Antimicrobial resistance in commensal *Escherichia coli* isolated from poultry along a gradient of intensification of poultry production in the Northern Zone of Tanzania

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ABSTRACT

Antimicrobial resistance is a threat to human and animal health. There is widespread, unregulated use of antimicrobials in Tanzania in poultry production, which may impose selective pressure on gastrointestinal commensals. The indiscriminate use of antimicrobials in health care and agriculture has exerted selective pressure among commensal bacteria such as *Escherichia coli* enabling them to acquire fitness against antimicrobials. Extensive research has been conducted in human and animal pathogens, but few studies have investigated antimicrobial resistance in enteric commensals. When enteric commensals share the same niche with pathogens, there is potential for lateral gene transfer between commensals and pathogens. One of the aims of the present study was to determine the prevalence of antimicrobial resistant commensal coliforms across four poultry farm types in Arusha and Moshi, Northern Zone, Tanzania. The second was to determine prevalence of AMR in commensal *E. coli* isolates using different thresholds for interpret resistance, and to examine the impact of methodology and thresholds on apparent AMR prevalence.

Samples were collected from Moshi and Arusha urban districts. Ten wards were randomly chosen in each district, with random selection of one representative farm in each ward per production system (extensive, semi-intensive, indigenous intensive and broiler intensive). In each farm, cloacal swabs were collected from 10 chickens. Resistance against four antimicrobial compounds was explored, selected based on common use (tetracycline) or importance to human health based on the World Health Organization's list of Critically Important Antimicrobials (ciprofloxacin, ceftazidime and imipenem). The breakpoint plate method was used in screening for presence of resistant coliforms in cloacal samples in Tanzania whilst confirmatory testing was conducted in Glasgow on a subset of plate weeps. Chromogenic agar was used in identification of individual *E. coli* isolates whilst *uid*A PCR was used for confirmation of the species. To analyse the susceptibility of individual isolates, disc diffusion testing was used. Inhibition zone diameters were interpreted using clinical breakpoints (CB), ecological cutoffs (ECOFFS) (provided by the European

Committee on Antimicrobial Susceptibility Testing (EUCAST) and normalised resistance interpretation based wild type cut-offs (CO_{WT}).

No correlation was found between prevalence data generated in Tanzania and Glasgow. Resistance to each of the four compounds was seen at least once in every farm. There was no consistent pattern revealing a clear association between intensification of poultry production and prevalence of resistance. Tetraycline resistance was consistently higher across farm types compared to other antimicrobials based on both disc diffusion method and the breakpoint plate method. According to the CB and CO_{WT} interpretation, the prevalence of AMR was low for ceftazidime and imipenem (< 4%), intermediate for ciprofloxacin, and high for tetracycline (> 67%). Relatively high prevalence was observed based on ECOFFS, e.g. 45.8% for ceftazidime and 64.4% for imipenem. These results suggest that interpretation of resistance can be impacted by the type of threshold used. Our study reveals that healthy poultry are reservoirs of resistant *E. coli*. Thus, there may be a risk of transmission of resistant bacteria in and out of the farms, for example, through contaminated water, use of poultry manure in crop production, or through the food chain. Control strategies need to be developed, including further studies to determine factors that may be contributing to the AMR problem in poultry farms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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AUTHOR'S DECLARATION

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

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CHAPTER ONE

PREVALENCE OF ANTIMICROBIAL RESISTANCE

1.1 Introduction

1.1.1 Prevalence of antimicrobial resistance

Antimicrobial resistance (AMR) is regarded as one of the major public health problems of the 21st century. According to a high profile report, AMR causes more than 700,000 deaths each year around the world (O'Neill, 2014). Genuine data of the worldwide burden of AMR is still missing, as the current information is not truly illustrative of the worldwide situation, especially in developing nations. At best, existing studies provide estimates largely based on collation of small scale or individual studies that vary greatly in setting, scope, sampling frame and methodology often compelling bold inferences to be made from very limited data. Due to such huge information gaps in the existing data on AMR, the status globally is regarded partial and quite tentative. Moreover, although there are a few existing reports in low-income countries (e.g. situation analysis in Mozambique (Sigauque and Saide, 2016) or the Tanzania AMR National Action Plan (Hakanen et al., 2017)), the available information, for the most part, addresses the situation in developed countries (European Centre for Disease Prevention and Control (ECDC), 2009) and US (Centers for Disease Control and Prevention (CDC), 2013) while underestimating the real condition in the developing countries. Despite being published five years apart, the European Centre for Disease Prevention and Control report (ECDC, 2009) and AMR review report (O'Neill, 2014), did not account for population-based surveillance data. Their inferences were largely dependent on data provided by the European Antimicrobial Resistance Surveillance network (EARS-Net) that only records invasive infections diagnosed in hospitals and for a variable proportion of the total number of hospitals in each country. This implies that significant information from some hospitals was left out. On the other hand, population-based surveillance which is clearly unrepresented in these reports has the advantage of providing additional information about asymptomatic carriage of resistant bacteria which could spill over to clinics during visits at any given opportunity. Asymptomatic carriage in the healthy population can provide an indication of the existence of other exogenous sources of AMR bacteria and determinants other than hospitals and this includes other stressors prompting the development of antimicrobial-resistant microorganisms in the community, which could have the potential of causing high levels of resistance in the healthy population. These may not be noticed unless the healthy population is screened.

As of late, there is clear evidence of acquisition of AMR through food, between animals and humans although the directionality has not been clearly established (Muloi et al., 2018). Ideally, the AMR prevalence seen in hospitals could be a function of the general population, which only involves reported cases of ill individuals whilst the healthy population carrying AMR remains unchecked. A systematic review that was conducted to determine the role of farm animals on the emergence and dissemination of AMR bacteria and their determinants to humans discovered that only 8 studies (18%) suggest a possible transmission of AMR from food animals to humans, 25 studies (56%) suggest transmission between animals and humans without a clear direction specified and 12 studies (26%) did not support transmission at all (Muloi et al., 2018).

Even with these limitations, the global situation of AMR is alarming. According to the US CDC, more than two million people every year are affected with antimicrobial-resistant infections, with at least 23 000 dying as a result of the infection in US (CDC, 2013). On the other hand, each year in Europe, it has been estimated that 400 000 infections and 25 000 deaths occur due to the most frequent multidrug-resistant (i.e. resistant to three or more different antimicrobial classes) bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) (ECDC, 2009). Common infections in neonatal intensive care are becoming extremely difficult and sometimes impossible to treat (Stoll et al., 2010). Hospital based studies in developing countries have shown that out of 834 pathogens causing neonatal sepsis in the first 3 days of life, resistant *Klebsiella* is the leading pathogen causing up to 26% of all infections followed by resistant *E. coli* and other gram positive bacteria such as *S. aureus* causing 13% to 17% of the infections (Nathoo et al., 1990; Sugandhi et al., 1993; Zaidi et al., 2009). Similarly, for community acquired neonatal infection resistant gram-negative bacteria are still the most commonly isolated pathogens beginning notably resistant *Klebsiella* spp. and resistant *E. coli* (Zaidi et al., 2009).

Antimicrobial resistance as mentioned below in developing countries is yet to be fully addressed, as there is quite significant lack of sufficient data on the pattern of resistance in most countries. Due to absence of effective surveillance systems, efficient point of care diagnostic tools to detect AMR,

standardised guidelines for selection of appropriate antimicrobial therapy and poor dissemination of research information on the AMR pattern, most of the health systems are left stranded with the lack of up to date information on the AMR pattern within their populations, subsequently leading to the difficulty of making a decision on the choice of antimicrobial for specific infection (Ayukekbong et al., 2017). Thus, health professionals in these countries use multiple or more broad-spectrum antimicrobials to treat infections caused by several bacteria or those for which establishing the aetiology agent of the disease is deemed difficult or takes a quite a while (Neu, 1992). This practice increases resistance as the drug applies selective pressure not only upon the causative agent, but also upon a vast fraction of the patient's microbiota including the commensals (Calva et al., 1996). Moreover, the lack of proper enforcement and implementation of regulatory systems and absence of stewardship programs in some countries provide favourable conditions for continuation of imprudent use of antimicrobials (e.g. National Action Plan in Tanzania). In developing countries, more than 1000 cases for every 100 000 individuals yearly were infected with multidrug resistant typhoid serotypes (Crump et al., 2003). It is thought that the development and dissemination of typhoid serotypes that are resistant to various antimicrobials such as ampicillin, chloramphenicol and co-trimoxazole (trimethoprim sulfamethoxazole), in part could be the reasons for persistence of the disease. On the other hand, in Pakistan and India, where carbapenems are used widely, outbreaks of carbapenem resistant *Escherichia coli* and *Klebsiella pneumonia* have frequently been reported (Poirel et al., 2011). To make matters worse, some of these resistant clones and their plasmids have spread to other countries including developed countries. A good example is the emergence of New Delhi metallo-ß-lactamase (NDM-1) plasmid mediated carbapenem resistance that spread from India to Europe, USA and Africa (Kumarasamy et al., 2010; Poirel et al., 2011). Another good example is multidrug resistant *Salmonella* Typhi, which emerged in 1987 and spread throughout the Indian subcontinent and South East Asia (Mirza et al., 1996). In that study, it was reported that 69% of *Salmonella* Typhi that were isolated from blood were multidrug resistant. Due to this observation, fluoroquinolones have become first line drugs for such *Salmonella* infections. However, epidemics of infections associated with ciprofloxacin resistant *S.* Typhi have been reported in Tajikistan (Ridley and Threlfall, 1998). These isolates

have been reported to carry large, self-transmissible plasmids, which encode resistance to each of the first line drugs and can be transferred to other pathogens.

Antimicrobial resistance in most African countries is quite widespread, particularly in aetiological agents of disease. For instance, in Kilifi, Kenya, over half of the non-typhoidal *Salmonella* isolates from children were multidrug resistant (Oundo et al., 2000). A review study conducted to capture the situation in the whole of Africa, despite of lack of data from more than 40% of countries in the African continent, revealed that the median resistance of *Escherichia coli* to trimethoprim and gentamycin was 88.1%, 80.7% respectively whilst resistance to other antimicrobials such as carbapenem was common in *P. aeruginosa* (Tadesse et al., 2017). Between the latter study and Ampaire et al. (2016), who conducted a review study in East Africa, there is a close agreement which reveals high levels of resistance to common antimicrobials with estimates ranging approximately between 50% and 100%, particularly for ampicillin and cotrimoxazole (Otage, 2015). Gram-negative bacteria, particularly *Klebsiella* spp. and *E. coli* appear to be commonly resistant to gentamycin with estimates ranging between 20% to 47% (Mugalu et al., 2006) whilst gram-positive bacteria have been reported to be commonly resistant to ampicillin (100%) (Mulatu et al., 2014), gentamicin and ceftriaxone (50% to 100%) (Muluye et al., 2014) with methicillin-resistant *Staphylococcus aureus* prevalences ranging from 2.6% to 4.0% (Demilie et al., 2018). Short clinical studies in Tanzania involving children and pregnant women have revealed higher resistance rates in *Klebsiella* spp. than *E. coli* (Festo et al., 2011). There is likewise clear evidence showing growing AMR of nosocomial pathogens. For instance, resistance against gentamicin in *E. coli* has been reported to range from 7% at Muhimbili National hospitals (MNH) in 2003 to more than 44% in the same hospital in 2011 (Lyamuya et al., 2011; Rimoy et al., 2008). Molecular characterization has enabled the detection of antimicrobial resistant clones in some countries where there was no past evidence of their existence. For instance, in Tanzania Mshana and colleagues (2011) reported the presence of Extended Beta-Lactamase producing (CTX - M positive) *E. coli*, sequence (ST) 131, which has also been found in Canada, India, Kuwait, France and Switzerland (Nicolas-Chanoine et al., 2007; Coque et al., 2008).

1.1.2 Use of antimicrobials in animal production

Antimicrobials have been used in animals for a long time for treatment of diseases, prevention and control of diseases and as growth promoters. Metaphylatically, because of infectious disease, the whole flock is usually treated to prevent the dissemination of illness in the flock, despite the exhibition of clinical symptoms in a few animals. The process involves the provision of high doses of antimicrobials for a short time frame whilst in prophylaxis, antimicrobials are administered in feed or drinking water in low doses for a longer period of time, usually for several weeks. During this time, animals are not manifesting any clinical signs, but the risk of infection exists (Ndashe et al., 2016). The use of antimicrobials as growth promoters stems back to the 1950s (Jukes et al., 1950), when Stokstad and Jukes found out that small subtherapeutic doses of penicillin and tetracycline could enhance weight gain. However, as of present a number of countries have made an effort to ban the use of antimicrobials for subtherapeutic purposes. For instance, in the European Union use of antimicrobials for growth promotion was banned in 2006 (Cogliani et al., 2011).

Due to the frequent use of antimicrobials, it is thought that food animals could be substantial reservoirs of antimicrobial-resistant bacteria as the food production chain is an ecosystem composed of different ecological niches, which involve numerous bacteria co-existing and conceivably being exposed to selection pressure (Acar and Moulin, 2006). They can be transmitted directly or indirectly to humans through food consumption or direct and indirect contact with colonised or infected animals or through contact with excreta, such as urine or faeces, or blood (Chuang et al., 2015). Occupationally exposed workers such as veterinarians, farmers, abattoir workers and food handlers, as well as those directly in contact with animals, are at high risk of being colonized or infected with antimicrobial-resistant bacteria (Marshall and Levy, 2011; Aubry-Damon et al., 2004). A study of French pig farmers and non-farmers showed that the pattern of co-resistance to ampicillin-streptomycin-cotrimoxazole was significantly more common among *E. coli* isolated from pig farmers compared with *E. coli* isolated from non-farmers (Aubry-Damon et al., 2004). Exposed workers and their families provide a likely route for entry of antimicrobial-resistant bacteria and AMR genes into the community and healthcare settings

where subsequent exchanges and the acquisition of resistance mechanisms are evident (Marshall and Levy, 2011). Moreover, a large proportion of antimicrobials are not transformed into inactive forms once administered in animals and subsequently get retained in the tissue of the animals or disseminated in the environment which is another important reservoir of antimicrobial resistant bacteria or antimicrobial residues (Zhu et al.,2013). Antimicrobial residues, such as fluoroquinolones, macrolides, aminoglycosides, and tetracyclines (Kümmerer, 2009) have been identified broadly in the environment. Studies demonstrate major sources of contamination for antimicrobial-resistant bacteria and resistance genes in the environment include wastewater from farms or hospitals and feacal waste (in some areas via open defecation), animal husbandry and wildlife (Ortiz et al., 2016; Bondarczuk et al., 2016; Husman and Larsson, 2016). High prevalence of residues in various ecological niches in the farm-to-fork continuum is thought to enhance the pool of antimicrobial resistant bacteria and AMR genes in the ecosystem because of exposure to sub-inhibitory concentrations of the residues (Acquaah-Mensah et al., 2012). There is also evidence indicating the possibility of transmission of antimicrobial resistant airborne pathogens (Huijbers et al., 2015), e.g. resistant *Coxiella* spp. in the air and dust particles, which can lead to Q-fever (Dorko et al., 2012).

In many countries, clear linkage of AMR in animals and humans has not been ascertained. A great part of evidence identifying the potential transfer of resistance problem from animals and humans originates from a consideration of the epidemiology of zoonoses, mainly *Salmonella* and *Campylobacter* or indicator organisms such as *E. coli* and enterococci, which can be carried asymptomatically by healthy humans and animals. However, the epidemiology of these diseases is far from simple since there are many possible sources other than food animal of animal origin (Phillips et al., 2003). When antimicrobials are used in animals, resistance is likely to be selected in commensal and pathogenic intestinal flora and other colonized or infected body sites leading to an increase in prevalence (Aarestrup et al., 2000; Bager et al., 2002). Humans and other animals can acquire resistant pathogens and commensal organisms by ingesting them. Contaminated meat and other cross-contaminated foods cause millions of cases of gastrointestinal illnesses such as salmonellosis and campylobacteriosis each year in the USA alone (Scallan et al., 2011). The threat that antimicrobial

use in food-producing animals poses to human health via this route has been estimated using microbial risk assessment models (Mcewen, 2012). Using an exposure-based model, one study assessed how many cases of *Campylobacter jejuni* infection (i.e. resistant cases) could arise from contaminated ground beef. The study estimated 12 cases in the USA after one year of fluoroquinolone use in cattle, rising to 44 cases and one death after 10 years (Anderson et al., 2001). Another good example is the vancomycin-resistant enterococci (VRE), which normally colonize the gut, and have been noted to acquire resistance to multiple antimicrobials over time, making the glycopeptide vancomycin one of the last therapeutic options. The epidemiology of VRE differs substantially between the USA and Europe. In Europe, *Enterococcus faecium* carrying the *vanA* element for vancomycin resistance was commonly found in the intestinal flora of farm animals as well as healthy people but carriage of VRE in farm animals and healthy people was absent in the USA until 2008 (Bonten et al., 2001). This difference was ascribed to the widespread agricultural use of avoparcin, which is a glycopeptide used in Europe since the 1970s but was never approved for use in the USA in agriculture. The problem in Europe was addressed through the prohibition on the utilization of avoparcin in 1997 and other antimicrobials as growth promoters in animal feed. By contrast, in the USA, selection for vancomycin resistance was later discovered to be primarily due to human-to-human transmission in hospitals; therefore, control had to be conducted differently. Avoparcin, which confers cross-resistance to vancomycin, has been shown to select for VRE in animals (Aarestrup et al., 1996). A large reservoir of VRE in animals presents many opportunities for human infection and the potential for resistant bacteria to colonize the human niche. Molecular epidemiologic studies have also provided strong evidence on the possibility of human to animal transmission and vice versa (Woodford, 2009; Freitas et al., 2011).

1.1.3 The role of commensals as reservoirs of antimicrobial resistance

One of the pitfalls of controlling AMR for many decades stems from the hidden role of commensals in the emergence, amplification, dissemination and maintenance of AMR genes. It is only recently that this phenomenon has been uncovered. In various studies, molecular characterization of multidrug-resistant commensal *E. coli* has revealed considerable gene diversity, thereby highlighting

the importance of *E. coli* strains as reservoirs of a wide array of transferable genetic determinants (Karczmarczyk et al., 2011; Schink et al., 2011). While commensal bacteria may be a hidden reservoir of AMR genes, which can serve as an early and potent more accurate indicators of the resistance status of the microbiota, dominant AMR carriers vary among ecosystems, antimicrobials and even the specific AMR genes within the same host or environmental microbiota (Feßler et al., 2011). For instance, in fermented milk, the main AMR gene carriers are lactic acid bacteria and in infants, *Staphylococcus*, not *E. coli* was found to be the primary commensal in the gastrointestinal tract shortly after birth (Wang et al., 2006). Aside from their role as transmitters of resistance genes to potentially pathogenic bacterial species under certain resistance conditions or changes in microbial niches, the non-pathogenic commensal bacteria may pick up the status of pathogens (Miskinyte et al., 2013).This could happen through horizontal gene transfer of virulence genes, which could co-occur with resistance genes (Shan Lu *et al.,* 2016), and these genes could be translocated into the recipient bacterial chromosome and transmitted vertically through to the progeny. Secondly, it could happen through colonization of new environments, e.g. intestinal commensal *E. coli* could colonise the urinary tract or the blood system stream (Miskinyte et al., 2013). Thirdly, it may occur through the reprogramming of cellular transcription without genetic changes by upregulation of virulence genes (Koli et al., 2011). This phenomenon is apparent in quorum sensing in which gram-negative and gram-positive bacteria co-ordinate communal behaviour that involves regulation of specific genes in response to population density in which certain chemical compounds (signal molecules called autoinducers) accumulate in the cell of the bacteria with increase in bacteria population. This activates the transcription of quorum-sensing-regulated genes (Eboigbodin, et al., 2006; Henke and Bassler, 2004). The phenomenon is also employed in AMR resistance in which genes that code for efflux pumps are over expressed to enable the pumping out of antimicrobials (Pearson et al., 1999; Soto, 2013).The two systems also play a role on antimicrobial resistance in gram-positive bacteria (Singh and Ray, 2014)

The acquisition of AMR genes in commensals may occur through horizontal gene transfer or selective pressure because of consistent use of antimicrobials in animal production and this may create therapeutic problems. Some of the genes

acquired by commensal bacteria are thought to co-select for other resistance against other types of drugs. For instance, tetracycline resistance genes have been found to be associated with other types of resistance genes including extended ß-lactamase resistance genes (Hammerum and Heuer, 2009). Reservoirs of resistance may be present in healthy humans and animals (Choudhury et al., 2012). *E. coli* as a commensal bacterium is found in the digestive system of most animals (e.g. poultry, cattle, dogs) (Caudell et al. 2018) and can contaminate food products during slaughter or food handling and subsequently increase the risk of ingestion of AMR bacteria by humans. According to van den Bogaard et al. (2001), three isolates of AMR *E. coli* found in contaminated turkey meat belonged to identical types as isolates from turkey faeces. Results from an experimental study demonstrated that it is possible for antimicrobial-resistant *E. coli* strains of animal origin to establish as part of the flora of healthy human after being ingested (Linton et al., 1977). Different animal model studies have also demonstrated that the intestine is a hot spot for horizontal transfer of resistance genes between *E. coli* (Jacobsen et al., 2007; Wang et al., 2006). There was a study which detected the same AMR genes in animals, meat and in humans, suggesting horizontal transfer (Overdevest et al., 2011; Silva et al., 2012). Hart et al. (2006) used a chicken and mouse intestine model to detect the transfer of a tetracycline resistance gene from *E. coli* of animal origin to *E. coli* of human origin. As an important and ubiquitous commensal in the enteric system of chicken, this study intends to screen for the prevalence of AMR in commensal *E. coli* obtained from chicken cloacal samples. This is fundamentally based on the possibility of commensal *E. coli* being a source of resistance genes that could be transferred to other species that are pathogenic to humans.

