Poultry	5637595	4650020	155
F12G2CX	Sheep/Goat	5622626	4709006	112
F12HOUSEA	Environment	5446127	4652044	112
F12P3CZ	Poultry	5378647	4708724	124
F12S1CX	Sheep/Goat	5307585	4709005	89
F12S2B	Sheep/Goat	5243568	4650859	89
F12S2C	Sheep/Goat	5243568	4643102	113
F13HOUSECZ	Environment	5185791	4708244	135
F13P3CX	Poultry	5174394	4708632	22
F13WATERCZ	Environment	5174394	4708025	141
F14C3CZ	Cattle	5160598	4708637	141
F15P5CX	Poultry	5157245	4663297	124
F15S2CX	Sheep/Goat	5157245	4709007	108
F16C9B	Cattle	5099785	4644768	108
F16C10A	Cattle	5078123	4709912	122
F18C1CX	Cattle	5056281	4708167	92
F18C6CX	Cattle	4990032	4679690	92
F18HOUSECX	Environment	4990032	4649517	27
F18P2CX	Poultry	4963740	4663611	139
F19C4CZ	Cattle	4940177	4709108	141
F19H3CX	Human	4940177	4679112	144
F19HOUSEA	Environment	4884411	4820056	121
F20P5CZ	Poultry	4864582	4708144	121
F22G1CX	Sheep/Goat	4862929	4709006	140
F22G1CZ	Sheep/Goat	4862929	4684077	140
F23H3CZ	Human	4815181	4645235	117
F24H2CX	Human	4802866	4760295	96
F26P2CZ	Poultry	4802866	4684195	156
F27C1CX	Cattle	4790867	4709005	101
F27G2CX	Sheep/Goat	4790867	4707945	101
F27P7CX	Poultry	4783647	4709005	24

Isolate	Bacterial Host	Number of sequences	Genome Size	Mean Coverage
F28P1E	Poultry	4783647	4672601	110
F28P2A	Poultry	4726257	4708300	110
F28P3A	Poultry	4712673	4655050	152
F29C4A	Cattle	4663312	4698851	138
F29H2CZ	Human	4647731	4684319	148
F29P1A	Poultry	4608338	4660184	148
F30C2C	Cattle	4589108	4711185	109
F30H2CZ	Human	4589108	4708143	119
F30HOUSEA	Environment	4577186	4710879	204
F31G1B	Sheep/Goat	4527916	4709099	150
F31G2C	Sheep/Goat	4456531	4675650	164
F31G3B	Sheep/Goat	4443808	5139067	103
F31H2A	Human	4410936	4708813	23
F31H3D	Human	4410936	4651169	118
F31PI1E	Pig	4406693	4643290	118
F31PI2A	Pig	4355539	4703054	90
F32C1A	Cattle	4355539	4670876	90
F32C3A	Cattle	4353151	4710507	146
F32C4B	Cattle	4307653	4643718	189
F32C5A	Cattle	4298492	4710991	189
F32G1B	Sheep/Goat	4289152	4701600	92
F32H1A	Human	4289152	4642900	92
F32H2A	Human	4269760	4710777	92
F32P1A	Poultry	4269760	4649764	117
F32P3A	Poultry	4260231	4708397	107
F32P4B	Poultry	4256438	4709527	112
F33C4B	Cattle	4240203	4642448	86
F33G2B	Sheep/Goat	4202885	4698326	86
F33H2A	Human	4177115	4772753	29
F33PI1A	Pig	4177115	4864770	98
F33PI2A	Pig	4128577	4708741	94
F33PI4B	Pig	4128399	4643172	129
F33PI5A	Pig	4128399	4645330	100
F34G1A	Sheep/Goat	4103397	4709726	98
F34H2B	Human	4094342	4697866	98
F35C3E	Cattle	4081643	4648923	187
F35H1A	Human	4076991	4642826	187
F35P2C	Poultry	4031911	4648021	178
F35P3D	Poultry	4009892	4675175	141
F36G1E	Sheep/Goat	4009892	4699816	82
F36H2A	Human	3998512	4644351	137
F36H3B	Human	3976247	4709878	130
F37C1A	Cattle	3964061	4709562	117
F37C2A	Cattle	3962990	4647542	144
F37P1C	Poultry	3962990	4900040	144
F37P2A	Poultry	3960940	4646114	126
F37P3B	Poultry	3960940	4708835	126
F37PI1B	Pig	3947606	4658093	78
F37PI2A	Pig	3916495	4699680	78
F37PI3A	Pig	3859723	4655711	68
F38C3A	Cattle	3848138	4699545	105
F38P4A	Poultry	3834335	4648288	106
F40G2A	Sheep/Goat	3783005	4691471	127