Currently, in Tanzania there is no study that has investigated the prevalence of AMR in commensal *E. coli* across all poultry meat production systems. Existing studies in Tanzania have either investigated prevalence of resistant bacteria in one type of poultry farm (e.g. extensive poultry systems) or compared between two poultry systems (e.g. broiler and extensive systems). Comparison of resistance patterns and prevalence across multiple regions and production systems (See sections 2.1 and 2.2.2 for details) is pertinent to gaining a deeper understanding as to whether geographical differences and an

intensification of poultry production could have an impact on AMR patterns and prevalence. By analysing resistance profiles of *E. coli* isolates across the four farm types, we may find relevant associations that can lead to a further understanding of the epidemiology of resistant bacteria (Harwood et al., 2000) and hence allow appropriate interventions to be carried out. In short, this study has as a main goal of contributing to a better understanding of carriage of antimicrobial resistant *E. coli* in chickens in the four poultry production systems, a phenomenon which could have an implication for public health risks associated with consumption of poultry meat or direct contact with the animals.

1.1.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is normally conducted to determine if bacteria are resistant or not. There are two major ways in which this can be conducted, i.e. using phenotypic methods or genotypic methods. Whether or not genotypic methods are superior to phenotypic methods is debatable (Cockerill, 1999). There are several potential advantages of genotyping over phenotyping, e.g. when organisms under study are slow growers. Some organisms cannot be cultured or are not easily cultured and so only genotypes can be determined in these cases (Cockerill, 1999). On the other hand, the resistance of a microorganism to a specific microbial agent may occur via different mechanisms associated with different resistance genes or a large array of single or coincidental mutations. With genetic methods one only gets what they are specifically looking for. This is in contrast to culture-based methods which are more comprehensive in assessing antimicrobial resistance. By using the same culture-based assay, different forms of resistance can be detected. Due to their capacity to survey different forms of resistance, culture-based methods are also helpful for detecting emerging or new forms of antimicrobial resistance (Cockerill, 1999). Genetic methods may detect resistance genotypes that are expressed at levels that may not be clinically relevant. Examples of this include lower level vancomycin resistance encoded by *van* genes (i.e. vanB and vanC) and poorly expressed extended spectrum ß lactamase resistance (Cockerill, 1999). The focus of this study will be directed towards phenotypic methods.

1.1.5 The breakpoint AST technique and Individual Isolate assays

For the past few decades, phenotypic methods have been the predominant methods used for screening and monitoring resistance in bacteria populations. Given that these methods are cheap and readily available, they have a widely been used in clinical settings in routine tests, both in developed and low-income countries. As of late, many epidemiologists have taken a keen interest in AMR. Scientists in this field developed a new method that focuses on screening populations of bacteria, whereas clinical scientists prefer traditional individual isolate screening methods. The population-focused method is a breakpoint method. It is an abbreviated form of agar dilution procedure (Piddock, 1990). This method is now gaining popularity among epidemiologist and has been used by some researchers in Tanzania (Rugumisa et al., 2016; Lyimo et al., 2016). Like the conventional individual isolate assays, this method utilizes breakpoint concentrations that are set up based on microbiological data. In this method the antimicrobial is added to or integrated into the agar at a specific breakpoint concentration. Growth on the plate is compared to growth on an antimicrobial-free control (Piddock, 1990). No growth indicates presence of susceptible strains and growth indicates presence of resistant strains. The advantage of this method over individual isolates assays is that it evaluates a huge number of microorganisms within a sample at a once while the individual isolates assay requires selecting only a few isolates which may or may not represent an entire population of microorganisms in a sample. The individual isolate assays have the advantage of assessing the resistance of a single isolate against several antimicrobials on one plate while the breakpoint method requires inclusion of just one antimicrobial in a media at any given time. For instance, in disc diffusion testing six or more different antimicrobials can be tested against an organism in one petri dish (Piddock, 1990).

1.1.6 Individual isolate assays for susceptibility testing

Some of the most common and widely recognised assays for AST of individual isolates are the disc diffusion test on agar and microdilution methods in liquid media (Piddock, 1990). These methods will be further discussed in Chapter 3.

1.1.7 Background on interpretive breakpoints

Breakpoints were initially set up to guide therapy (Kronvall et al., 2011). They had to evolve to try and satisfy both the need to guide therapy and the need to detect biological resistance. Moreover, due to a lack of harmonisation of breakpoints between different countries and within the same country, AST methods had to go through major phases of development including setting up appropriate breakpoints that can help in objectively determining resistant organisms (Kronvall et al., 2011). Minimum inhibitory concentration (MIC) breakpoints for microbroth dilution assays were formed based on various aspects including pharmacokinetics and microbiological data. These MIC were then translated into zone diameter breakpoints for agar-based assays using the regression line between MIC values and inhibition zone diameters based on multiple bacterial species (Turnidge and Paterson, 2007). The calculated zone diameter breakpoints for an antimicrobial agent were intended for use irrespective of bacterial species. The two major standards were CLSI and EUCAST. The method was improved by the introduction of the use of the error rate bound method of Metzler and DeHaan (1974). Subsequent studies suggested that interpretation criteria should be species-specific for improved accuracy (O'Brien et al., 1977). Chapter 3 discusses the use of different breakpoints to determine resistance of individual isolates.

1.2 Research Objectives

Two major objectives of this study include;

1. To determine the prevalence of antimicrobial resistant coliforms across four poultry production systems in Arusha and Moshi.
2. To determine the prevalence of AMR *E. coli* isolates using clinical breakpoints and epidemiological cut-off value methods.

CHAPTER TWO

PREVALENCE OF ANTIMICROBIAL RESISTANT COLIFORMS IN POULTRY CLOACAL SAMPLES ACROSS FARM TYPES IN MOSHI AND ARUSHA DISTRICTS

2.1 Introduction

Poultry, especially chickens, are the most commonly kept and most numerous livestock species in the world (Perry, 2002; Morek et al., 2010) Backyard chickens are widely distributed in rural and peri-urban areas where they play important roles in income generation, food production and social interactions (Minga et al., 2001; Thornton, 2002; Morek, 2010). In Tanzania, the per capita consumption of poultry meat is estimated at about 15 kg per annum (Tanzania Country Brief, 2016). It accounts for about 25% of the meat demand. Backyard chickens make up over 70% of the total chicken population and supply the vast majority of the poultry meat and eggs for residential markets (Covarrubias et al., 2012). In 2016, the chicken population in Tanzania consisted of 69 million birds, of which 37 million were backyard and the remaining 32 million were commercial, including 24 million broilers and 8 million layers (Tanzania Country Brief, 2016). Poultry production for commercial purposes is mostly practised in urban and peri-urban areas. The Tanzania National Panel Survey (NPS) of 2008 - 2009 showed that only 10% of rural farm households are market oriented (i.e. sell more than 50% of their produce (Covarrubias et al., 2012) but consumption patterns are changing and production systems are becoming more intensive (Sindiyo et al., 2018; Wilson, 2015).

In Tanzania, chickens are reared under different production systems, some of which involve scavenging (free-range and semi-intensive systems), intensive systems which mainly involve indigenous breeds and broilers, which are imported meat-specific breeds (Sindiyo et al., 2018). The free-range system (also referred to as extensive) is the dominant system in most rural areas and has been practised for many years (Sonaiya, 1990; Kitalyi, 1998). It requires minimal resource input and is generally considered secondary to other agricultural activities by farmers. This type of production has many limitations, including high disease prevalence, exposure to predators, poor nutrition and poor growth rate (Mwalusanya et al., 2002; Mutayoba et al., 2012; Goromela et al., 2006). The birds are owned mostly by women and children for home consumption, small cash income, social and cultural activities. Birds are left to scavenge around the homesteads during the day, feeding on household leftovers, waste products as well as insects, worms, seeds and plants. The birds are not regularly provided with water and other inputs such as supplementary feeds,

housing or treatment (Goromela et al., 2006). In semi-intensive systems, birds are partially confined within enclosures made from local materials, in overnight shelters or in fenced yards (Sonaiya, 1990). In this system, there is a regular provision of water, grains and household wastes, vaccination but little medication to control disease and parasites. There may be exchange of cockerels between the farms (Sonaiya, 1990; Goromela et al., 2006). Because of better management, compared to free range systems, mortality is less and there is greater egg production (50 to 150 per hen per year) and higher growth rates (10 to 20 gram/day) (Goromela et al., 2006). Products are used for home consumption, family cash income and as a source of micro-credit.

Confined systems (intensive) with indigenous chickens are operated by some families, particularly those living in peri-urban and urban areas where there are markets for eggs and meat, while broiler systems are mostly businesses rather than family-run operations. Broiler farmers rear imported breeds predominantly for meat production. The choice of production system depends on the availability of resources and inputs needed for a particular production system (Guèye, 2000). Intensive and broiler poultry keepers are more likely to use the recommended standard practices such as appropriate housing, feeding, and disease control programmes, and yield around 250 to 300 eggs per hen per year and growth rates of 50 to 55 grams/day (Sonaiya, 1990).

The wide use of antimicrobials in poultry production to control and manage disease in chickens, particularly in semi-intensive, intensive and broiler systems may select for antimicrobial resistant commensal organisms in chickens. Faecal *E. coli* is often considered as a good indicator for selection pressure imposed by an antimicrobial use and it is common in the chicken intestine (Alekshun and Levy, 2006). This study, therefore, investigates the prevalence of AMR in coliforms in extensive, semi-intensive, intensive and broiler farm types, focusing particularly on AMR profiles in commensal *E. coli* using phenotypic methods coupled with species confirmation using uidA PCR. *E. coli* is the organism of choice because it can easily acquire AMR when exposed to antimicrobial agents. In addition, *E. coli* can be found in almost all ecological niches and are important human, environmental and animal reservoir of AMR genes. *E. coli* is also capable of transferring these resistance genes to other

bacteria, including pathogenic bacteria. Consequently, *E. coli* is of substantial clinical importance in both human and veterinary medicine (Agersø et al., 2014).

2.2 Materials and methods

2.2.1 Study locations

The study was conducted in urban Arusha and Moshi districts (Figure 2.1). The two urban districts were selected because of the presence of the four production systems of interest: extensive, semi-intensive, intensive and broiler. Arusha urban district (or Arusha City Council) is one of the seven districts of the Arusha region of Tanzania and contains the district and economic capital. Moshi urban is one of the seven districts of the Kilimanjaro region of Tanzania and contains the regional capital, Moshi.

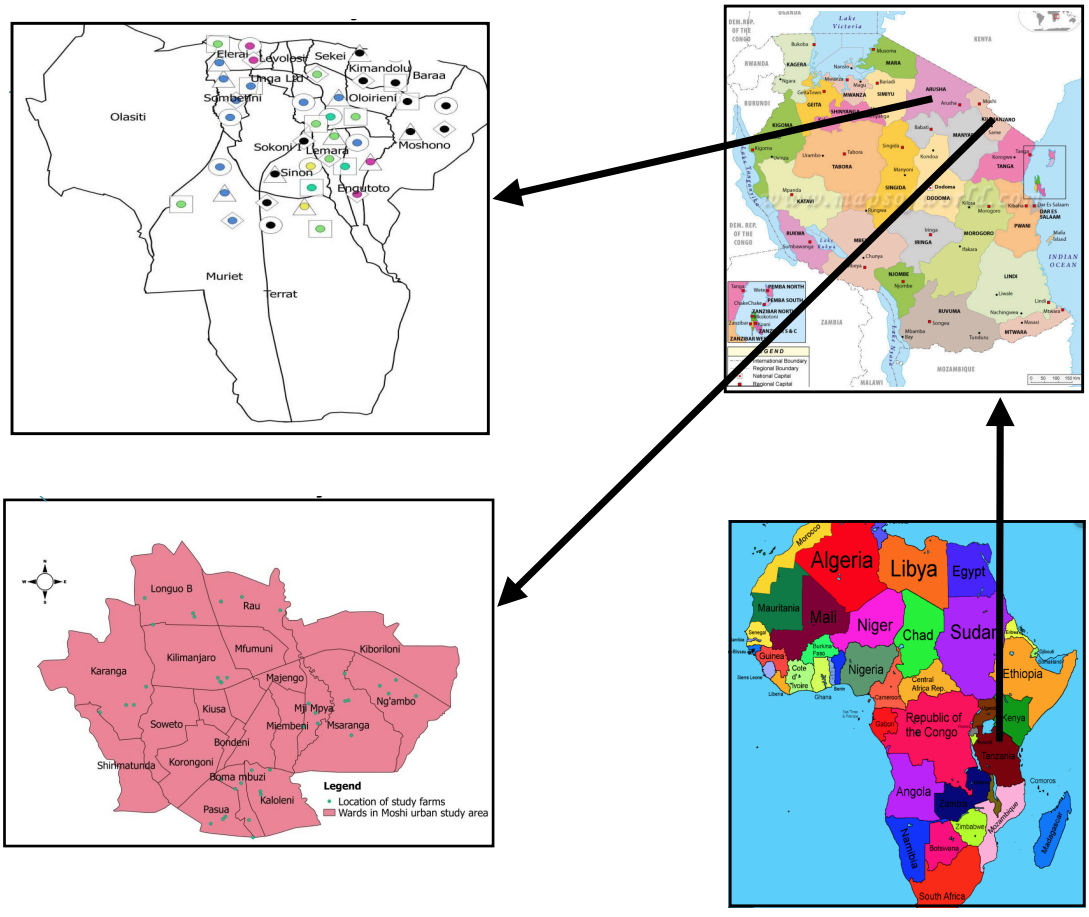

Figure 2.1 Map of Africa (bottom right), showing location of Tanzania (top right), including Arusha urban district (top left) and Moshi urban district (bottom left). Location of the study farms is marked with symbols and dots (top left) and green dots (bottom left). Source: Map of United Republic of Tanzania (2014) and map of Africa (2016) from http://thefutureofeuropes.wikia.com (maps allowed for public use). Site accessed on 30/8/2018. Map of Arusha reproduced from Sindiyo et al. (2018).

2.2.2 Categorization of farming systems

Farm types were categorised using the following criteria: type of chicken; supplementation of feed; degree of confinement of the chickens; use of veterinary services; use of labour; flock size and number of poultry houses. Farm types are described below with examples shown in Figure 2.2.

2.2.2.1 Extensive systems

Extensive systems were characterised by indigenous chickens kept under free-range conditions. Chickens obtained food through scavenging around the homestead. These systems involved little input in terms of management time, provision of water, feeding, housing and disease control. Natural breeding was common on the farm. Flock sizes ranged from 5 to 50 birds.

2.2.2.2 Semi-intensive systems

Semi-intensive systems were characterised by indigenous chickens mostly contained in enclosed facilities, but also, at some point during the day, released to scavenge. Thus, the system involved partial confinement of the birds. Supplementary feeding was involved, for example via provision of kitchen waste containing remnants of household meals such as bananas or cassava. In addition, commercial supplements were regularly incorporated into feed that was mixed by farmers on site from independently bought ingredients. Veterinary services were provided when necessary. Flock sizes ranged from 50 to 200 birds.

2.2.2.3 Intensive systems

Intensive systems were characterised as being high-input urban and peri-urban commercial systems in which indigenous chickens were reared for meat and egg production. Crossing of local breeds was common practice in order to improve the yield of poultry products (meat and eggs). Feed and feed supplements were provided. Farms obtained feed and foundation stocks from large-scale commercial poultry farms. Chickens were confined full time in constructed facilities. The system involved the use of veterinary services for prevention and management of disease and involved full-time labour. Flock sizes ranged from 50 to 1000 birds.

2.2.2.4 Broiler systems

Broiler operations were characterised by the use of imported broiler breeds and focused exclusively on meat production. Crossing was done regularly to enhance the breeds and ensure a high production of poultry meat. Broiler systems comprised highly intensified units in which chickens were confined full time in constructed facilities. As for the intensive systems, broiler systems involved the use of commercial feeds, supplements and extensive use of veterinary services. The system invested in full-time labour and the flock sizes exceeded 200 birds.

Figure 2.2 Examples of poultry production systems in the northern zone of Tanzania (pictures taken during field work): intensive broiler (top left); semi-intensive (top right); intensive indigenous (bottom left) and extensive (bottom right).

2.2.3 Selection of wards and farms

A list of administrative wards containing 25 and 21 wards for Arusha and Moshi, were obtained from the Arusha and Moshi municipal councils, respectively. Subsets of wards containing all four production systems were identified, resulting in 18 wards for Arusha and 12 wards for Moshi. Ten wards for each district were then randomly selected from these subsets. The selection was done by writing the name of each of the 18 wards in Arusha and 12 wards in Moshi on pieces of paper and folding to avoid disclosure and prevent bias during selection. Separately for Moshi and Arusha, the pieces of paper were randomised by tossing in a bowl. Then, five individuals each picked a piece of paper from the bowl without replacement. This procedure was done independently for Moshi and Arusha and repeated to generate a final list of 10 wards for each district. Ward locations and farm locations are shown in Figure 2.1.

2.2.4 Study design and sample size

A cross-sectional study was conducted in which a target number of 100 cloacal swabs would be collected from per farm type (n = 4) per district (n = 2), or 800 samples in total. Use of 100 samples allows for estimation of prevalence of 50% (worst case scenario for sample size calculation, for any lower or higher prevalence the number of samples needed would be smaller) with a confidence level of 95% and precision of 10%. The confidence level is described as "the probability of accepting the null hypothesis when it is true" and the precision of the estimate (or acceptable error in the estimate) is described as "half the width of the desired confidence interval". The confidence interval is the interval around a parameter estimate (here: the prevalence), such that "if an experiment was repeated an infinite number of times, the interval generated would contain the true value of the parameter in the proportion of trials set as confidence level" (here: 95%) (http://epitools.ausvet.com.au/). Per production system, one farm was selected (randomly or purposively) in each ward, followed by a convenience sampling of 10 birds in each of the farms. Random selection was conducted if there were more than 10 farms of a specific production system in a given ward. Each sampling day involved visiting a single ward and sampling chickens from farms in all four production systems.

The selection of chickens in non-intensive production systems (i.e. extensive and semi-intensive) was conducted without regard to the age of chicken, whereas in intensive production systems (i.e. intensive and broiler systems) the selection of chickens depended on how chickens were sorted in their cages by age. For instance, the majority of farms would separate chickens with an age gap of two weeks into different cages. To maximise diversity in our selection, we chose cages corresponding to different ages and randomly picked 3 chickens from each cage (making 9 samples) and then selecting the 10^{th} chicken to be sampled arbitrarily from any cage.

2.2.5 Collection and laboratory handling of cloacal swabs

Cloacal swabs were collected using Amies swabs (MML Diagnostics, Troutdale, OR). The process involved inserting a swab into the cloaca of the chickens and gently swabbing the mucosal wall taking any fluid or faecal material around the cloaca (Figure 2.3). The swabs (Figure 2.4) were transported

in ice packed cool boxes to the Kilimanjaro Clinical Research Institute (KCRI) where they were stored at –80 ˚C in 1000 µl mixture of 85% Brain Heart Infusion and 15% glycerol.

Figure 2.3 From left to right, poultry cloacal sample collection, labelling, temporary storage in cooler boxes and shipment to the zoonosis laboratory at Kilimanjaro Clinical Research Institute.

Figure 2.4 Poultry cloacal swab in a transport media (Amies swab)

2.2.6 Laboratory procedures for isolation and enumeration of coliforms on MacConkey agar

Screening was conducted to identify and enumerate the overall coliform population and resistant coliforms contained in cloacal samples using MacConkey agar with or without antimicrobials, respectively, using a modified breakpoint plate protocol based on Caudell et al. (2018). MacConkey agar as a selective and differential medium contained bile salts and crystal violet that selectively inhibit the growth of gram-positive bacteria such as *S. aureus*, whilst lactose and a neutral red indicator in the medium allowed the differentiation of gram-negative bacteria based on lactose fermentation. Coliforms are generally capable of fermenting lactose and producing acid, forming pink to red colonies, whilst other gram-negative bacteria such as *Salmonella, Shigella and Pseudomonas* spp. are incapable of fermenting lactose, leading to the formation of white colonies. Antimicrobial susceptibility breakpoint assays were used to identify coliforms that were resistant to particular antimicrobial compounds at specific concentrations (as described below) in accordance with CLSI standards.

Media preparation of MacConkey agar (Oxoid Thermofisher, Basingstoke, UK) was conducted by suspending approximately 50 grams of dehydrated media

in 1000 ml purified/distilled water and heating to boiling point to dissolve the media completely. The dissolved media was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes and cooled to 45 - 50 °C. Working stocks of the antimicrobials were prepared at specific concentrations (tetracycline (16 µg/ml), ciprofloxacin (4 µg/ml), ceftazidime (8 µg/ml) and imipenem (4 µg/ml)) and mixed well with cooled but still liquid media before pouring into sterile petri plates (Greiner Bio-One, cat. no. 662160).

The cloacal swab samples, which were stored in a mixture of Brain Heart Infusion (BHI) (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), were thawed at 2 °C overnight. Samples were homogenised and a volume of 50 µl was aliquoted and mixed with 450 µl of maximum recover diluent (MRD) (Oxoid Thermofisher, Basingstoke, UK), and vortexed. The mixture was plated using a spiral plater (Spiral System, Inc. Cincinnati, Ohio) which was set to dispense 50 µl of the mixture in a logarithmic dilution on plain MacConkey, as well as onto MacConkey plates with antimicrobials. Briefly, this method involved inoculation of the liquid sample on a rotating MacConkey agar with the spiral plater. The volume of the cloacal sample suspension was dispensed as the dispensing stylus moved from the centre to the edge of the rotating plate. This was followed by incubation at 37 ± 3 °C overnight.

Lactose fermenting colonies were identified as pink colonies (Caudell et al., 2018) on the media (Figure 2.5) and assumed to be coliforms (*E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.), even though exceptions to that classification may exist. Colonies that appeared yellow or white were not enumerated. Coliforms that grew on MacConkey plates with antimicrobials were considered resistant. After incubation, enumeration of coliforms was conducted on plain MacConkey agar and MacConkey agar with antimicrobials using the spiral plater grid method that has been standardised for use at KCRI. The method involved placing a grid on each plate, positioned on a level surface and adjusted so that the centre of each grid matched that of the plate on the viewer. This position was maintained while counting colonies. The grid was divided into segments in which colonies were enumerated from the outer edge of the segment toward the centre allowing the corresponding

microbial concentration (bacterial count/ml) on the whole plate to be estimated according to the KCRI standardized protocol.

2.2.7 Collection and storage of plate sweeps

Sweeps of bacteria were taken from plain MacConkey agar plates and archived at -80 °C. Aliquots of the plate sweeps were later prepared for shipment to Glasgow where further analysis was conducted. Plate sweeps were only collected if a plate contained pink colonies, i.e. presumed coliforms. In brief, the process involved demarcating the plate into two segments using a marker on the lower surface of the plate. Through the use of cotton swabs, sweeps were taken from one segment by placing the cotton swab at the centre of the plate where growth was most concentrated and then carefully dragging while rolling the swab across the surface towards the edge of the plate. Two vials of plate sweeps were taken from each plate. One vial was archived and the other was stored for shipment to Glasgow. Swabs were stored in tubes containing 15% glycerol mixed with MRD media, organised in cardboard boxes and stored at -80 °C. Plates sweeps were shipped frozen on dry ice to the One Health Research in Bacterial Infectious Diseases (OHRBID) laboratory at Glasgow University where further work was conducted as detailed below.

2.2.8 Additional testing in Glasgow

Further work in Glasgow was conducted to assess whether results found in Tanzania based on the breakpoint plate method were reproducible. For this process, one sample per farm was selected from each of the 74 farms (of 79 sampled) on which coliforms were found.

Plate sweeps from the selected samples that were shipped from Tanzania and stored in Glasgow at -80 °C were processed as follows. The steps involved taking 40 µl of the plate sweep and inoculating in Luria-Bertani broth (Oxoid, Canada) and then incubating overnight at 37 °C. The optical density (OD) of the resulting broth was measured by adding 100 µl of the culture to a microwell plate then measured using a spectrophotometer at 570nm (OD570) and adjusting until the desired concentration was reached (i.e. 0.5 MacFarland). From the desired broth concentration, serial dilutions from 10^{-1} to 10^{-7} of culture suspension were prepared on a microwell plate. Per dilution, three drops of 20

µl were inoculated on plain MacConkey agar (MAC) and MacConkey agar with a breakpoint concentration of tetracycline (TET) (Oxoid Thermofisher, Basingstoke, UK) and incubated overnight at 37 °C. The purpose of the serial dilutions was to achieve a concentration at which colony forming units could be enumerated on the plates, whereby the ideal number of colonies per drop would be between 5 and 50 to allow for accurate enumeration.

In Tanzania, colony counts on breakpoint plates were determined for suspensions of cloacal swabs whereas colony counts on breakpoint plates in Glasgow were determined for dilutions of plate sweeps that were harvested from MacConkey plates in Tanzania. Thus, direct comparison of colony counts was not meaningful. As an alternative means of comparison, the ratio of tetracycline resistant coliforms to total coliforms was calculated for cloacal swab suspensions and plate sweeps as follows:

$$\text{Ratio} = \frac{\text{Log (cfu/ml) on tetracycline plates}}{\text{Log (cfu/ml) on plain MacConkey plates}}$$

2.2.9 Statistical analysis

Statistical analysis was conducted using the computing environment (R version 3.6.1). A Chi-squared test was used to test the association between presence of coliforms and district (i.e. Arusha and Moshi), farm type and antimicrobial classes (tetracycline, ciprofloxacin, imipenem and ceftazidime). The coefficient of determination (R-squared value) was used to provide the proportion of variance in the dependent variable (coliform counts resistant to one antimicrobial) explained by the independent variable (coliform counts resistant to another antimicrobial). This quantity was used to assess the strength of the relationship between counts of coliforms resistant to different pairs of antimicrobials. Pearson's correlation coefficient was used to assess correlation between the proportions of tetracycline resistant isolates derived in Tanzania and Glasgow. Distributions of counts were compared between groups (farm type, district, and antimicrobial type) using the Kruskall-Wallis test and medians were compared using the Mann-Whitney U Test.

2.3 Results

2.3.1 Prevalence of coliforms

Out of 800 target samples, 746 cloacal swabs were collected (Table 2.1). This was fewer than planned because some farms lacked a sufficient number of chickens to sample 10 chickens per farm. In addition, cloacal samples were collected from only 19 extensive farms in Moshi and Arusha urban districts, out of 20 target farms, as one farm could not be sampled at the time of sample collection. Thus, samples were collected from 79 of the 80 farms visited. Of the 746 samples that were collected, 648 (86.8%) contained coliforms (Table 2.1) corresponding to 74 of the 79 sampled farms. There was no significant difference in the overall prevalence of coliforms between Moshi (86.4 %) and Arusha districts (87.3%) (Chi-squared = 0.060, df = 1, p-value = 0.81).

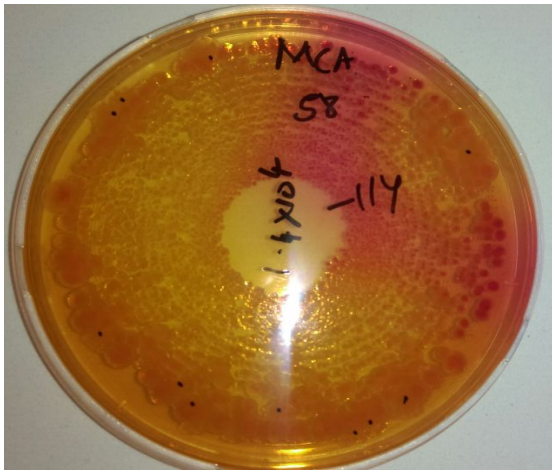

Figure 2.5 Growth on MacConkey agar. Pink colonies (lactose fermenters) were considered to be coliforms.

Table 2.1 Number of cloacal swab samples positive for coliforms (% in brackets) across four farm types in Arusha and Moshi districts in Northern Tanzania.

District	Farm	Number of samples		
		Coliforms absent	Coliforms present	Total
Arusha	Extensive	19 (22.6)	65 (77.4)	84
	Semi Int.	11 (12.4)	78 (87.6)	89
	Intensive	16 (17.8)	74 (82.2)	90
	Broiler	2 (2.2)	88 (97.8)	90
	Total	48 (13.6)	305 (86.4)	353
Moshi	Extensive	11 (11.1)	88 (88.9)	99
	Semi Int.	12 (12.1)	87 (87.9)	99
	Intensive	21 (21.9)	75 (78.1)	96
	Broiler	6 (6.1)	93 (93.9)	99
	Total	50 (12.7)	343 (87.3)	393
Combined	Extensive	30 (16.4)	153 (83.6)	183
	Semi Int.	23 (12.2)	165 (87.8)	188
	Intensive	37 (19.9)	149 (80.1)	186
	Broiler	8 (4.23)	181 (95.8)	189
	Total	98 (13.1)	648 (86.9)	746

There was a difference between farm types in the prevalence of coliforms within the Arusha district (Chi-squared = 17.19, df = 3, p-value < 0.001) and within the Moshi district (Chi-squared = 11.46, df = 3, p-value < 0.01), but no consistent pattern in the prevalence across the farm types could be observed in either district. However, combining data across districts and across non-broiler farmers, showed that broiler farms had significantly higher prevalence of coliforms (95.8%) than the other farm types combined (83.8%) (Chi-squared = 16.56, df = 1, p-value < 0.0001). Between districts, the extensive farm types showed the greatest difference (of almost 11.5%), but this difference was not statistically significant (Chi-squared = 3.5914, df = 1, p-value = 0.058).

2.3.2 Distribution of presence of resistant coliforms by antimicrobial type

Table 2.2 Number of cloacal samples (% in brackets) positive for antimicrobial resistant coliforms by antimicrobial and farm type in Arusha and Moshi districts.

District	Farm type	Tetracycline		Total	Ciprofloxacin		Total	Imipenem		Total	Ceftazidime		Total
		Yes	No		Yes	No		Yes	No		Yes	No	
Arusha	Extensive	59 (90.8)	6 (9.2)	65	40 (61.5)	25 (38.5)	65	39 (60)	26 (40)	65	50 (76.9)	15 (23.1)	65
	Semi Int.	73 (93.6)	5 (6.4)	78	38 (48.7)	40 (51.3)	78	54 (69.2)	24 (30.7)	78	63 (80.8)	15 (19.2)	78
	Intensive	72 (97.3)	2 (2.7)	74	55 (74.3)	19 (25.6)	74	63 (85.1)	11 (14.9)	74	65 (87.8)	9 (12.2)	74
	Broiler	87 (98.9)	1 (1.1)	88	80 (90.9)	8 (9.1)	88	60 (68.2)	28 (31.8)	88	78 (88.6)	10 (11.4)	88
Moshi	Extensive	82 (93.2)	6 (6.8)	88	41 (46.6)	47 (53.4)	88	63 (71.6)	25 (28.4)	88	71 (80.7)	17 (19.3)	88
	Semi Int.	84 (96.6)	3 (3.4)	87	75 (86.2)	12 (13.8)	87	79 (90.8)	8 (9.2)	87	81 (93.1)	6 (6.9)	87
	Intensive	69 (92)	6 (8)	75	53 (70.7)	22 (29.3)	75	57 (76)	18 (24)	75	59 (78.7)	16 (21.3)	75
	Broiler	90 (96.8)	3 (3.2)	93	82 (88.2)	11 (11.8)	93	68 (73.1)	25 (26.9)	93	79 (84.9)	14 (15.1)	93
	Total	616 (95.0)		648	464 (71.6)		648	483 (74.5)		648	546 (84.3)		648

The total number of samples containing resistant coliforms is summarized by region and by farm type in Table 2.2 whilst the variation in prevalence at farm type level is shown in Figure 2.6. Resistance to each of the four antimicrobial types was detected at least once in every extensive farm and every broiler farm, and in 18 of 19 semi-intensive and intensive farms. Neither district had a consistently higher prevalence of resistance. The prevalence of tetracycline resistance across all farm types (95.0%) was higher than the prevalence of resistance to other antimicrobial types (71.6% had ciprofloxacin resistant coliforms; 74.5% had imipenem resistant coliforms and 84.3% had ceftazidime resistant coliforms), and a significant difference between the resistant proportions (Chi-squared = 144, df = 3, p-value < 0.0001) was found. Similar patterns were seen within each district with significant differences between the overall proportions of resistance to each antimicrobial type in Arusha (Chi-squared = 84.0, df = 3, p-value < 0.001) and Moshi (Chi-squared = 62.7, df = 3, p-value < 0.001) and in both districts the prevalence of resistance to tetracycline was greater than the prevalence of resistance to the other antimicrobial types.

There was no consistent increase or decrease in prevalence of resistant coliforms with intensification of farm types (Table 2.2; Figure 2.6). Prevalence of tetracycline resistance was generally high across all farm types. Within any given farm type and district, the prevalence of tetracycline resistance was higher than the prevalence of resistance to any other antimicrobial type. However, there were differences in prevalence of resistance across farm type for antimicrobials other than tetracycline. In Arusha, there were significant differences between farm types for ciprofloxacin (Chi-squared = 37.9, df = 3, p-value < 0.001) and imipenem (Chi-squared = 11.4, df = 3, p-value < 0.01) and in Moshi for ciprofloxacin (Chi-squared = 50.1, df = 3, p-value < 0.001), imipenem (Chi-squared = 11.8, df = 3, p-value < 0.01) and ceftazidime (Chi-squared = 7.88, df = 3, p-value = 0.049). There is evidence of an interaction between farm type and district for prevalence of ciprofloxacin resistant coliforms (Figure 2.6), i.e. a decrease with intensification in Arusha (with the exception of broiler farms) and an increase with intensification in Moshi.

There were no consistent effects of district on the distribution of resistance. The prevalence of imipenem resistant coliforms appears higher in Moshi although the difference is only significant for the semi-intensive farms (Chi-squared = 10.9, df = 1, p-value < 0.001) whilst no effect of district was seen on the prevalence of tetracycline (Chi-squared = 0.042, df = 1, p-value = 0.84), ciprofloxacin (Chi-squared = 0.73, df = 1, p-value = 0.39) and ceftazidime (Chi-squared = 0.01, df = 1, p-value = 0.92) resistant coliforms.

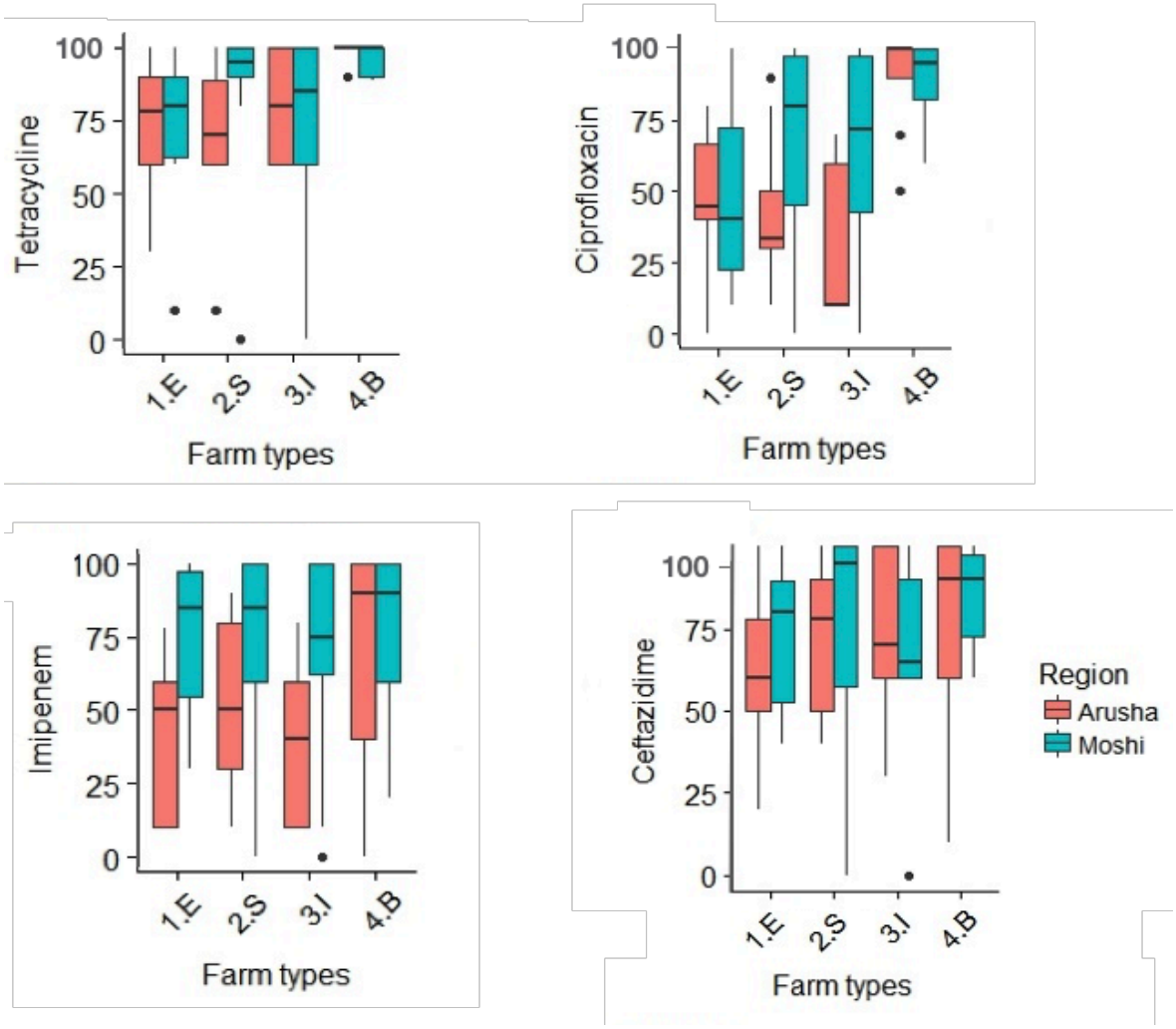

Figure 2.6 Distribution of farm-level prevalence of resistant coliforms in poultry cloacal swabs (n = 648) where up to 10 samples were taken per farm per production system in Moshi district (green) and Arusha district (orange) for the four farm types: extensive (1.E), semi- intensive (2.S), intensive (3.I) and broiler (4.B) and the four antimicrobial types: tetracycline (TET), ciprofloxacin (CIPRO), imipenem (IMI) and ceftazidime (CEFT).

2.3.3 Distribution of counts of resistant coliform per antimicrobial type

2.3.3.1 The distribution of antimicrobial resistance per antimicrobial type within farm types across two districts in northern Tanzania

When comparing data between farm types, the lowest median colony counts across all farm types are for ciprofloxacin resistant colonies (Figure 2.7). Median counts for tetracycline, imipenem and ceftazidime resistant colonies are similar across compounds and farm types for the semi-intensive, intensive and broiler farm types. The distributions of counts are skewed towards lower counts for ciprofloxacin in extensive, intensive and broiler farms, and for ceftazidime in extensive farms.

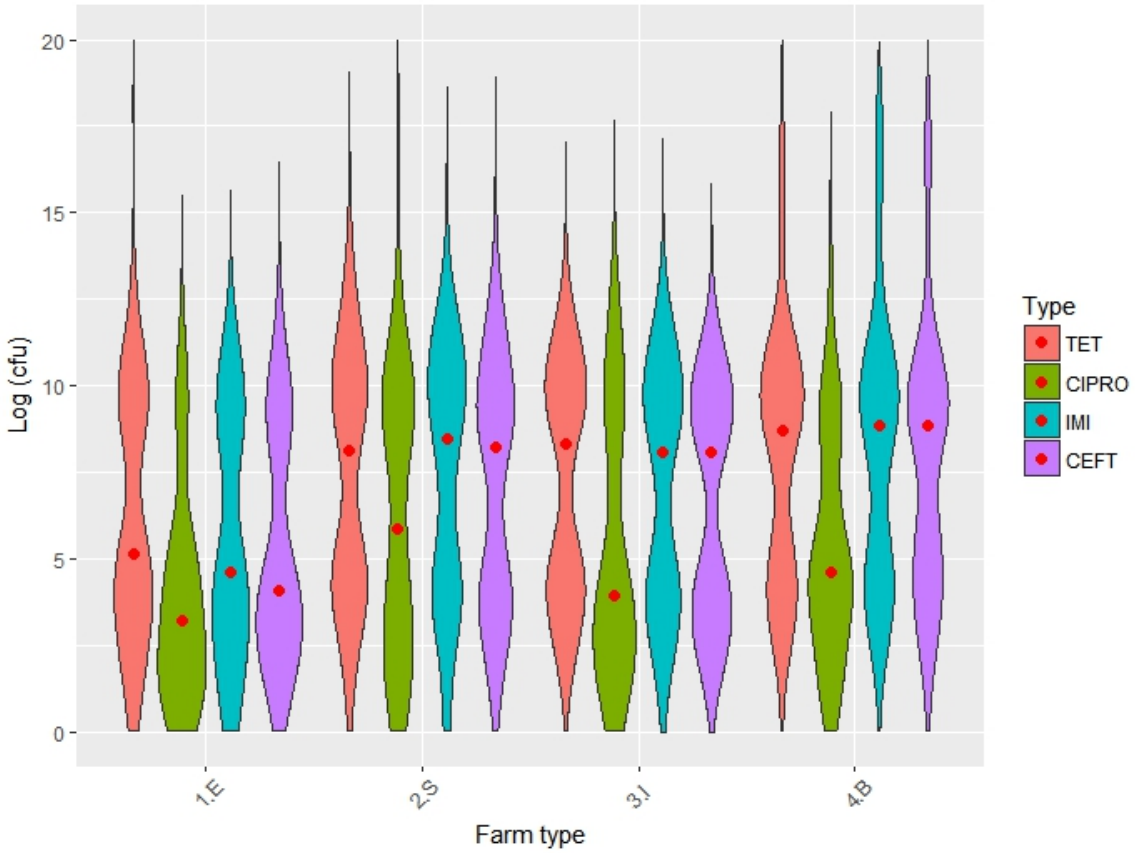

Figure 2.7 Violin plot of coliform colony counts on culture positive MacConkey plates containing one of four antimicrobial compounds, i.e. tetracycline (TET), ciprofloxacin (CIPRO), imipenem (IMI), or ceftazidime (CEFT) and streaked with cloacal swabs from the four farm types: extensive (1.E), semi-intensive (2.S), intensive (3.I) and broiler (4.B). Data combined for Arusha and Moshi districts. Red dots indicate median values. Red dots indicate median values.