Isolate	Bacterial Host	Number of sequences	Genome Size	Mean Coverage
F40P2A	Poultry	3743488	4700698	127
F41G1B	Sheep/Goat	3743488	4645860	101
F41G3E	Sheep/Goat	3730659	4645625	116
F41G4A	Sheep/Goat	3692884	4645337	104
F41H3A	Human	3676919	4690463	104
F41P1E	Poultry	3676919	4708128	79
F41P5A	Poultry	3646594	4709156	79
F42C1A	Cattle	3612171	4709333	81
F42HOUSEA	Environment	3592044	4658099	81
F42PI3D	Pig	3592044	4646941	179
F43G1A	Sheep/Goat	3535438	4710040	179
F43H1B	Human	3535438	4656035	115
F43P1A	Poultry	3498326	4655524	124
F43PI1D	Pig	3464240	4657165	124
F44C2B	Cattle	3464240	4649352	154
F44C4C	Cattle	3424858	4657244	154
F44C5A	Cattle	3408439	4659978	130
F44H2C	Human	3404504	4645261	155
F44P1A	Poultry	3388692	4851928	155
F44P3B	Poultry	3361927	4642551	100
F44P4A	Poultry	3350862	4644377	151
F44P5A	Poultry	3335785	4644884	174
F44PI1A	Pig	3335785	4709883	128
F45C2C	Cattle	3320111	4665743	170
F45G1A	Sheep/Goat	3320111	4709100	170
F45G2C	Sheep/Goat	3258101	4659978	117
F45P1A	Poultry	3238373	4709020	117
F45P2E	Poultry	3231920	4644812	169
F45P3B	Poultry	3178216	4700566	92
F46C1A	Cattle	3127357	4656277	133
F46G2B	Sheep/Goat	3127357	4779860	118
F46H1A	Human	3082237	4665507	79
F46P3A	Poultry	3024047	4652783	185
F47C1A	Cattle	3019330	4709401	124
F47P1E	Poultry	3019330	4643357	124
F48C1A	Cattle	3005253	4709890	122
F48G3A	Sheep/Goat	3005253	4648107	106
F48P3B	Poultry	2940394	4651511	106
F49C1A	Cattle	2940394	4710009	223
F49C3A	Cattle	2930597	4644146	87
F49P7A	Poultry	2930597	4708679	87
F51C2C	Cattle	2902327	4656087	144
F51P2A	Poultry	2902327	4645120	144
F52G1A	Sheep/Goat	2898710	4709508	81
F52G2B	Sheep/Goat	2898710	4649446	78
F52G3B	Sheep/Goat	2867122	4708548	91
F52H3C	Human	2855451	4645950	183
F52P2A	Poultry	2855451	4709018	162
F53H3A	Human	2768818	4643605	80
F53PI1A	Pig	2768818	4643471	113
F55G3A	Sheep/Goat	2725448	4649339	152
	Average	**4276582.08**	**4691652**	**118**

Figure II-iii Slanted maximum likelihood phylogenetic tree of all WGS isolates. Tree is slanted to indicate bootstrap support values clearly and is split across two pages. All nodes are in the same order as **Figure 4.3.**





Appendix III

Material concerning Chapter 5

Table III-i Standard metrics of 187 sequences obtained from whole genome sequencing of faecal E. coli isolated from slaughterhouse workers.