Figure 2.8 shows analysis of the count data by district and includes counts from plain MacConkey (total coliforms) as well as the breakpoint plates. The distributions of total and resistant coliform counts were clearly bimodal and

sometimes trimodal, e.g. counts for total coliforms, tetracycline resistant coliforms, imipenem resistant coliforms and ceftazidime resistant coliforms for broiler farms in Moshi district.

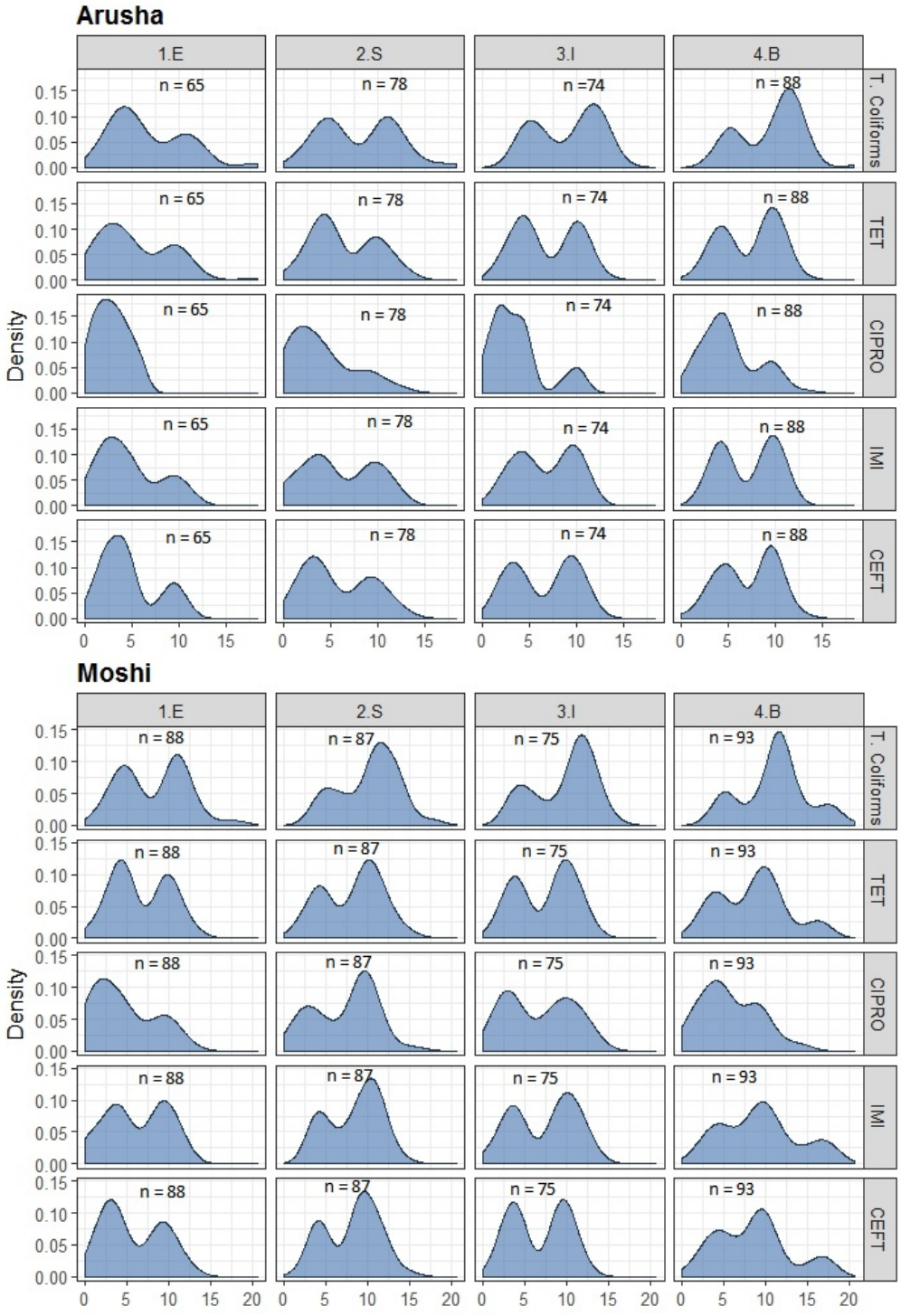

Figure 2.8 Density plots for the total and resistant coliform counts (in log cfu) in cloacal swabs for the four types of antimicrobials (tetracycline (TET), ciprofloxacin (CIPRO), imipenem (IMI), ceftazidime (CEFT)) per farm type in the Arusha and Moshi urban districts. 1.E = extensive, 2.S = semi-intensive, 3.I = intensive, 4.B = broiler.

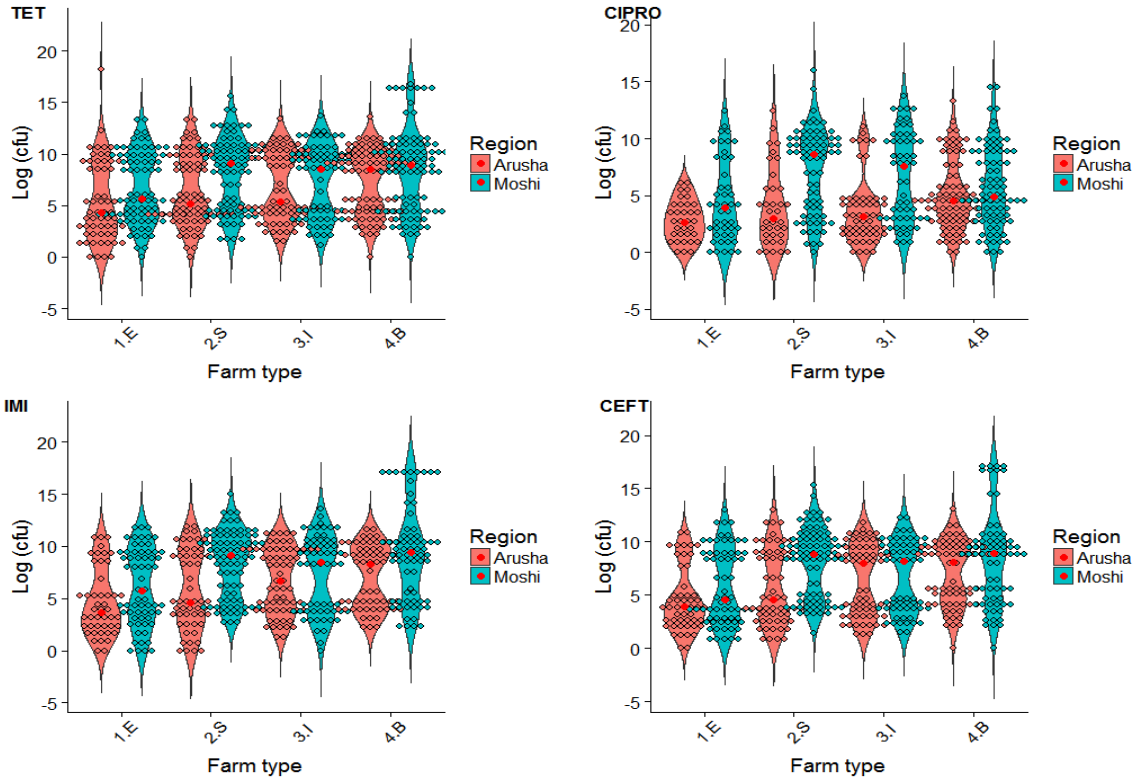

Figure 2.9 Colony counts on culture positive plates containing four antimicrobial types (tetracycline (TET), ciprofloxacin (CIPRO), imipenem (IMI), ceftazidime (CEFT)) and streaked with cloacal swabs for Arusha district (orange) and Moshi district (green) from the four farm types: extensive (1.E), semi-intensive (2.S), intensive (3.I) and broiler (4.B). Red dots indicate median values. Each blue or orange dot represents an individual poultry cloacal swab.

The individual counts contributing to the density distributions shown in Figure 2.8 are shown explicitly in Figure 2.9. Median counts (in log(cfu) ranged from just under 5 to just under 10, with no consistent differences between antimicrobial compounds. These patterns could also be observed for individual antimicrobial types. In general, there was a significant difference between districts for tetracycline (Kruskal-Wallis = 11.2, df = 1, p-value < 0.001), for ciprofloxacin (Kruskal- Wallis = 24.3, df = 1, p-value < 0.0001), for imipenem (Kruskal - Wallis = 19.5, df = 1, p-value < 0.0001) and for ceftazidime (Kruskal-Wallis = 8.15, df = 1, p-value < 0.01).

As well as variation between districts, there was variation in the distribution of coliform counts across farm types. There was a significant difference in the distribution of resistant coliform counts across the four antimicrobial types (Kruskal-Wallis = 192, df = 3, p-value < 0.0001). A significant

difference was seen in the distribution of coliform counts across all the four farm types (Kruskal - Wallis = 43.6, df = 3, p-value < 0.0001).

Most farms exhibited high within-farm variation in coliform counts (Figure 2.10) although the patterns depended on the antimicrobial types as well as the farm identity. Whilst for some farms some antimicrobial types had counts centred around the median (e.g. A-I5), others had highly dispersed coliform counts (e.g. farm A-I6). There were also farms which only had high counts (e.g. M-B10) and farms with only low counts (e.g. A-I8). No consistent patterns or differences between farm type or district could be observed.

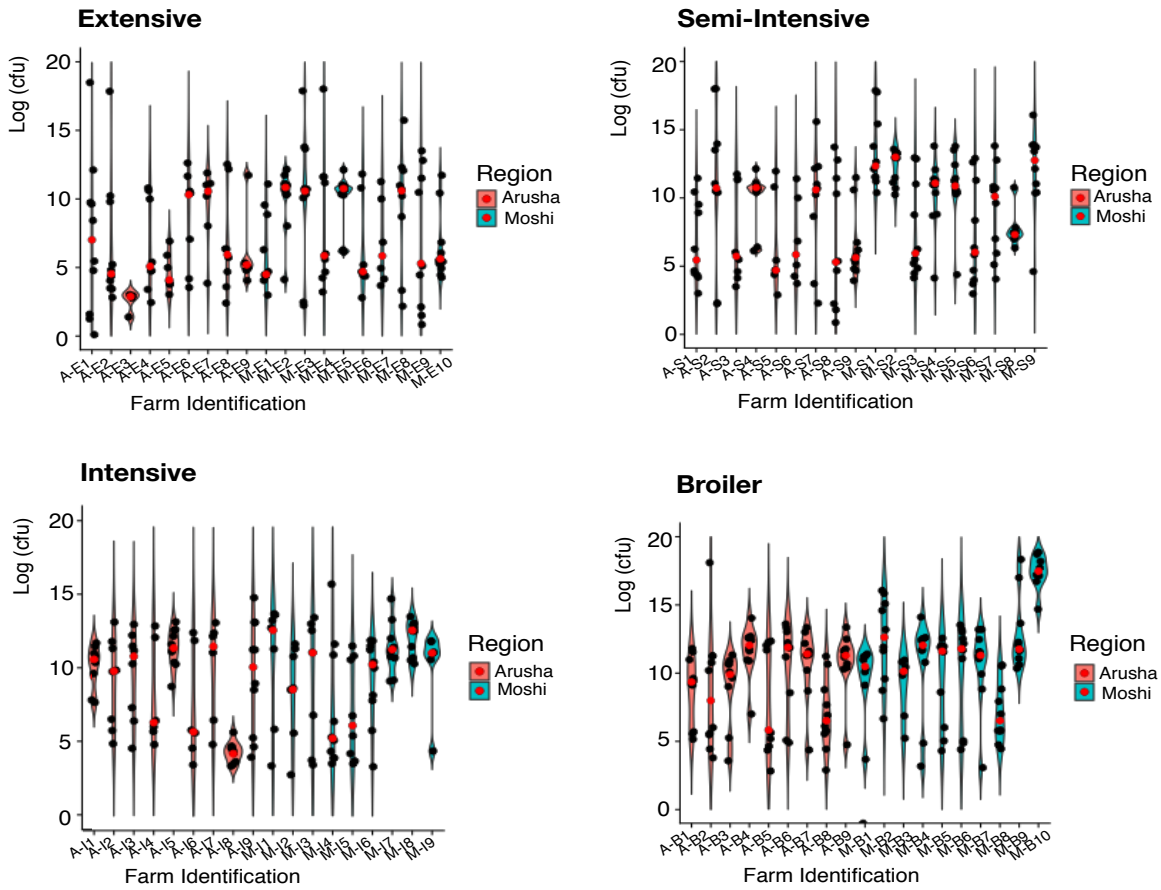

Figure 2.10 Distribution of total coliform counts within farms grouped by farm type (extensive, semi- intensive, intensive and broiler) in Arusha and Moshi districts. Each black dot represents a cloacal swab (n = 648). Red dots represent the median counts for individual farms (up to 10 samples per farm).

Distributions of counts for individual birds within farms were also examined for each of the breakpoint plates (Figure 2.11 through 2.14). Generally, there was high variability in coliform counts within individual farms

with a few exceptions. For example, in counts of tetracycline resistant coliforms (Figure 2.11), farm M-E1, showed substantial variability while farm A-E3 had minimal variability. There were some farm and antimicrobial types for which there were only high counts (ciprofloxacin resistant coliforms on farm M-B10, Figure 2.12) while other farms contained only low coliform counts (tetracycline resistant coliforms on farm A-E3, Figure 2.11). These patterns, however, were not consistent between antimicrobial types. Overall, there was significant difference in the distribution of resistant coliform counts between individual farms for all antimicrobial types; tetracycline (Kruskal-Wallis = 235, df = 73, p-value < 0.0001), ciprofloxacin (Kruskal-Wallis = 346, df = 73, p-value < 0.0001), imipenem (Kruskal-Wallis = 339, df = 73, p-value < 0.0001), ceftazidime (Kruskal-Wallis = 288, df = 73, p-value < 0.0001).

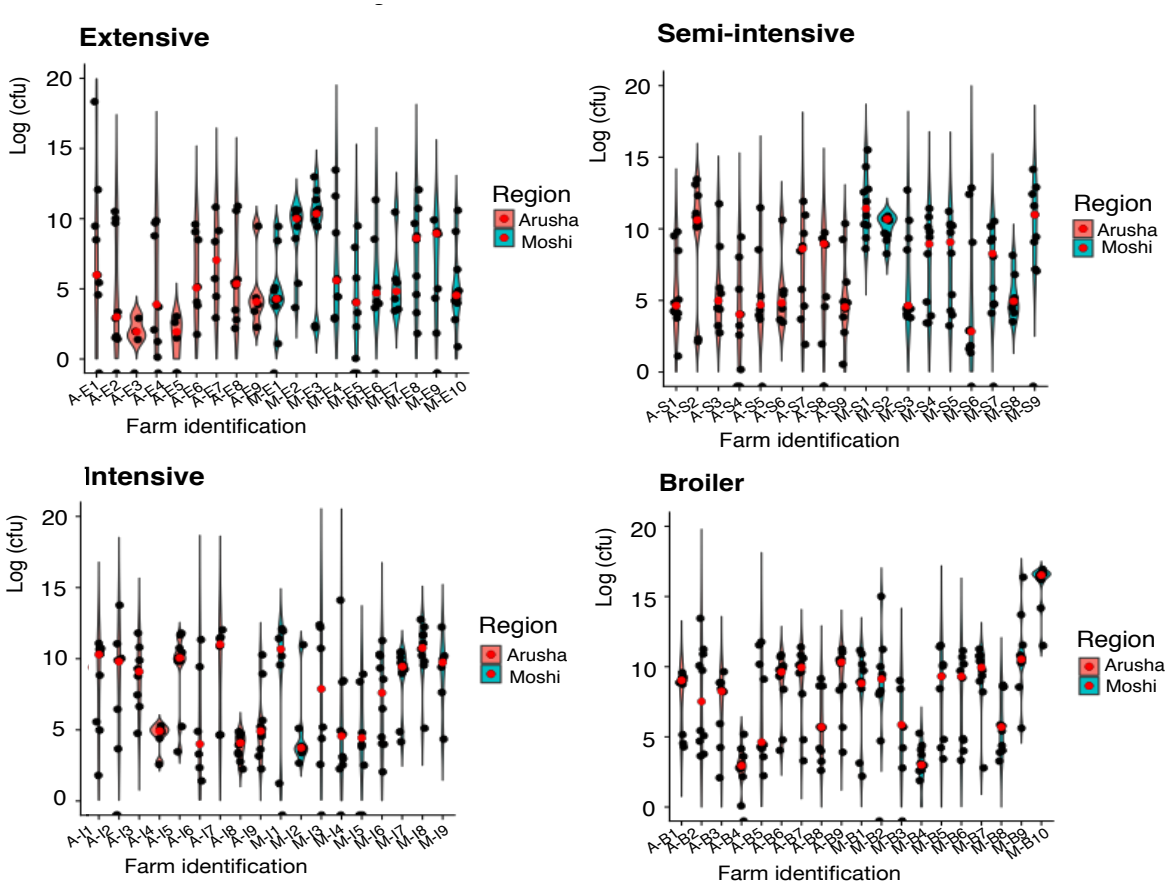

Figure 2.11 Distribution of tetracycline resistant coliform counts within farms grouped by farm type (extensive, semi- intensive, intensive and broiler) in Arusha and Moshi districts. Each black dot represents a cloacal swab (n = 616). Red dots represent the median counts for individual farms (up to 10 samples per farm).

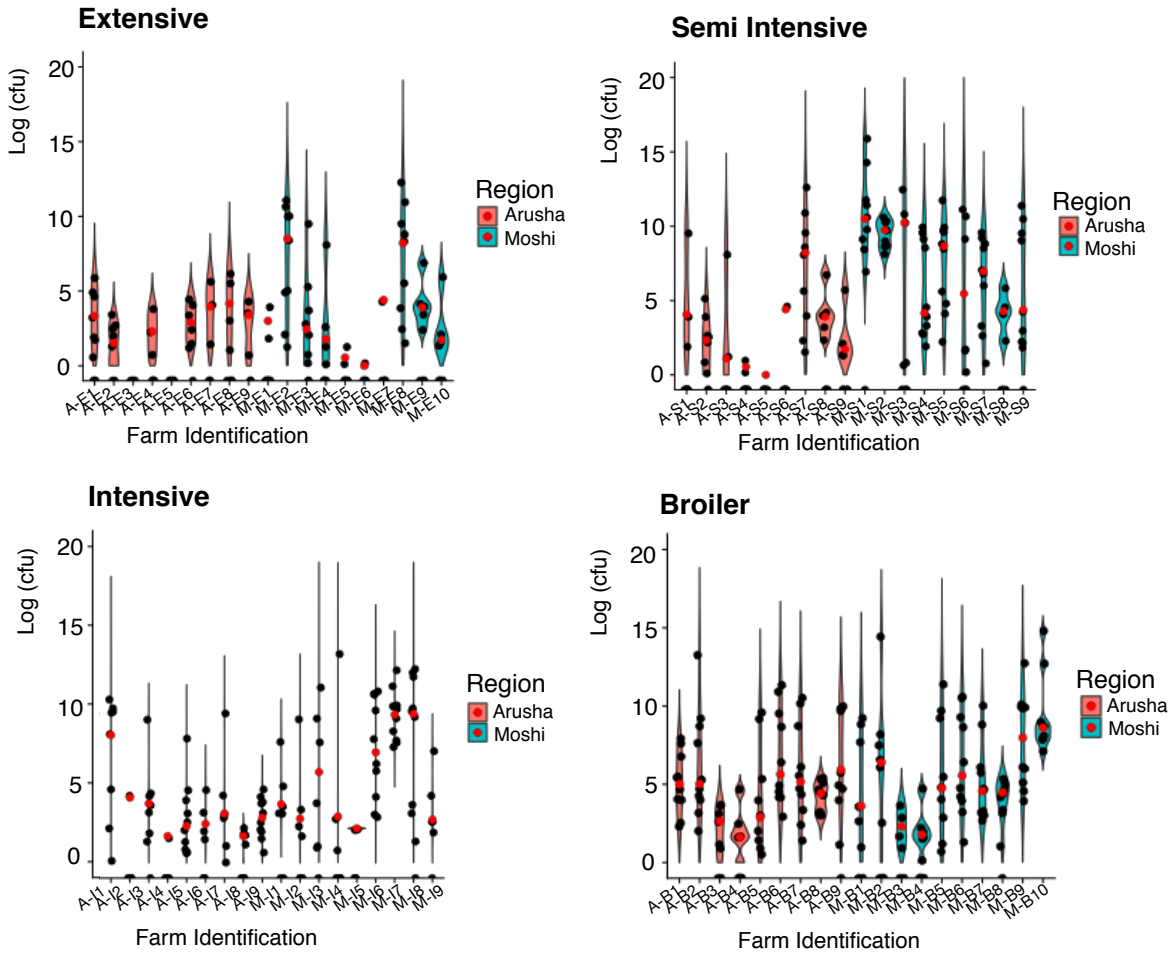

Figure 2.12 Distribution of ciprofloxacin resistant coliform counts within farms grouped by farm type (extensive, semi- intensive, intensive and broiler) in Arusha and Moshi districts. Each black dot represents a cloacal swab (n = 464). Red dots represent the median counts for individual farms (up to 10 samples per farm).

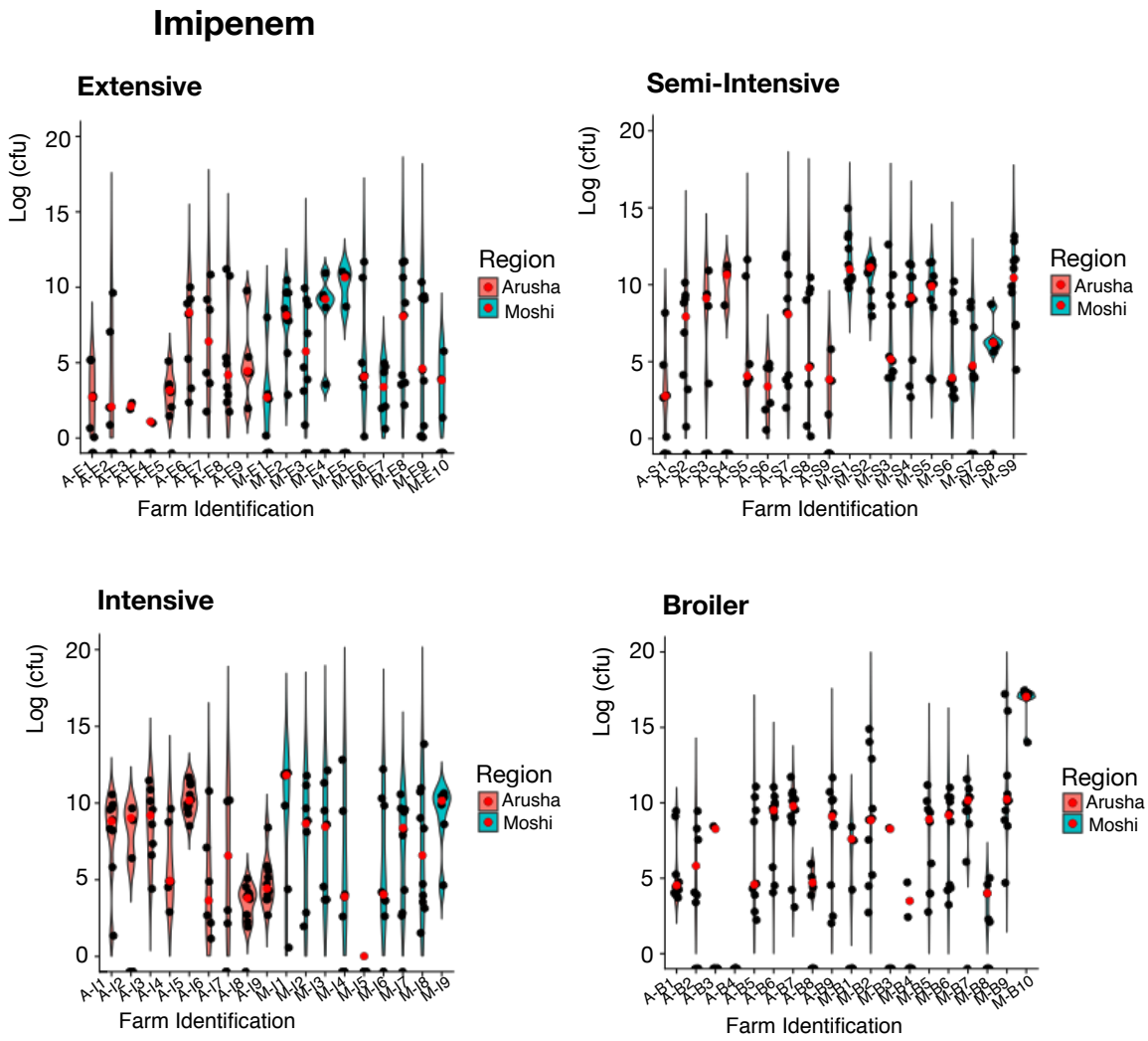

Figure 2.13 Distribution of imipenem resistant coliform counts within farms grouped by farm type (extensive, semi- intensive, intensive and broiler) in Arusha and Moshi districts. Each black dot represents a cloacal swab (n = 483). Red dots represent the median counts for individual farms (up to 10 samples per farm).

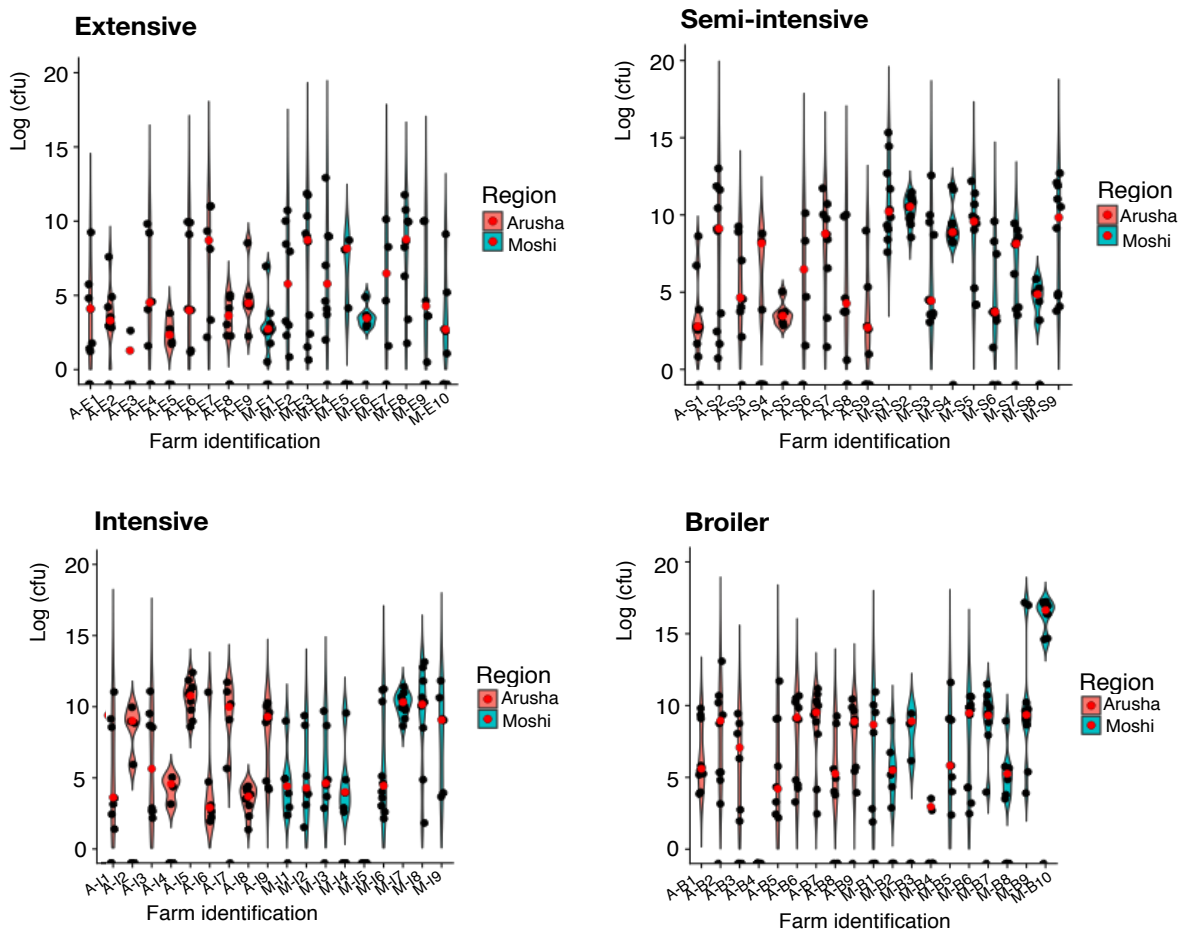

Figure 2.14 Distribution of ceftazidime resistant coliform counts within farms grouped by farm type (extensive, semi- intensive, intensive and broiler) in Arusha and Moshi districts. Each black dot represents a cloacal swab (n = 564). Red dots represent the median counts for individual farms (up to 10 samples per farm).

2.3.4 The relationship between counts of coliform resistant to different antimicrobial compounds

The regression lines (Figure 2.15) illustrate the relationship in each cloacal sample between the number of colony forming units resistant to each antimicrobial pair. The strength of the relationship varies. For example, only 29% of the variation in coliforms counts resistant to ciprofloxacin could be explained by change in the imipenem coliform counts (a weak association) whereas 55% of the variation in coliform counts resistant to ceftazidime could be explained by a change in the count of tetracycline resistant colonies (a strong association).

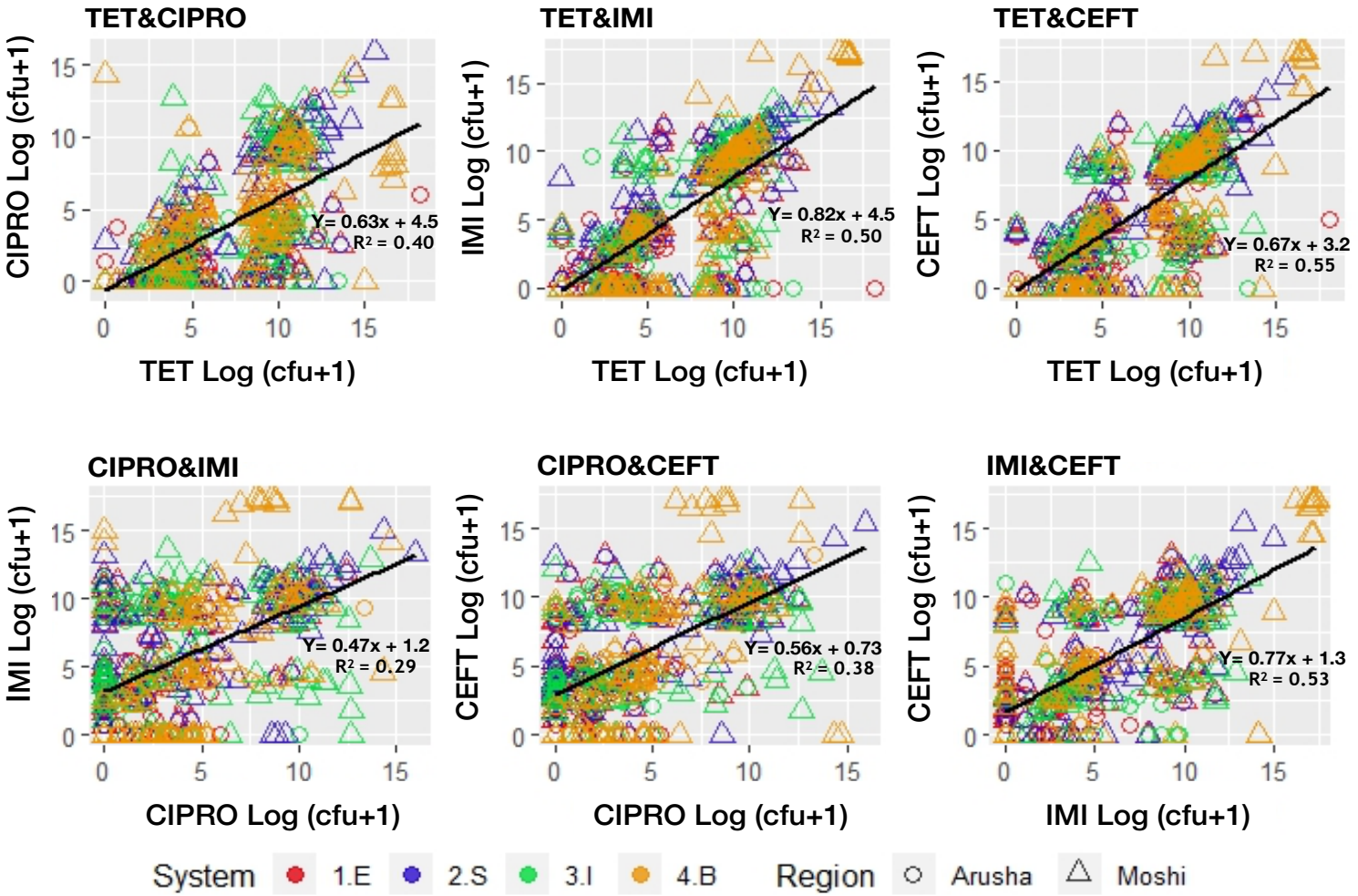

Figure 2.15 The pairwise relationships between coliform counts (data transformed using log(cfu+1) resistant to the four antimicrobial types (tetracycline (TET), ciprofloxacin (CIPRO), imipenem (IMI) and ceftazidime (CEFT)) in Arusha and Moshi districts for the four farm types: extensive (1.E), semi- intensive (2.S), intensive (3.I) and broiler (4.B).

2.3.5 Growth on plain MacConkey agar and breakpoint agar for plate sweeps

For the 74 plate sweeps transferred from Tanzania to Glasgow, growth was observed on plain MacConkey and tetracycline plates (Figure 2.16) whilst no growth was observed on ciprofloxacin, ceftazidime and imipenem plates. Therefore, these data were excluded from further analysis and comparisons were restricted to tetracycline and plain MacConkey counts. Dilutions that were used for analysis on MAC were between 10^{-4} to 10^{-7} whilst dilutions that were used for analysis on TET plates were between 10^{-1} to 10^{-7}.

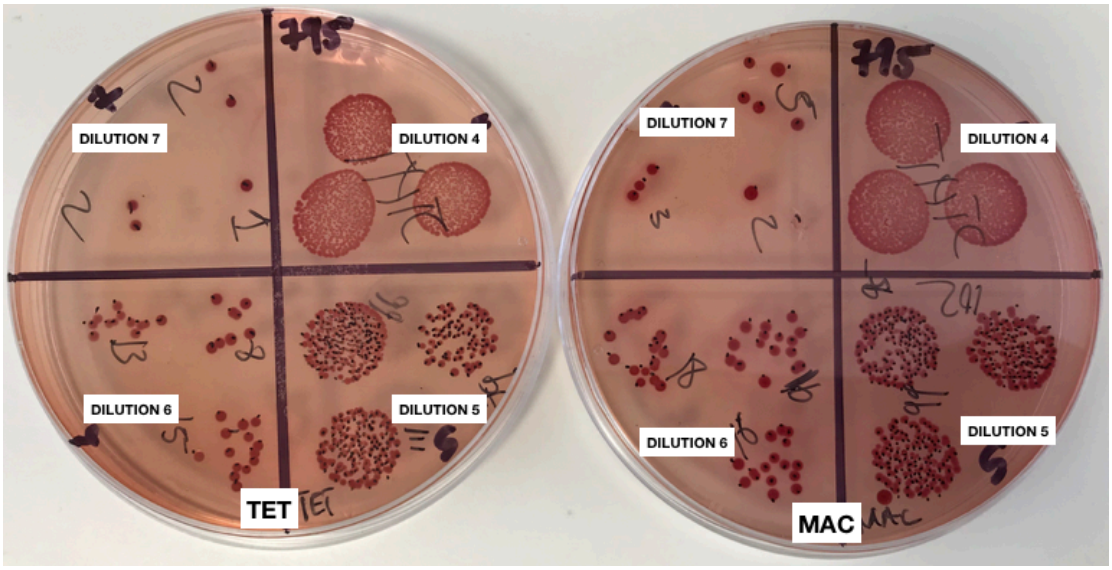

Figure 2.16 Dilution series of coliforms on plain MacConkey (MAC) and a MacConkey plate with breakpoint concentration of tetracycline (TET). Comparison of counts allows determination of the proportion of coliforms that is resistant to tetracycline, enabling comparison of data derived from plate sweeps with those derived from the original samples.

Direct comparison between count data derived in Tanzania and that in Glasgow was not meaningful because the data in Tanzania were obtained from the cloacal swab samples whereas the data in Glasgow were obtained from plate sweeps. As an alternative, the ratio of the number of tetracycline resistant isolates to the number of all coliform isolates was calculated for each sample in the Glasgow and Tanzania derived datasets. There was no association the between the ratios in the 64 samples (R^2 = 0.0385; Figure 2.17).

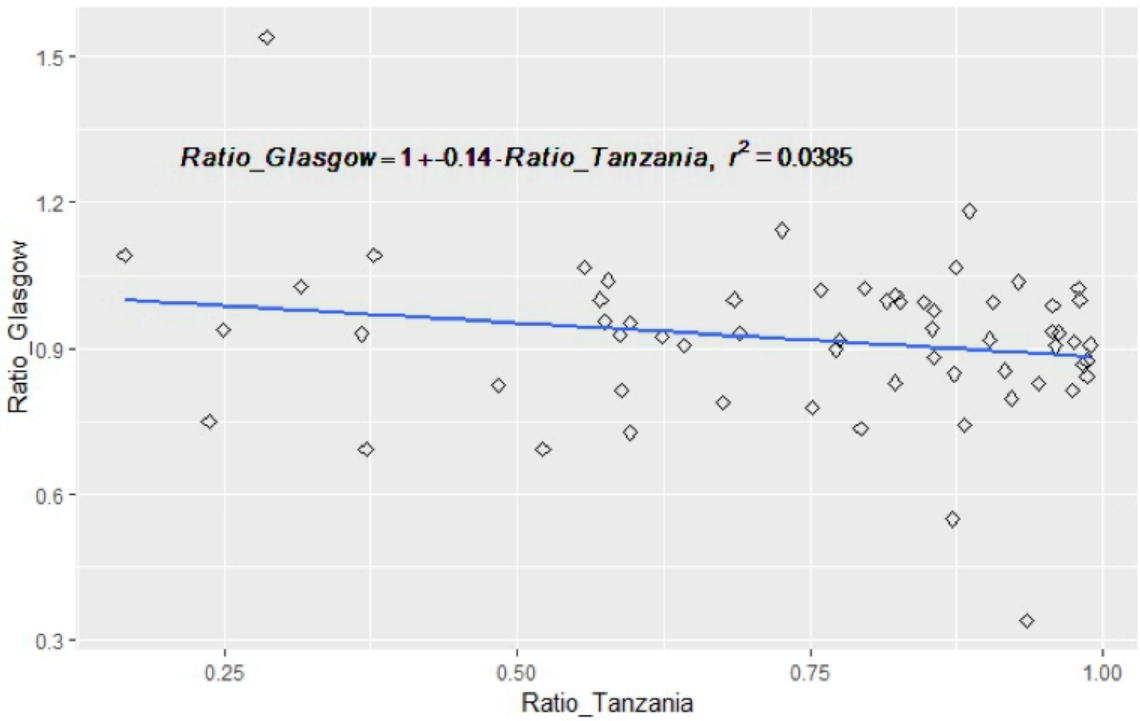

Figure 2.17 The ratio of coliform counts on tetracycline-containing MacConkey breakpoint plates to coliform counts on plain MacConkey plates as determined from suspensions of cloacal swabs in Tanzania and from plates sweeps from the same swabs that were frozen, shipped and retested in Glasgow.

2.4 Discussion

Our findings indicate that chickens can be reservoirs of resistant coliforms. Resistance to each of the four antimicrobials was detected at least once in every farm on which coliforms were found. No significant difference in prevalence of resistant coliforms was seen between Arusha and Moshi districts, which may reflect similar antimicrobial use in these districts. These results differ from other studies on antimicrobial resistance in this area of Northern Tanzania which have found differences in antimicrobial usage patterns and resistance levels between the major ethnic groups in these districts (Caudell et al., 2017; Caudell et al., 2018). However, the Caudell studies investigated rural communities with distinct ethnic groups and livelihoods in each district. Thus, the Caudell work encompassed many types of livestock husbandry whereas the present study focuses on chicken farming as the livelihood of interest.

The prevalence of tetracycline resistance was consistently higher in all farm types in both districts. Unlike the other antimicrobials, tetracycline use is quite common (Sindiyo et al., 2018). Both districts have been reported to use antimicrobials, particularly tetracycline, for non-therapeutic purposes (i.e. prophylaxis and growth promoters) (Caudell et al., 2017; Sindiyo et al., 2018). Tetracycline is one of the most ubiquitous antimicrobials used in animal production worldwide (Dahshan et al., 2015) especially in low-income countries where heavy use is driven by its accessibility, low price, wide spectrum and shelf life. Coupled with its ability to co-select for other resistance, these factors all act in favour of widespread tetracycline resistance (Al-Ghamdi et al., 2000; Kazuki and Tetsuo, 2010). Other countries have also consistently revealed high tetracycline resistance (Fahrenfeld et al., 2014; Tacão et al., 2014; Hamisi et al., 2014; Chee-Sanford et al., 2001; Sunde and Norström, 2006).

Although prevalence of resistance to the other antimicrobials was lower than for tetracycline, apparent resistance to ceftazidime, imipenem and ciprofloxacin was nevertheless very common. Differences in prevalence of resistance to these other antimicrobials could potentially be associated with variation in usage between farms and farming systems in use of those antimicrobials, but data on usage was not collected as part of this study. Resistance to imipenem and ceftazidime was not expected as these

antimicrobials are not used in poultry production (Sindiyo et al., 2018). Reservoirs of cephalosporin resistance were reported in the past in water sources (i.e. tap and open water sources) within these same districts (Lyimo et al., 2016). Moreover, these water sources are commonly used in poultry production (Sindiyo et al., 2018) hence may provide an explanation for the presence of cephalosporin resistance in poultry. By contrast, no direct evidence to date on imipenem use or resistance in poultry or environmental sources has been reported in these districts to help us understand the source of resistance to this compound in poultry. When plate sweeps were harvested in Tanzania and re-tested in Glasgow, the observations of cephalosporin, ciprofloxacin and imipenem resistance could not be reproduced so experimental methods may also have contributed to the apparent resistance. Confirmation of the presence of AMR based on testing of individual isolates would be desirable, although interpretation criteria could still affect prevalence estimates when using such an approach (see Chapter 3).