Isolate	Type of Slaughterhouse	Number of sequences	Genome Size	Mean coverage
120336	Cattle and Sheep/Goats	280444	4647468	19
120233	Cattle only	801789	4642033	28
120375	Cattle only	818581	4887208	29
120418	Cattle only	173901	4820417	26
120431	Cattle and Sheep/Goats	792986	4820343	25
120500	Cattle and Sheep/Goats	216922	4644879	29
120504	Cattle and Sheep/Goats	510637	4712205	22
120505	Cattle and Sheep/Goats	508273	4659989	22
120510	Cattle and Sheep/Goats	418541	4764593	19
120511	Cattle and Sheep/Goats	1180995	4660451	53
120515	Cattle and Sheep/Goats	537141	4651390	23
120516	Cattle and Sheep/Goats	344653	4697450	13
120519	Cattle only	1164967	4647188	54
120520	Cattle and Sheep/Goats	593661	4677960	25
120531	Pigs only	690268	4657096	30
120534	Pigs only	785925	4681720	32
120540	Cattle only	894226	4645678	39
120544	Cattle only	1042161	4647103	48
120546	Cattle only	407029	4650145	16
120547	Cattle only	5312475	4694562	243
120548	Cattle only	1190524	5085209	55
120550	Cattle only	422153	5083874	19
120561	Cattle only	252654	4690411	29
120564	Cattle only	1028585	4656472	30
120595	Cattle and Sheep/Goats	2252194	4787402	89
120596	Cattle and Sheep/Goats	368273	4787437	15
120597	Cattle and Sheep/Goats	809933	4680078	36
120633	Cattle only	1657061	4647460	77
120637	Cattle only	736931	4692297	33
120655	Cattle only	6921496	4657801	319
120689	Pigs only	1617929	4658892	76
120721	Cattle and Sheep/Goats	1357007	4677039	58
120724	Cattle and Sheep/Goats	1279570	4650381	60
120725	Cattle and Sheep/Goats	2671485	4715062	125
120726	Cattle and Sheep/Goats	2456962	4779760	118
120748	Cattle and Sheep/Goats	621427	4759651	28
120798	Pigs only	1920259	4713344	86
120802	Cattle only	523779	4645326	24
120811	Pigs only	523779	4830662	24
120819	Cattle only	815485	4657034	35
121231	Cattle and Sheep/Goats	1476998	4658311	70
121236	Cattle and Sheep/Goats	732522	4711302	35
121250	Pigs only	2084868	4646355	91
121317	Cattle and Sheep/Goats	1745880	4730711	84
121340	Cattle only	2270720	4734044	104
121368	Cattle only	767913	4661015	35
121369	Cattle only	2276807	4692192	100
121426	Cattle only	1131480	4706178	53
121427	Cattle only	1485164	4645347	69