Our study revealed strong correlations (with an R^2 exceeding 0.5 in some cases) in the number of coliforms resistant to antimicrobial pairs (i.e. tetracycline and ceftazidime; tetracycline and imipenem; imipenem and ceftazidime). Although this data does not confirm presence of the two types of resistance in individual isolates, the high correlation suggests the possibility of co-selective pressure between different antimicrobials. This ability is well established for tetracycline. This is mostly facilitated by a co-transfer of tetracycline resistance genes along with genes responsible for conferring resistance to other types of antimicrobials in the same genetic elements (Al-Ghamdi et al., 2000; Harada and Asai, 2010; Jong-Mi and Gun-Jo 2015) Further investigation is needed to establish whether this phenomenon exists between ceftazidime and imipenem.

The distribution of coliform counts not only showed considerable variation between samples but also revealed bimodal and trimodal distributions. Various studies have attributed bimodal or trimodal distribution of bacterial populations to different forms of resistance mechanisms (Shah et al., 2006; Smith and Christofilogiannis, 2007). Bacteria can form separate populations based on expression levels of resistance (Mazzariol et al., 2000; Mcmurry et al., 1998).

The bimodal distribution could also be attributed to the presence of a varying resistance mechanism (Mazzariol et al., 2000). Wistrand-Yuen et al. (2018) demonstrated that microorganisms have the capacity of developing different mechanisms of resistance when subjected to varying levels of exposure to antimicrobials. When exposed to sub-therapeutic levels of antimicrobials, microorganisms are capable of evolving novel mechanisms that are different from those observed during lethal selection (i.e. treatment). The study also showed that that under sub-therapeutic conditions, microorganism can develop degrees of resistance in a stepwise process over time through accumulation of several resistance mutations, which independently have small effects. There is therefore the possibility that poultry in the current study were constantly exposed to sub-therapeutic levels of antimicrobials, whether from the environment or through direct use of antimicrobials, which may have led to differing mechanisms of resistance or levels of resistance within the same species, generating the tri or bimodal distributions.

A bimodal distribution can also be caused in certain bacterial populations by phenotypic switching between latency (or sometimes slow growth) of bacterial cells. This happens when bacteria are subjected to antimicrobial stress or other environmental stresses. These slow growing cells are called persister cells and it is believed they can maintain this phenotype for a long time (Kussell et al., 2005; Balaban et al., 2004). Thus, genetically identical bacterial populations can respond heterogeneously to antimicrobial treatment, creating multimodal distributions (Radzikowski et al., 2016; Kussell et al., 2005; Balaban et al., 2004). However, further investigations need to be conducted to determine reasons for this phenomenon in the present study.

There was a striking difference in results obtained in Tanzania compared to the results obtained in Glasgow for a selection of 74 samples. First, there was loss (7%) of plate sweeps where growth on MacConkey agar was not seen when plate sweeps were re-cultured in Glasgow. This may have been due to loss of viability during shipping of coliforms. Viability of coliforms can be affected by storage at -20 ºC (Schukken et al., 1989) and temperatures in that range may have occurred during shipment. Second, no resistance was found to ceftazidime, imipenem or ciprofloxacin in the plate sweeps analysed in Glasgow. The

subsequent comparisons therefore focused on a comparison of the ratio of tetracycline resistant colony counts to total colony counts between the Glasgow and Tanzania analysed samples but no correlation was found between the proportion of tetracycline resistant colonies in the Glasgow and Tanzania analyses.

The observed differences could be due to several various reasons. One is the loss of antimicrobial efficiency during shipping of reagents to Tanzania. Some antimicrobials such as imipenem are quite sensitive to temperature fluctuation. Temperature fluctuations or a breach in the cold chain during shipment is quite possible, particularly when ice is used for refrigeration as this melts very quickly in hot countries like Tanzania. For instance, the recommended storage temperature for imipenem is -70 ºC; once this temperature is exceeded and temperature rises above -10 ºC the compound losses its efficiency. Loss of quality may also have occurred for ciprofloxacin and ceftazidime, leading to the apparent resistance observed in Tanzania, but not in Glasgow where quality of reagents would not have been affected by ambient temperatures. Control strains should have been included for each compound in each testing round. This is common in MIC testing but wasn't practiced in this project when focusing on the breakpoint plate method.

2.5 Conclusion

Our investigation suggests the widespread presence of resistant coliforms in poultry in poultry production systems in two districts in Northern Tanzania. Such information is valuable both for poultry farm workers and for the community as a whole as it provides information on the potential risk of exposure to resistant coliforms. More broadly, this information can help aid awareness amongst health professionals and policy makers about the extent to which AMR could be entering the food chain. However, our study also highlights limitations which led to the lack of reproducibility of results between two different laboratories. This study highlights the importance of maintaining appropriate temperatures during shipment of reagents as the potency and viability of fragile compounds such as imipenem could be affected. Although this was not verified in the current study breaks in cold chain could be detrimental to the functionality of certain antimicrobials and could cause them to be less

effective. Quality control strains with known resistance profiles should be included routinely in breakpoint plate investigations.

CHAPTER THREE

ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* ISOLATES FROM POULTRY USING CLINICAL BREAKPOINTS, ECOLOGICAL CUT-OFFS AND NORMALISED RESISTANCE INTERPRETATION

3.1 Introduction

AST is based on measurement of the concentration of antimicrobials that inhibit bacterial growth. This can be done in two major ways, i.e. in liquid media or on solid media and yields data on minimal inhibitory concentrations (MIC) in liquid media or inhibition zone diameters (IZD) on solid media. Regardless of how the data is generated, the laboratory measurements in mg/L (for MIC) or mm (for IZD) need to be interpreted in terms of susceptibility versus resistance. For this classification, thresholds, also known as breakpoints or cut-off values, are needed to distinguish between the categories. There are two major ways of setting those thresholds, i.e. based on clinical breakpoints or using ecological cut-offs (ECOFFS). For clinical breakpoints, *in vivo* data and PK/PD data are used. For ECOFFS, *in vitro* data are used, with thresholds set by international standardisation bodies like the Clinical and Laboratory Standards Institute (CLSI) in the USA or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or by the researcher based on normalised resistance interpretation (NRI). In this introduction, methods of measurement and methods of interpretation will be described, including their strengths and weaknesses, as well as the role of standardisation bodies.

3.1.1 Measurement

3.1.1.1 Minimum Inhibitory Concentrations

By definition, the MIC is the lowest concentration of an antimicrobial which can prevent visible growth of bacteria (Koeth et al., 2004). MICs have often been criticized because of the unnatural conditions through which they are generated (Turnidge and Paterson, 2007). Moreover, they have been reported (Koeth et al., 2004) to lack reproducibility. Repeat results should differ by no more or less than a two-fold dilution but often they are less consistent. Factors that could contribute to this variability include the method used in generating MICs (e.g. broth macro-dilution, broth microdilution, or agar dilution) (Koeth et al., 2004) as well as the choice of medium (e.g. Mueller Hinton, Iso-Sensitest, or Sensitest medium, lot to lot variation, divalent cation concentrations and effects of additives such as blood), inoculum size and concentration, incubation conditions (temperature and duration), and precision in the preparation of different concentrations of the antibacterial being used (Koeth et al., 2004). Thus, MIC are only meaningful when the methods for determining them are

standardised. Despite their limitations, MIC measurements are widely used in clinical microbiology and in research, using standardisation of methods and interpretation criteria to limit variability (see section 3.1.2).

3.1.1.2 Inhibition Zone Diameters

Inhibition zone diameters provide an estimate of the lowest concentration of an antimicrobial, which can prevent visible growth of bacteria on solid media with readings taken in millimetres (mm). The most common methods is the disc diffusion test where paper discs with known quantity of antimicrobials are applied to the surface of a test agar (Kassim et al., 2016). The antimicrobial diffuses away from the disc forming a concentration gradient which inhibits the growth of bacteria at a certain point and hence causes a zone of inhibition (Turnidge and Paterson, 2007). The zone extends until the concentration of the drug is insufficient to inhibit the growth of the organism (Koeth et al., 2004). An alternative method is the antimicrobial gradient method or elipsometer test (E-test), which is based on growth inhibition on solid media by a strip that contains a gradient of antimicrobial concentrations rather than just a single concentration as in the disc diffusion test (Reller et al., 2009). There are several factors that have been reported to influence IZD. The zone of inhibition can be affected by the rate at which the drug diffuses through the agar and the rate of growth of bacteria (Koeth et al., 2004). The rate of diffusion of the antimicrobial through the media depends on the concentration of the antimicrobial, molecular weight of the antimicrobial, solubility properties of the antimicrobial, pH, ionization, incubation temperature and binding on the agar. Aside from the rate of diffusion of the antimicrobial, the choice of agar is also believed to influence IZD (Koeth et al., 2004). The depth of agar recommended for use is 4 mm ± 0.5 mm (Barry and Fay, 1973). Plates with shallow agar may produce false positive results for susceptibility, as the antimicrobial compound will diffuse further than it should. The size of the inoculum is another factor (Koeth et al., 2004; Turnidge and Paterson, 2007). If the size of the inoculum is too small, the zone of inhibition will be larger than it is supposed to be and if the inoculum is large, the zone of inhibition will be smaller. Some assays may be affected by excessive thymidine or thymine, which inhibits the effects of sulphonamide and trimethoprim resulting in smaller zones of inhibition or no zones at all. The incorrect concentration of divalent cations

(calcium and magnesium) will affect the results of aminoglycoside and tetracycline tests against *P. aeruginosa*. Excess cation concentration will result in reduced zone sizes and low concentration will increases zone sizes (Koeth et al., 2004). There are media that have been specifically formulated to prevent the effect of these factors (e.g. Mueller Hinton agar). The method is well suited for water soluble antimicrobials (Turnidge and Paterson, 2007). A limitation of the method is that zones of inhibition do not always have clear or regular boundaries, making measurement subjective and introducing error.

3.1.2 Interpretation and standardisation

3.1.2.1 History and activity of standardising bodies

The CLSI and EUCAST, which was established later, have a common goal of developing standards, formulating guidelines to ensure the quality of laboratory testing, harmonising interpretation, and improving patient care (EUCAST, 2015; Kassim et al., 2016). CLSI was first established in 1968, then known as the National Committee for Clinical Laboratory Standards (NCCLS) (Barry, 2007). The organisation came into existence because of a discussion that was held by 15 organisations that sat with a common goal of finding ways to improve what laboratories are doing for patients and subsequently developing a consensus process for standardisation. The organisation was accredited by the American National Standards Institutes (ANSI) as a voluntary consensus standardisation organisation. In 2005, the name NCCLS was changed to CLSI to encompass and embrace the international focus of this organisation (Lovgren et al., 2007). The CLSI governance structure is comprised of a board of directors in which there is a consensus council and an expert panel for each specialty (e.g. medics, veterinarians, policy makers, microbiologists, educators and pharmacists). Within the consensus council there are document development committees, subcommittees and working groups (e.g. Antimicrobial Resistance Subcommittees). EUCAST was formed in 1997 (Kahlmeter et al., 2015). The current EUCAST structure incorporates different professional bodies from Europe, e.g. the pharmaceutical industry, veterinarians, medics, microbiologists and media manufacturers. Subcommittees within EUCAST (e.g. the EUCAST Veterinary Subcommittee Testing (VetCAST) were formed to cover various aspects of susceptibility testing, including terminology, breakpoint setting and methodology, and to develop guidelines (Kahlmeter et al., 2015). The Steering

Committee makes the final decision and the rest of the consultation is done by the General Committee.

While these two organizations seem to have the same mission, they differ as far as operation, decision making, delivery of outputs and structure are concerned. For example, in contrast to EUCAST the pharmaceutical industry has an upper hand in decision making in CLSI (Kassim et al., 2016). The Food and Drug Administration (FDA) makes decisions on clinical breakpoints before they are adopted by CLSI and this raises significant concerns on the likelihood of bias which favours the interests of the FDA (Kassim et al, 2016). In EUCAST, the industry has no formal position and there is no seat for industry on either the Steering Committee or the General Committee, although there is a working relationship between the industry and EUCAST. The industry is frequently consulted on technical issues and vice versa (Kassim et al., 2016). Moreover, open public consultations are an integrated part of the EUCAST decision-making process. When it comes to financial support, CLSI relies greatly on income from sales of documents, membership dues and the industry while the industry is not allowed to contribute financially to any of the activities offered by EUCAST (Kassim et al., 2016). EUCAST is financed by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and to some degree by the European Centre for Disease Prevention and Control (ECDC) (Kassim at al., 2016). This is done to prevent commercial interests overruling critical considerations (e.g. health-related) in decision making. Due to such financial reasons, CLSI guidelines are only accessible annually through subscription at a cost of US $350 for members and a cost of US $500 for non-members, which may be a problem for microbiology laboratories in resource-poor settings (Kassim et al., 2016). On the other hand, EUCAST guidelines and other information (e.g. MIC distributions, clinical breakpoints, ECOFFS and rationale documents) are openly accessible online. Finally, CLSI may only include breakpoints for antimicrobials that are registered in the US (Kassim et al., 2016). This is in contrast to EUCAST, which has made an effort to include clinical strains from countries with epidemiology that differs widely from Europe and also for new antimicrobials (Kassim et al., 2016). Many countries are now shifting from the utilization of CLSI to EUCAST guidelines. For example, at the ESCMID conference in Amsterdam (April 2019), representatives from Brazil and China presented on

adoption of EUCAST guidelines by their countries (RN Zadoks, personal communication). However, in the future, collaboration between the two organizations will be needed for truly harmonizing breakpoints.

3.1.2.2 Clinical breakpoints

Clinical breakpoints refer to those concentrations that separate strains where there is a high likelihood of treatment success from those bacteria where treatment is bound to fail (Turnidge and Paterson, 2007). These breakpoints are derived from prospective human clinical studies comparing outcomes with the MICs of the infecting pathogen. Clinical breakpoints can also be calculated from knowledge of pharmacodynamic (PD) parameters and the dimension of that parameter that predicts efficacy *in vivo* (Turnidge and Paterson, 2007). Some scientists refer to them as pharmacokinetic/pharmacodynamic (PK/PD) breakpoints, where data that have been generated in animal models are extrapolated to humans by using mathematical or statistical techniques (Kahlmeter et al., 2015; Turnidge and Paterson, 2007).

3.1.2.3 Ecological cut-offs

Ecological cut-offs are threshold concentrations (also called microbiological breakpoints) that distinguish wild type (WT) populations of bacteria from those with acquired or selected resistance mechanisms (Kronvall et al., 2011), with the same rationale applying to IZD data. Data used in deducing this type of threshold are generated from moderate to large numbers (at least 50 observations) of MIC or IZD tests, sufficient to describe the WT population (Kahlmeter etal., 2015). In this context, a WT isolate is defined as an isolate that does not harbour any acquired or selected resistance to the particular antibacterial being examined or to antimicrobials with the same mechanism/site of action (Turnidge and Paterson, 2007). To reduce confusion about the meaning of the term breakpoint, EUCAST proposed the use of the term 'epidemiological (or WT) cut-off value' to replace the term 'microbiological breakpoint' (Turnidge and Paterson, 2007). Epidemiological cut-off values were created to describe biological phenomena of phenotypic resistance rather than simply detecting or classifying the presence or absence of resistance or resistance genes or predicting clinical outcomes. They are useful when there is no consensus on clinical breakpoints or when resistance has not yet been

described (Kahlmeter et al., 2015). However, EUCAST has not provided a clear method as to how their thresholds are established (Kronvall, 2011).

3.1.2.4 Normalised Resistance Interpretation

Normalised Resistance Interpretation (NRI) can be used to estimate ECOFFS (Kronvall et al., 2011). The NRI method works under the assumption that, for as long as the WT on the sensitive side is not affected by resistance development, a normalised peak can be reconstructed for MIC or IZD distributions. This method has been widely used in defining the WT population in IZD histograms and for calculating ECOFFS (Kronvall et al., 2011; Ruane et al., 2007; Smith and Christofilogiannis, 2007). It eliminates the need to estimate cut-off values by inspection from distributions provided by EUCAST, although it can be applied to EUCAST data to provide an objective estimate of the cut-off value. Through the use of the NRI method, it was discovered that estimated EUCAST ECOFFS for some species-drug combinations were not accurate as they did not include all WT isolates (Kronvall et al., 2011). The conclusion was that EUCAST had adjusted ECOFF values and underestimated the proportion of WT isolates in some of the reference populations (Kronvall et al., 2011). Moreover, for some pathogens (e.g. *S. aureus*) they found bimodal distributions which indicated that the EUCAST distributions could contain non-WT isolates with some kind of AMR mechanisms (Kronvall et al., 2011). The NRI method can be applied for calculation of EUCAST thresholds, whereby the organisation would need to give formal endorsement of proposed thresholds before they could be considered official EUCAST thresholds, as well as for the calculation of cut-off values for other datasets and by other organisations and individuals. To differentiate between EUCAST-approved and unofficial cut-offs, the terms ECOFF and cut-off for WT (CO_{WT}) will be used, respectively.

3.1.3 Aim of this chapter

This chapter will focus on calculating CO_{WT} values for *E. coli* from poultry in Tanzania from samples described in Chapter 2 and on determining the prevalence of AMR using different criteria for interpretation, i.e. clinical breakpoints, ECOFF and CO_{WT}. The aim is to determine whether the prevalence of AMR will vary depending on interpretation criteria.

3.2 Materials and Methods

3.2.1 Phenotypic identification of *E. coli* colonies using Chromogenic Agar

3.2.1.1 Preparation of Chromogenic agar medium

Chromogenic agar (CHROMagar™ ECC, Sigma Aldrich, UK) was prepared by mixing 32.8 grams of CHROMagar powder in one litre of sterile distilled water and heating to boiling temperature. Agar was then poured into each plate and allowed to dry overnight before use.

3.2.1.2 Phenotypic Identification using Chromogenic agar (CHROMagar)

CHROMagar (Sigma Aldrich) was used for simultaneous identification and differentiation of *Escherichia coli* from other bacteria. The media contains chromogenic substrates (Salmon-GAL) which upon interaction with ß-glucuronidase in *E. coli* isolates cleave and release a chromophore that makes *E. coli* colonies appear blue. Coliforms that lack this enzyme (*Enterobacter*, *Klebsiella* and *Citrobacter*) are expected to be mauve, while non-coliforms appear white or are completely inhibited from growing. Per poultry farm, a single isolate was used, obtained from plate sweeps that were grown on MacConkey agar as described in section 2.2.8. Prior to inoculation on CHROMagar, individual isolates (a single isolate per plate) with characteristic pink-dry appearance were picked from MacConkey plates and inoculated in Luria-Bertani broth (Oxoid, Canada), followed by incubation overnight at 37 ºC. Then, 50 µl of the pure culture was inoculated on CHROMagar, spread evenly using a sterile L-shaped spreader (VWR, UK, catalogue number 6121560P) and incubated overnight at 37 ºC. Afterwards, phenotypically blue colonies (i.e. pure colonies) were picked from each individual CHROMagar plate (one per plate) and subjected to further species confirmation using quantitative *uid*A PCR.

3.2.1.3 Reference strains

Reference strains were obtained from the Veterinary Diagnostic Services laboratory at the University of Glasgow. Isolates originated from samples taken from dogs. The identity of isolates was determined using the API 20E strips (API system by Biomerieux-https://www.biomerieux.co.uk/product/apir-id-strip-range). *Escherichia coli* was used as a positive control and *Klebsiella* as a negative control for both phenotypic and genotypic confirmation of *E. coli* Isolates. Both reference isolates were confirmed to be resistant to all

antimicrobials used in this study by the Veterinary Diagnostic Services laboratory.

3.2.2 Molecular detection of *E. coli* using quantitative uidA PCR

3.2.2.1 DNA extraction

DNA extraction was conducted using a QIAamp DNA mini Kit (Qiagen, UK) as described by Khan and Yadav (2004) with an optimised standard operating procedure. The procedure involved taking a single isolate from each CHROMagar plate described in section 3.2.1.2 and resuspending the isolate in 1 ml of Luria-Bertani media (VWR, UK). Then, 50 µl of the suspension was taken and added into a 1.5 ml microcentrifuge tube and centrifuged at 5000 x *g* (7500 revolutions per minute (rpm) (Horizon Model 642E, Fisher Health Care, Fair Lawn) for 10 minutes. The remaining portion of the culture suspension was incubated at 37 ºC overnight and used for susceptibility testing in the next steps as explained in section 3.2.4. Meanwhile, the supernatant of the centrifuged aliquot was discarded and the bacterial pellet resuspended in 180 µl buffer ATL. To remove any contaminating proteins, a broad-spectrum serine protease (proteinase K) was added to the pellet and the mixture was incubated at 56 ºC for 1 hour. A protein precipitating solution, buffer AL (200 µl), was added, then pulse-vortexed for 15 seconds and incubated at 70 ºC for 10 minutes. Ethanol (200 µl) was added and the mixture transferred to a QIAamp Mini spin column and centrifuged at 6000 x *g* (8000 rpm in Horizon Model 642E)) for 1 minute. This was followed by two wash steps: the addition of buffer AW1 and centrifugation at 6000 x *g* for 1 min; and then addition of W2 and centrifugation at 20,000 x *g* (14,000 rpm in Horizon Model 642E) for 3 minutes. The filtrate was discarded in each step. Buffer AE (200 µl) was used for final elution followed by incubation for 1 minute and centrifugation at 6000 x *g* (8000 rpm) in Horizon Model 642E for 1 minute. The extracted DNA concentration was measured with a Nanodrop (Nanodrop-2000 Spectrophotometer, NanoDrop Technologies).