Isolate	Type of Slaughterhouse	Number of sequences	Genome Size	Mean coverage
121428	Cattle only	831785	4659560	40
121460	Cattle only	1216605	4686061	58
121464	Cattle only	837529	4661237	41
121466	Cattle only	779504	4643650	34
121528	Pigs only	3222557	4687117	150
121560	Cattle only	1027236	4655576	50
121568	Cattle only	1431653	4650325	70
121595	Cattle and Sheep/Goats	1864989	4647465	87
121613	Cattle only	1532070	4650753	74
121617	Pigs only	892608	4680259	42
121644	Cattle only	875612	4679800	41
121650	Cattle only	1276962	4646246	59
121680	Cattle only	2189915	4646676	106
121712	Cattle only	1899829	4656115	85
121721	Cattle and Sheep/Goats	5065805	4646160	199
121723	Cattle and Sheep/Goats	678257	4645923	20
121736	Cattle and Sheep/Goats	787708	4688050	37
121766	Cattle and Sheep/Goats	767120	4656603	33
121772	Cattle and Sheep/Goats	1028589	4709581	50
121921	Cattle and Sheep/Goats	648519	4669160	31
121949	Pigs only	1126183	4661094	53
121967	Pigs only	1859777	4712927	88
123519	Cattle and Sheep/Goats	728122	4756079	34
123549	Cattle and Sheep/Goats	1263567	4658544	57
123559	Cattle and Sheep/Goats	642201	4713353	30
120172a	Cattle and Sheep/Goats	928172	4674699	45
120172b	Cattle and Sheep/Goats	1863447	4644767	88
120346a	Cattle and Sheep/Goats	1065411	4649141	51
120346b	Cattle and Sheep/Goats	2809256	4674378	121
120347a	Cattle and Sheep/Goats	1109773	4651050	48
120347b	Cattle and Sheep/Goats	1892940	4656820	92
120347c	Cattle and Sheep/Goats	878163	4691546	41
120354a	Cattle only	632581	4651001	29
120354b	Cattle only	1321223	4675349	62
120360a	Cattle only	2038289	4642708	99
120360b	Cattle only	872777	4645045	39
120502a	Cattle and Sheep/Goats	654552	4662467	31
120502b	Cattle and Sheep/Goats	1078722	4642064	51
120506a	Cattle and Sheep/Goats	1748033	4656303	83
120506b	Cattle and Sheep/Goats	1722343	4656505	81
120690a	Pigs only	1722343	4690405	81
120690b	Pigs only	1587380	4799692	76
120741a	Cattle and Sheep/Goats	1376318	4706221	64
120741b	Cattle and Sheep/Goats	1139716	4706311	55
120741c	Cattle and Sheep/Goats	1888328	4706333	90
120749a	Cattle and Sheep/Goats	1019141	4690637	46
120749b	Cattle and Sheep/Goats	2778074	4657171	132
120750a	Cattle and Sheep/Goats	1018063	4657089	43
120750b	Cattle and Sheep/Goats	4381797	4788506	191
121608a	Cattle only	1024489	4875078	50
121608b	Cattle only	1401990	4756010	59
121615a	Cattle only	1180697	4682967	57
121615b	Cattle only	1786210	4791783	86

Isolate	Type of Slaughterhouse	Number of sequences	Genome Size	Mean coverage
121649a	Cattle only	1106527	4649688	53
121649b	Cattle only	1517529	4662305	70
121919a	Cattle and Sheep/Goats	1741276	4714564	84
121919b	Cattle and Sheep/Goats	1254488	4644707	58
121930a	Cattle and Sheep/Goats	2918074	4644271	141
121930b	Cattle and Sheep/Goats	2326000	5083875	114
121972a	Pigs only	1564150	4646940	74
121972b	Pigs only	1693830	4639706	77
58-263A	Cattle and Sheep/Goats	1566089	4646537	71
58-286C	Cattle and Sheep/Goats	2144176	4684738	103
58-294D	Cattle and Sheep/Goats	2276536	4657564	102
58-323C	Cattle and Sheep/Goats	1889368	4645351	80
58-337B	Cattle and Sheep/Goats	2628689	4684732	109
58-340C	Cattle only	2100408	4656779	96
58-363CX	Pigs only	2100408	4887207	96
58-367C	Cattle only	2967843	4647573	124
58-377C	Cattle only	2415277	4684939	100
58-454A	Pigs only	280444	4887499	10
58-454C	Pigs only	801789	4656520	28
58-454CX	Pigs only	818581	4657251	29
58-454CZ	Pigs only	173901	4645897	26
58-457CZ	Pigs only	792986	4887206	25
58-464A	Cattle and Sheep/Goats	216922	4657075	29
58-464CX	Cattle and Sheep/Goats	510637	4644490	22
58-464CZ	Cattle and Sheep/Goats	508273	4666249	22
58-465CX	Cattle only	418541	4719106	19
58-467A	Cattle only	1180995	4656748	53
58-468CX	Cattle and Sheep/Goats	537141	4763529	23
58-468CZ	Cattle and Sheep/Goats	344653	4810327	13
58-477CX	Pigs only	1164967	4669584	54
58-490B	Pigs only	593661	4759767	25
58-498CZ	Cattle and Sheep/Goats	690268	4661468	30
58-503A	Pigs only	785925	4759726	32
58-523C	Pigs only	894226	4643670	39
58-533A	Cattle and Sheep/Goats	1042161	4667241	48
58-533CZ	Cattle and Sheep/Goats	407029	4767895	16
58-539CX	Cattle and Sheep/Goats	5312475	4645507	243
58-539CZ	Cattle and Sheep/Goats	1190524	4683177	55
58-546A	Pigs only	422153	4900032	19
58-546CXa	Pigs only	252654	4657077	29
58-546CXb	Pigs only	1028585	4657080	30
58-546CZ	Pigs only	2252194	4961227	89
58-547CX	Cattle only	368273	4666141	15
58-549C	Cattle and Sheep/Goats	809933	4642313	36
58-554A	Cattle only	1657061	4708600	77
58-558CZ	Cattle only	736931	4769144	33
58-560CX	Cattle only	6921496	4657075	319
58-562CZ	Cattle only	1617929	4669369	76
58-570CX	Cattle only	1357007	4659212	58
58-578CX	Cattle only	1279570	4853074	60
58-579C	Cattle only	2671485	4756270	125
58-583CZ	Cattle only	2456962	4657081	118
58-586CX	Cattle only	621427	4643601	28

Isolate	Type of Slaughterhouse	Number of sequences	Genome Size	Mean coverage
58-588CX	Cattle only	1920259	4657076	86
58-596D	Cattle only	523779	4687187	24
58-606A	Cattle and Sheep/Goats	523779	4655363	24
58-630CZ	Pigs only	815485	4657076	35
58-639CZ	Pigs only	1476998	4782516	70
58-644B	Pigs only	732522	4692279	35
58-651C	Cattle only	2084868	4648121	91
58-663A	Cattle only	1745880	4657018	84
58-671A	Cattle and Sheep/Goats	2270720	4919764	104
58-672B	Cattle and Sheep/Goats	767913	4656904	35
58-676A	Cattle only	2276807	4670850	100
58-676CZ	Cattle only	1131480	4657551	53
58-687A	Cattle and Sheep/Goats	1485164	4680830	69
58-697D	Cattle and Sheep/Goats	831785	4679672	40
58-702CX	Pigs only	1216605	4677663	58
58-704A	Pigs only	837529	4684738	41
58-706E	Pigs only	779504	4760361	34
58-716CX	Pigs only	3222557	4645200	150
58-716CZ	Pigs only	1027236	4656512	50
58-718D	Pigs only	1431653	4887210	70
58-724A	Pigs only	1864989	4657856	87
58-724CX	Pigs only	1532070	4645384	74
58-725CX	Pigs only	892608	4661280	42
58-725CZ	Pigs only	875612	4643117	41
58-733D	Pigs only	1276962	4657544	59
58-740CZ	Pigs only	2189915	4657563	106
58-742CX	Pigs only	1899829	4693275	85
58-747CX	Pigs only	5065805	4656512	199
58-747CZ	Pigs only	678257	4656510	29
58-751CX	Pigs only	787708	4656503	37
58-751CZ	Pigs only	767120	4657006	33
58-754B	Pigs only	1028589	4656793	50
	Average	**1398731**	**4698734**	**64**

Figure II-i Slanted maximum likelihood phylogenetic tree of all WGS isolates. Tree is slanted to indicate bootstrap support values clearly and is split across two pages. All nodes are in the same order as **Figure 5.3.**