3.2.2.2 Quantitative uidA PCR

Confirmation of *E. coli* species identity was performed via real-time quantitative polymerase chain reaction (qPCR), which targets the *uid*A gene in *E. coli*, a common gene found in almost all *E. coli* with a coding region of about 1809 base pairs (Jefferson et al., 1986). DNA from reference isolates of *E. coli*

and *Klebsiella* was used for positive and negative controls, respectively. All qPCR assays were performed using the Rotor gene system (Applied Biosystems, Foster City, CA, USA). The *uid*A qPCR primers and probe used for detection were as described in Frahm and Obst (2003). The forward primer was 5'-GTG TGA TAT CTA CCC GCT TCG C-3', the reverse primer was 5'-AGA ACG GTT TGT GGT TAA TCAGGA-3' and the probe ***FAM*** – TCG GCA TCC GGT CAG TGG CAG T – ***BHQ1*.** The probe was labelled with 56-FAM as a reporter fluorescent dye at the 5' end and the 3' end with BHQ_1 as the quencher dye. Reactions for *uid*A qPCR were performed as described in Frahm and Obst (2003). Briefly, qPCR reactions were performed in a 15 µL reaction volume using 2 × Quantitect Probe PCR master mix (Qiagen, Valencia, CA, USA), 0.4 µM of each primer, 0.2 µM of probe (Intergrated DNA Technology, Belgium), and 5 µl of template DNA from presumptive *E. coli* isolates. PCR conditions were 95 ºC for 2 minutes, 95 ºC for 5 minutes, followed by 45 cycles of 95 ºC for 5 seconds and 60 ºC for 5 seconds. Results were analysed using Rotor gene software.

3.2.3 Preparation of Mueller Hinton agar

The Mueller Hinton agar was prepared by suspending 38 g of medium in one litre of distilled water. The mixture was then autoclaved at 121 ºC for 15 mins, allowed to cool at room temperature to 60 ºC and poured into plates in a biosafety cabinet to avoid contamination. After the media solidified, the plates were placed in an incubator at 37 ºC overnight to check whether there would be any growth on the plates which would indicate contamination in media potentially arising from unsterile conditions while pouring the media in plates or that the media was insufficiently autoclaved. Upon confirmation that there was no contamination, the plates were temporarily stored in the refrigerator before use.

3.2.4 Culture and susceptibility testing using disc diffusion test

AST was conducted using standardised disc diffusion testing (Bauer et al., 1966). Antimicrobial susceptibility of *E. coli* was tested against four (4) antimicrobials at standard disc quantity based on EUCAST recommendations, i.e. ceftazidime (30 µg), ciprofloxacin (5 µl), imipenem (10 µg) and tetracycline (30 µg).The procedure involved taking the remaining aliquot of a culture suspension from section 3.2.2.1 above, which contained a single isolate that was verified

through *uidA* PCR to be *E. coli* in section 3.2.2.2 and testing it for susceptibility. The culture suspension was diluted with distilled water to a density that was equivalent to 0.5 MacFarland. Large plates (90 mm in diameter) were used with Mueller Hinton agar (4 to 6 mm in depth). Plates were air-dried for about 30 minutes before inoculation. Bacterial suspensions at 0.5 MacFarland were streaked evenly onto the surface of the medium with a plate spreader (VWR, UK, catalogue number 6121560P). After the inoculum dried for 3 to 5 mins, the four antimicrobial disks were placed on agar with flamed forceps and gently pressed down to ensure contact. Plates were then incubated at 37 ºC under aerobic conditions. After overnight incubation, the zone diameters were measured with a vernier caliper on the under surface of the petri dish near the agar surface.

3.2.5 Data analysis

For each antimicrobial tested, inhibition zone diameters were analysed to determine the prevalence of susceptible and resistant isolates using clinical breakpoints, ECOFFS or CO_{WT} values based on NRI. Clinical break points were derived from CLSI guidelines (CLSI 2016). ECOFFs were taken from EUCAST guidelines (EUCAST, 2017). Estimates for the EUCAST ECOFF for 30 µg tetracycline were not found on the EUCAST website hence an alternative which is a tetracycline analogue (i.e. tigecycline) with the desired concentration was used instead. All calculations of the CO_{WT} were conducted according to specifications in a published protocol by Kronvall and Smith (2016) using a spreadsheet made available by the authors (European patent No 1383913, US Patent No. 7,465,559; https://doi.org/10.1111/apm.12624).This spreadsheet was used to generate histograms of IZD and to calculate CO_{WT} values for each compound. The prevalence of resistance among poultry *E. coli* isolates from Tanzania was calculated based on each of the three thresholds, i.e. clinical breakpoints, ECOFFS and CO_{WT}.

3.3 Results

3.3.1 Phenotypic and molecular detection of *E. coli*

Of the 74 plate sweeps that were harvested from plain MacConkey agar in Tanzania, frozen and shipped to the UK and re-cultured, 69 samples showed growth while 5 samples did not (Table 3.1) demonstrating loss to follow up (7%) after storage and handling.

Table 3.1 Number of plate sweeps by farm type showing growth on MacConkey plates after re-culture in Glasgow of frozen material shipped from Tanzania.

Farm type	Sample	Growth	No Growth
Extensive	20	20	0
Semi intensive	17	15	2
Intensive	18	17	1
Broiler	19	17	2
Total	74	69	5

69 presumptive *E. coli* isolates taken from MacConkey and subcultured on CHROMagar, all showed growth. Not all presumptive *E. coli* colonies from MacConkey agar (i.e. pink dry colonies) were blue on CHROMagar, which is supposed to be sensitive and specific for *E. coli* (Figure 3.1). Based on subsequent confirmation with *uid*A qPCR, 11 isolates that were white, mauve or blue-and-white were confirmed to be *E. coli* and six isolates that appeared blue on CHROMagar were confirmed not to be *E. coli* (Table 3.2).

Table 3.2 Confirmation of *E. coli* species via *uid*A PCR for individual isolates from plate sweeps grown on CHROMagar

Colour on CHROMagar	Total	Positive	Negative
Blue	49	43	6
Mauve	6	2	4
White	6	4	2
White and blue	8	5	3

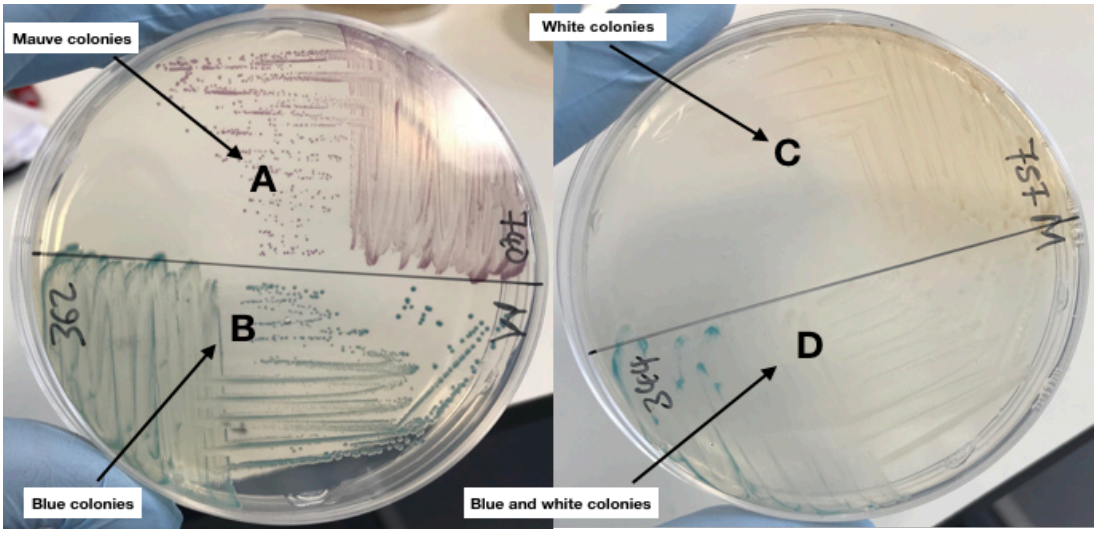

Figure 3.1 Phenotypes observed on CHROMagar. Each phenotype was found among *E. coli* and non-*E. coli* isolates based on *uid*A-based genotypic species confirmation

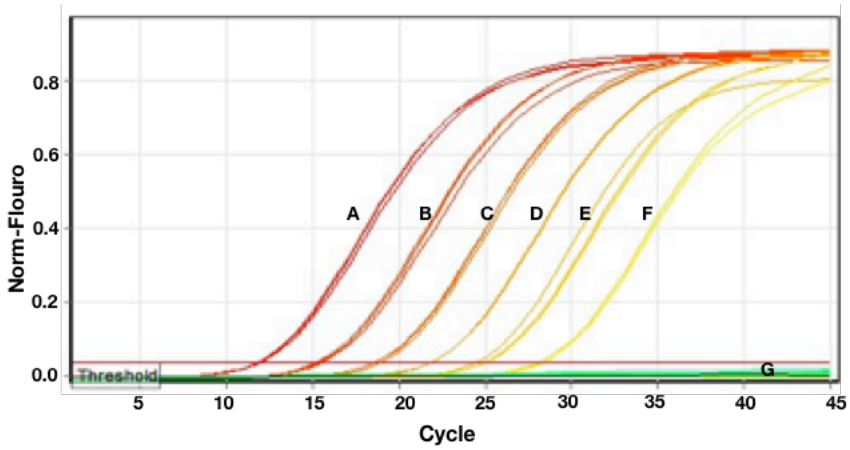

Figure 3.2 *E. coli* (*uid*A gene) standard curves obtained after amplification of reference strain DNA from serial dilution of A: 1.0E+01; B:1.0E+02; C:1.0E+03; D:1.0E+04; E: 1.0E+05; F: 1.0E+06; G: Negative control - *Klebsiella* spp. DNA

3.3.2 Susceptibility testing

The range of IZD values differed between antimicrobials (Figure 3.3). At least one isolate per antimicrobial was observed to have a zone diameter of 6 mm (Table 3.3). Except for tetracycline, the wild type cut-off (CO_{WT}) was lower than clinical breakpoints (CB) and ecological cut-offs (ECOFF).

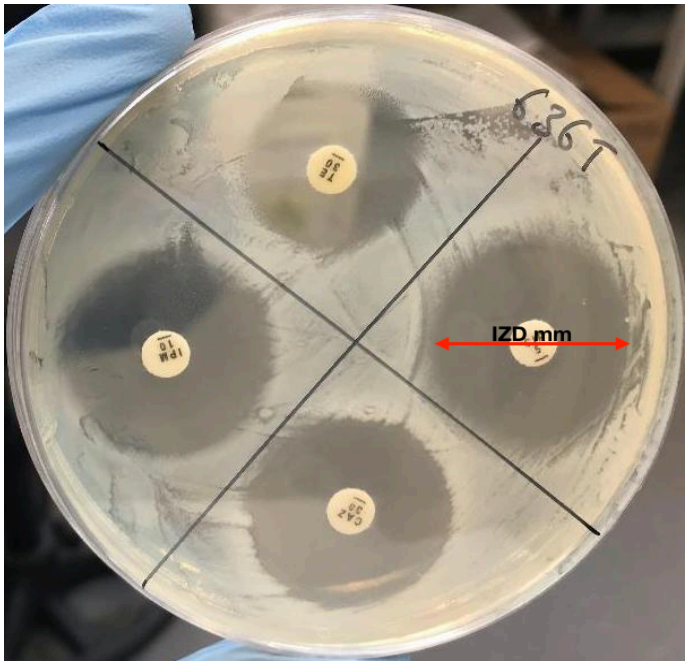

Figure 3.3 Growth of *Escherichia coli* on Mueller Hinton agar with inhibition zones seen around ceftazidime (CAZ), ciprofloxacin (CIP), imipenem (IPM) and tetracycline (TE). The red arrow demonstrates how readings of the inhibition zone diameters (IZD) were taken.

Analysis of the IZD distributions using the NRI method was used to estimate the mean zone size and standard deviation (SD) for WT isolates based on normalised histograms (Figure 3.4) and to estimate CO_{WT} for each compound (Table 3.3).

Table 3.3 Observed range of inhibition zone diameters and output from Normalised Resistance Interpretation, including the functional peak, standard deviation (SD) of the functional peak, and cut-off values for wild type (CO_{WT}).

Antimicrobial	**Range (mm)**	**Functional peak (mm)**	**SD (mm)**	**CO_{WT} (mm)**
Ceftazidime	6 – 38	29	4.8	15
Ciprofloxacin	6 – 40	26.5	5.1	14
Imipenem	6 – 38	24.5	4.7	13
Tetracycline	6 – 21	18	2.1	14

Except for tetracycline, the calculated SD values for IZDs of ciprofloxacin, imipenem and ceftazidime were larger than the recommended 4mm limit (Smith et al., 2012). According to Smith and colleagues, higher SD values maybe a result of fewer number of WT strains in a distribution or lack of homogeneity which may manifest in situations where significant numbers of Non-WT strains with low

level resistance are present in a strain set (Smith et al., 2012), hence affecting the derivation of an accurate CO_{WT}. As a result, it is recommended that CO_{WT} produced from data that generates normalised distribution of putative WT strains with SD > 4mm should be rejected or treated with caution (Smith et al., 2012).

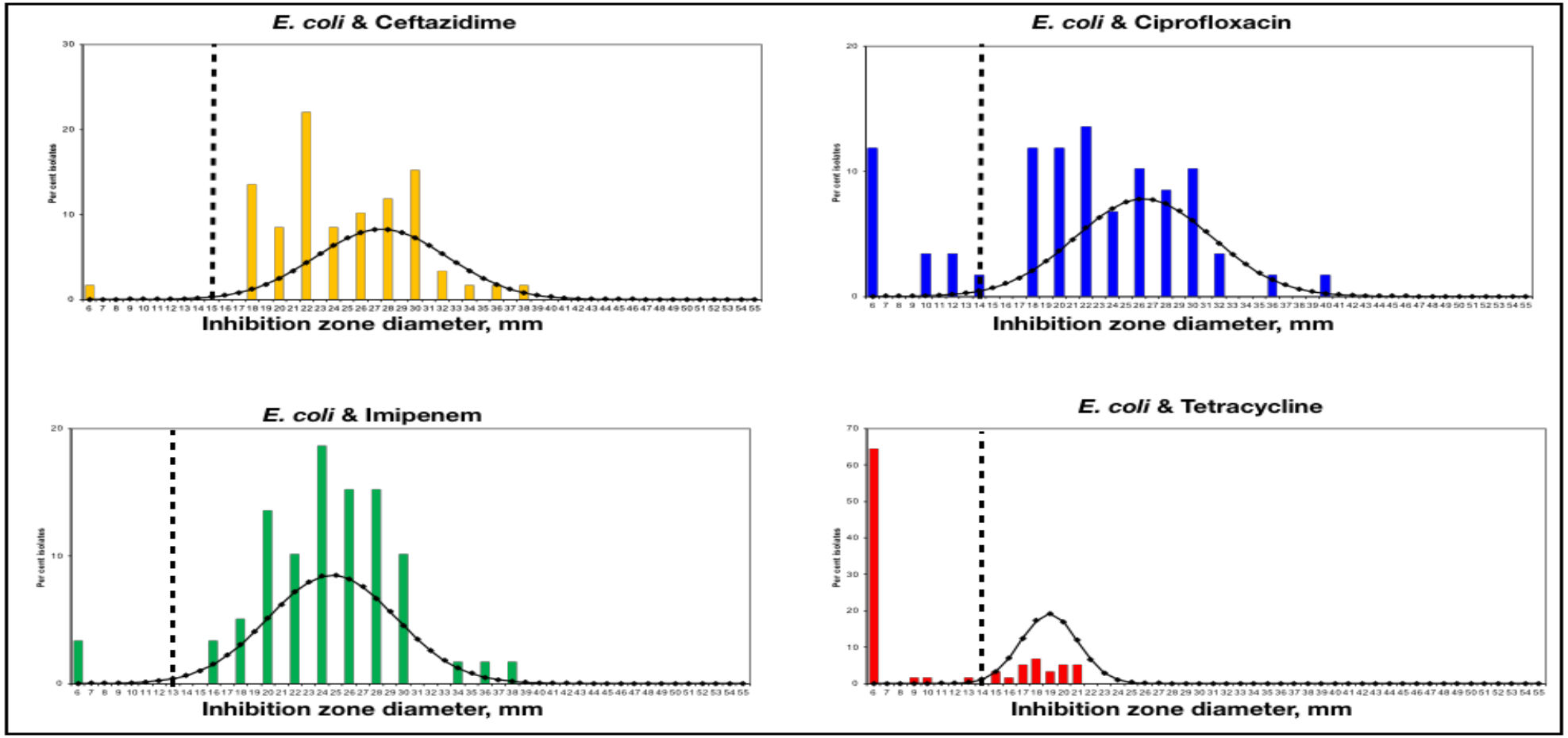

Figure 3.4 Histograms of the distribution of inhibition zones produced by discs containing 30µg of ceftazidime, 5µg of ciprofloxacin, 10 µg of imipenem or 30µg of tetracycline. The continuous black curved line represents the 4-point rolling mean, the dashed line is the wild type cut-off (CO_{WT}) calculated from the data using the Normalized Resistance Interpretation method. Graphs were prepared in Microsoft Excel, using the spreadsheet made available by P. Smith, W. Finnegan, and G. Kronvall (European patent No 1383913, US Patent No. 7,465,559).

3.3.3 Comparison of EUCAST reference data and Tanzanian poultry data

Comparisons of the distribution of IZDs for each antimicrobial compound based on EUCAST data and data generated in this study are shown in the histograms in Figure 3.5 below, together with CB, ECOFF and CO_{WT}-based threshold values for interpretation.

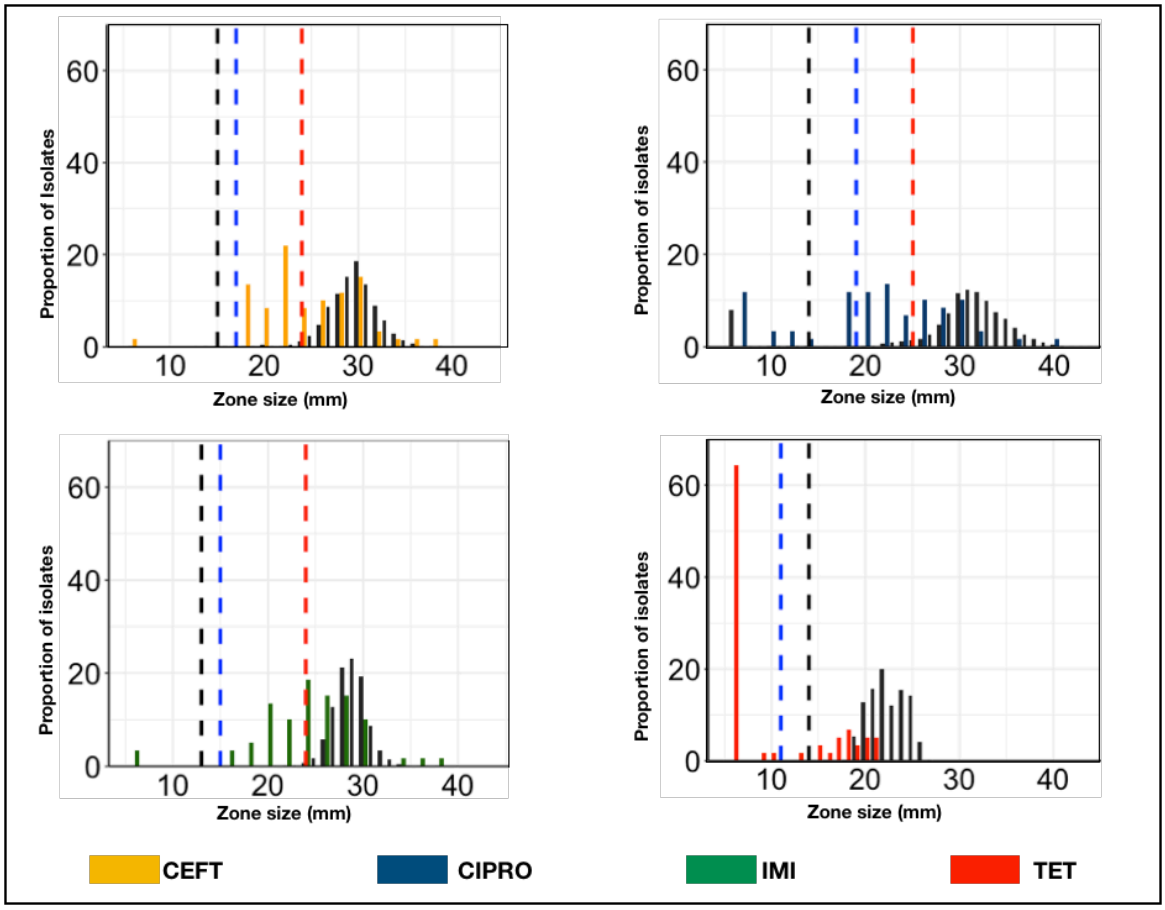

Figure 3.5 Distribution of inhibition zone diameters produced by 30 µg ceftazidime, 5 µg ciprofloxacin, 10 µg imipenem, or 30 µg tetracycline discs against *Escherichia coli*. The coloured bars indicate results for *E. coli* from poultry cloacal swabs from Tanzania (n = 59). Black bars indicate the distribution of IZDs from *E. coli* isolates from EUCAST data (n = 11,875, 36,774, 4,600 and 326 for ceftazidime (CEFT), ciprofloxacin (CIPRO), imipenem (IMI) and tetracycline (TET), respectively). Dashed lines represent CLSI clinical breakpoints (blue), EUCAST ecological cut-off values (red; not available for TET) and cut-off wild type values (black) based on normalised resistance interpretation of the data from Tanzanian poultry.

All four distributions revealed a shift to the left for IZD values of *E. coli* from Tanzanian poultry when compared to the EUCAST reference data, indicating lower levels of susceptibility. The EUCAST distribution for tigecycline (an analogue for tetracycline) was used since the distribution for tetracycline with the desired concentration (30 µg) was not available in the EUCAST website. It is only for tetracycline that the CB was smaller than CO_{WT}. For all compounds, CB differed from CO_{WT} by 5 mm or less whilst ECOFFS were much higher than CO_{WT} values.

3.3.4 Prevalence of AMR in *Escherichia coli* from Tanzanian poultry

Prevalence of AMR was calculated based on three thresholds for resistance (Table 3.4). Clinical breakpoints were acquired from 2016 CLSI guideline and EUCAST ECOFF from the EUCAST website. The CLISI guideline used was *Performance Standards for Antimicrobial Susceptibility Testing*. 26th edition, CLSI supplement M100S, ISBN 1-56238- 924-6 [Electronic]). Estimates of the EUCAST ECOFFS for TET 30 µg distribution were not available on the EUCAST website hence tigecycline data was used.

Table 3.4 Proportion of resistant (R), non-wildtype (non-WT),susceptible (S) or wild type (WT) *Escherichia coli* isolates from poultry cloacal swabs from Tanzania based on clinical breakpoints (CB), EUCAST ecological cut-off (ECOFF) and wild-type cut-off (CO_{WT}) as calculated using normalised resistance interpretation of the data generated in the current study.

Antimicrobial	CB	S (%)	R (%)	ECOFF (mm)	WT (%)	R (%)	CO_{WT} (mm)	WT (%)	Non-WT (%)
Ceftazidime	17	98.3	1.7	24	54.2	45.8	15	98.3	1.7
Ciprofloxacin	19	67.8	32.2	25	35.6	64.4	14	81.4	18.6
Imipenem	15	96.6	3.4	24	64.4	35.6	13	96.0	4.0
Tetracycline	11	32.2	67.8	-	-	-	14	30.5	69.5

With the exception of ciprofloxacin, estimates for the prevalence of resistance or non-WT were similar for CB and CO_{WT}-based interpretation. By contrast, prevalence of non-WT was much higher based on EUCAST ECOFF-based interpretation.

3.4 Discussion

In this study, prevalence of AMR in *E. coli* was estimated using two standard criteria, i.e. CB and ECOFF, and bespoke CO_{WT} values calculated from the data. Based on CB and CO_{WT}, prevalence of AMR was low for ceftazidime and imipenem (< 4%), intermediate for ciprofloxacin, and high for tetracycline (> 67%). High prevalence of tetracycline resistant isolates in the current study matches findings in previous studies conducted in the northern zone of Tanzania (Caudell et al., 2017; Hamisi et al., 2014; Rugumisa et al., 2016). Prevalence of ciprofloxacin resistance ranged from 18.6% to 64.4% in the current study depending on the criteria used. In previous studies in the northern zone of Tanzania, Hamisi et al. (2014) also reported high ciprofloxacin resistance (54.5%) in poultry whereas relatively limited resistance to ciprofloxacin (3.5%) was reported by Rugumisa et al. (2016). As in the current study, Hamisi et al. (2014) used the Kirby-Bauer method to analyse susceptibility of the isolates and clinical breakpoints for interpretation yet they found different estimates suggesting true differences in the AMR prevalence in the study populations. By contrast, Rugumisa et al. (2016) used the breakpoint plate method (as used in Chapter 2) and clinical breakpoints to determine the resistance of *E. coli* isolates so that differences in prevalence estimates between their study and the current study may partly be due to differences in methodology. Imipenem and ceftazidime were seldom used in poultry production according to qualitative survey data on antimicrobial use in Arusha (Sindiyo et al., 2018) and Moshi (see Chapter 4). Therefore, it was not anticipated that there would be resistance against these antimicrobials. Nonetheless, studies that were conducted within the same districts in the past revealed the existence of isolates that were resistant to 3rd generation cephalosporins in poultry. For instance, in a study by Hamisi et al. (2014), 29.8% of poultry isolates were observed to be resistant to cefotaxime (a 3rd generation cephalosporin) whilst Rugumisa et al. (2016) found relatively lower prevalence of ceftazidime resistance estimated at 6.5%. In the present study, resistance to ceftazidime was lower but it was detected. Though this observation may not be linked with direct use of these antimicrobials at farm level, it suggests that alternative sources could be present which introduce resistant bacteria in poultry farms. A study by Lyimo et al., (2016) in the northern zone in Tanzania, discovered bacteria harbouring bla_{TEM} genes (encoding broad spectrum ß-lactamase which hydrolyses many ß-lactams) and

bla$_{CTX-M79}$ (encoding enzymes which exert hydrolytic activity against ceftazidime) were present in closed (i.e. tap water) and open water sources (i.e. lakes or rivers). Since the majority of farmers in the northern zone use tap water in poultry production (Sindiyo et al., 2018), presence of *bla*$_{TEM}$ genes and *bla*$_{CTX-M79}$ in tap water may provide a potential explanation for ceftazidime resistance in *E. coli* isolates from animals that are not treated with those compounds. Prior to this study, no known study in Tanzania investigated imipenem resistance in poultry and therefore there is no direct evidence that could help understand the source of imipenem resistance in poultry. The use of imipenem in poultry production in Tanzania is not allowed officially, however, it may happen informally. Moreover, use in humans may result in imipenem resistant *E. coli* that can be acquired by poultry via human waste (e.g. faeces).

In contrast to AMR prevalence estimates based on CB values, prevalence estimates based on ECOFFS were very different from those based on CO_{WT}. The proportion of non-WT isolates was more than 35% for a carbapenem (imipenem), a 3rd generation cephalosporin (ceftazidime) and a fluoroquinolone (ciprofloxacin). All three categories are listed on the WHO's Critically Important Antimicrobials list, with the latter two included among the Highest Priority Critically Important Antimicrobials (HP-CIA) (WHO, 2017). Such a high prevalence of non-susceptibility in *E. coli* to HP-CIA could be of major public health concern. This shows that estimates of AMR prevalence and hence (apparent) public health concerns can be highly dependent on the choice of threshold. Similar dependence of AMR prevalence estimates on the choice of threshold has been reported in other studies with animal derived data, e.g. for *E. coli* from wild ungulates (Dias et al., 2015). On the one hand, this shows the importance of the NRI method as an objective method to generate cut-offs from specific datasets being examined including those of non-human or non-clinical origin. On the other hand, if (low level) resistance is highly prevalent in such specific datasets, use of the NRI method may fail to recognize it because it could fall within the normal distribution. One of the major limitations of the NRI-method is that cut-offs produced from small datasets may not be accurate or representative of a wider population. For instance, for three antimicrobials in our data, calculated SD values exceeded the recommended limit (Smith and Christofilogiannis, 2007). Indeed, for this reason, results from the current study

should be interpreted with caution. In addition, some of the distributions in the current dataset appeared bimodal rather than unimodal. The NRI method works from high IZD values to low IZD values and uses the highest peak in the data to estimate CO_{WT} values, limiting the impact of the second peak at lower IZD on the estimates. Still, the existence of a second peak, which could potentially indicate an intermediate population, violates the assumption of normal distribution of the data (Kronvall et al., 2011) and suggests that a larger dataset or further investigation of this phenomenon may be needed.

A third key feature of the results from this study is that the distribution of IZD of *E. coli* isolates was shifted to the left in comparison to the EUCAST reference data. This meant that poultry *E. coli* isolates had narrower zones and were less susceptible, even if they were still classified as susceptible or WT based on thresholds for categorical interpretation. Similar shifts have been observed in gram-negative isolates from wildlife compared to EUCAST data (Dias et al., 2015). This shift could be explained by various factors, including inherent differences between humans and animals. The gastrointestinal (GI) tract of animals, particularly ruminants and other herbivores, is different from the human GI tract, and more complex. It is possible, in theory, that this results in upregulation of generic efflux mechanism for toxins and antimicrobials. This mechanism has been suggested to explain a similar shift observed for gram-positive pathogens (*S. aureus*) on organic farms compared to conventional farms, where the IZD were smaller for isolates from conventional farms (Tikofsky et al., 2003). It is possible that exposure to antimicrobials from feed or water may have led to upregulation of generic detoxification mechanisms and efflux pumps in *E. coli* from poultry in Tanzania, leading to the observed shift in IZD distribution. Finally, considering that the poultry data and EUCAST data were generated in different laboratories, variation could be due to differences in methodology despite all attempts at standardisation (Smith and Christofilogiannis, 2007). Although the exact reason or the relative contribution of different reasons to the observed phenomenon is unknown, a comparison of wild-type distributions for *E. coli* from humans and birds may shed some light on the question (Sjölund et al., 2009). In Sjolund's study, data were generated in a single laboratory so there were no differences in methodology. Avian isolates in this study originated from wild birds. The wild-type distribution for the bird isolates did not differ from the

wild-type distribution for humans, suggesting that birds and humans do not have inherently different *E. coli* isolates. The birds in that study were all wild birds from the Arctic (Sjölund et al., 2009). Therefore, they would not have been exposed to antimicrobial treatment. Sjolund's data suggest that the resistance profiles of *E. coli* from birds is not inherently different from *E. coli* from humans and implies that antimicrobial use may be important in explaining the shift to the left for *E. coli* from poultry in Tanzania. There are many assumptions and uncertainties in this argument, but antimicrobial use is widespread on Tanzanian poultry farms. This will be discussed in Chapter 4, together with recommendations for future work.

The misclassification of coliform isolates from health poultry by CHROMagar may be linked to occurrence of different phylogroups of *E. coli* in humans and poultry (Logue et al., 2017). Variable growth and colony colouration of different *E. coli* strains on selective agar has been described before (Kase et al., 2015). Thus, commercially available bacterial indicator media give false positive or false negative results and should be described with caution in agricultural applications, as previously described for methicillin resistant *S. aureus* in bulk tank milk (Virgin et al., 2009). Although assessment of culture methods was not the aim of this chapter, the results show that methodology and results of both bacteriological culture and AST developed primarily for human use need to be interpreted with caution when applied in other settings.

CHAPTER FOUR

GENERAL DISCUSSION AND RECOMMENDATION

4.1 General Discussion

In the present study it was shown that healthy poultry are a reservoir of AMR bacteria in the Northern Zone of Tanzania (Chapter 2), indicated by presence of resistant *E. coli* in chicken cloacal swabs. This finding was expected as there is quite widespread use of antimicrobials in poultry farming in Arusha (Sindiyo et al., 2018), including evidence seen while collecting samples in extensive farms where inputs into poultry production are minimal but antimicrobial use was observed. A similar survey of poultry management and antimicrobial use practices was conducted in Moshi during collection of the cloacal swabs that are described in Chapter 2. However, only qualitative data on antimicrobial use was collected in the present study, presented in Figure 4.1 and Table 4.1. Verbal consent was obtained from each poultry farmer before the interview was conducted in front of a witness. Questions regarding antimicrobial use were open ended whilst questions on how antimicrobials were administered were closed questions (i.e. whether poultry keepers administered through feed or water). This survey revealed that the majority of poultry farmers administered antimicrobials through water whilst none of the poultry keepers added antimicrobials in feed (Table 4.1). Addition of antimicrobials in drinking water, together with the presence of AMR *E. coli* in water (see Chapter 3), would seem to provide the ideal situation for selection of resistant *E. coli* in the gastro-intestinal tract of poultry.

Table 4.1 Number of poultry farmers per farm type administering antimicrobials in water or feed in Moshi Urban District, Northern Zone, Tanzania

Farm type	**Adds antimicrobials in water**		**Adds antimicrobials in feed**	
	Yes	**No**	**Yes**	**No**
Extensive	7	3	0	10
Semi-intensive	10	0	0	10
Intensive	9	1	0	10
Broiler	8	2	0	10
Total	34	6	0	40

Poultry is one of the fastest growing sources of animal protein in Tanzania. Recent estimates suggest that poultry meat contributes to almost 25%

of the meat demand in Tanzania (Wilson, 2015). Therefore, finding resistance in poultry isolates raises critical concerns on the food safety since resistant micro-organisms can find their way into the food chain through meat contamination during slaughter and subsequently get consumed if poultry meat is not properly prepared. Moreover, poultry have the capacity of shedding resistant microorganisms into the environment ultimately transferring them to other animals and areas. The use of poultry manure is quite common amongst livestock-crop farming communities in the Northern Zone of Tanzania, and resistant *E. coli* from chickens could potentially end up contaminating fruits and vegetables. Presence of AMR *E. coli* on vegetables has been demonstrated in South Africa and Europe and was primarily attributed to different water sources (Jongman et al., 2016; Araújo et al., 2017) but the use of poultry manure may also contribute to the problem.

Tetracycline resistance was common, based on testing of samples on break point media (Chapter 2) and based on testing of individual samples with formal AST methods (Chapter 3). This was not unexpected because tetracycline resistance is common in human and animal isolates in Tanzania and globally (Chapter 3). Tetracycline is the most commonly used antimicrobial in poultry farms in Moshi (Figure 4.1) and the only compound reported to be used on all farm types, which may explain the high level of tetracycline resistance.

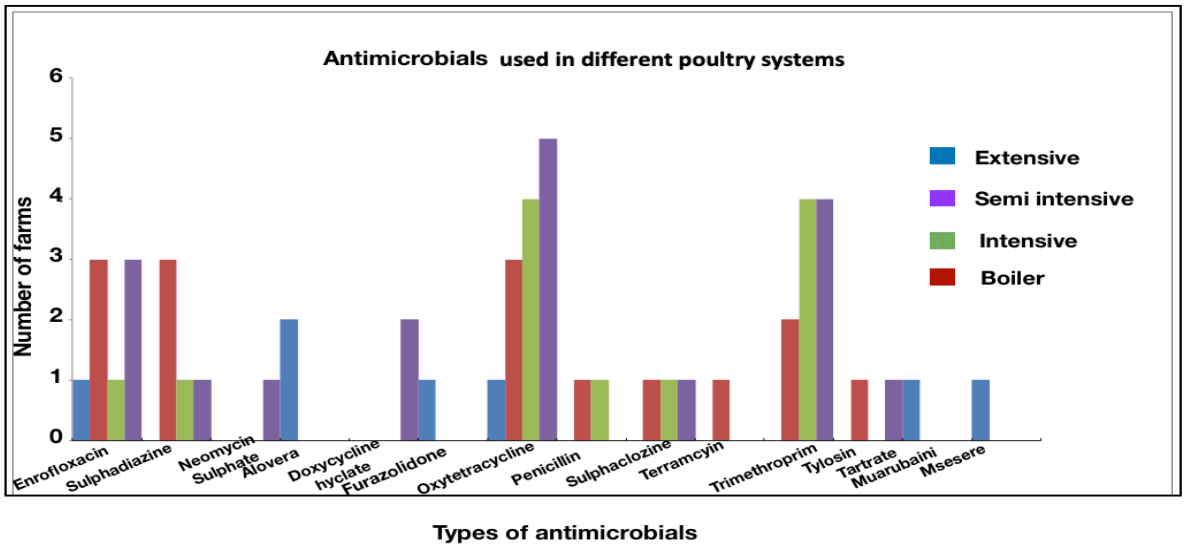

Figure 4.1 Antimicrobials used in different poultry farm types in Moshi Urban District in the Northern Zone of Tanzania based on qualitative surveys on the farms participating in this study.

Use of trimethoprim and sulfadiazine was relatively common too and reported on four farm types. Tetracycline is cheaper than ciprofloxacin, which may explain why it is used widely. Worryingly, however, three of ten participating broiler farmers reported use of ciprofloxacin, which is a quinolone and belongs to the HP-CIA. Such compounds should not be used in animal production. This practice may explain the relatively high prevalence of ciprofloxacin resistant *E. coli* in this region and is of major concern. Equally important, Chapter 3 shows that resistance also occurred against other compounds (i.e. imipenem and ceftazidime), even though farmers reported no use of those compounds (Figure 4.1). Carbapenems are known to have broad spectrum activity against gram-positive and gram-negative bacteria. They are also considered as the last resort for gram-negative bacteria (Köck et al., 2018). Thus, the emergence and spread of resistance to these antimicrobials constitute major public health concern. Based on the interviews we did with poultry keepers carbapenems were not used in poultry production. Therefore, this study highlights the necessity of exploring other sources through which AMR bacteria and their determinants could be introduced at farm level in the absence of use of antimicrobials. This is pertinent as AMR is a multifaceted problem. There are various factors that could contribute to the development and emergence of antimicrobial resistant bacteria. In extensive poultry farms, both traditional medicinal plants and other antimicrobials were used, both in Arusha (Sindiyo et al., 2018) and in Moshi. Frequently used medicinal plants were *Msesere*, *Muarubaini* and *Aloe vera*. Whether medicinal plants contribute to AMR or might be useful as alternatives to antimicrobials would need further study (Abdallah, 2011).

In contrast to numerous studies (Van Boeckel et al., 2015; Rugumisa et al., 2016; Gerber et al., 2008; Luangtongkum et al., 2006) findings from Chapter 2 did not support the view that intensification of poultry production is a risk for increased resistance. This was somewhat unexpected because utilisation of antimicrobials amongst poultry keepers was found to be associated with socio-economic status of the farmers (Sindiyo et al., 2018; Caudell et al., 2017) and the higher ciprofloxacin use among broiler farmers (Figure 4.1) suggests that this may also be the case in Moshi. Therefore, higher wealth could be expected to be associated with intensive farming practices, higher use of antimicrobials and

higher prevalence of AMR. The lack of an association between farm type and AMR prevalence suggests that a multifactorial approach may be needed to understand the dynamics of AMR prevalence in these poultry systems, including detailed analysis of potential sources of selection pressure, including the use of medicated feed, treatments, herbal remedies, water and socio-economic aspects. Based on conversations with the poultry farmers from Moshi and Arusha in this study, the majority of farmers did not seek professional help but rather sought advice from other sources, e.g. neighbours or friends (Sindiyo et al., 2018; personal observation). This may have affected the association between farm type and antimicrobial use practices because the source of advice may have been similar for all farm types. It is also a possibility that our findings were impacted by other factors, such as those associated with cultural-ecological practices, which were not quite distinct between the farm types. Lack of significant differences between regions and farm types may also be due to limited statistical power of the current study. The data from Chapters 2 and 3 could be used to inform sample size calculations for future studies.

Findings in Chapter 3 show potential implications of using different thresholds and how that could change the trajectory of interpretation of resistance or prevalence as whole. Most studies presented in Chapter 1 reported on prevalence of resistant bacteria without defining the type of thresholds which were used in interpretation of resistance. In Chapter 3, it was revealed that there is a possibility that the prevalence of resistance could be misinterpreted (i.e. elevated or underestimated) when ECOFFS or clinical breakpoints are applied as opposed to bespoke thresholds generated from specific datasets. When distributions of poultry derived *E. coli* isolates from the present study were aligned with EUCAST reference distributions, a move towards lower zone sizes was seen for poultry isolates rather than EUCAST reference isolates. This suggested that a proportion of isolates from the wild-type normal distribution could easily be misclassified as resistant based on EUCAST distributions. This is an important issue because there was quite significant difference in AMR prevalence as indicated by EUCAST ECOFFS compared to CO_{WT} values calculated from poultry data. In the EUCAST data, a significant portion of the data originated from SENTRY, a human centred dataset which has almost no representation of data from Africa. Thus, the EUCAST data may not reflect the

WT distribution for human or poultry *E. coli* isolates obtained from Africa and AMR prevalence in Tanzania may be overestimated if EUCAST thresholds are used. Likewise, the MYSTIC (Meropenem Yearly Susceptibility Test Information Collection) dataset combines data from medical centres and is also human focused and biased towards high income countries, resulting in potential misinterpretation of AMR data from LMICs. Clinical breakpoints are set for human therapeutic purposes, yet we have numerous studies that utilise the same breakpoints to interpret animal data. Despite similarities in prevalence based on clinical breakpoints and CO_{WT} in the current study, this progressively demonstrates that there is a general lack of understanding of origin, purpose and interpretation of these thresholds. Likewise, it demonstrates that none of these reference thresholds can be utilised as universal benchmarks in interpretation of resistance for animal and human studies across the world. Moreover, it raises concerns particularly in low income countries, where extremely stringent financial priorities have to be set based on research outputs yet potential misinterpretation of prevalence of resistance could occur. Going forward, critical investigation should be conducted on the current thresholds, in addition, to incorporation of data from low resource countries while generating these thresholds. When generating such data, stringent quality control of media and procedures, e.g. as described in Chapter 3 should be implemented to avoid some of the difficulties that were encountered in the current study, particularly in Chapter 2, and to ensure that differences in AMR prevalence are only due to differences in interpretation or true differences in prevalence, and not to differences in methodology.

4.2 Conclusion and Outlook

From Chapter 1, it is clear that AMR is of global concern and that animal agriculture may contribute to the problem. In Tanzania, the poultry sector could be a major contributor because of its rapid growth, which is linked to urbanisation and increasing consumer wealth in urban areas and drives intensification of poultry production. In Chapter 2, prevalence of AMR across four scales of intensification was explored for coliform organisms using the breakpoint plate method. Prevalence of AMR against a commonly used compound such as tetracycline was high and prevalence of AMR against compounds that should not be used in poultry production, such as cephalosporins

and imipenem was low but there was no obvious difference between production systems. In Chapter 3, using standard antimicrobial susceptibility tests of individual confirmed *E. coli* isolates, the difference in prevalence of AMR to different compounds was confirmed. Actual prevalence estimates were highly dependent on interpretation criteria. For future studies of the AMR problem in poultry in Tanzania, a larger sample size may be needed together with consideration of additional socio-economic and environmental drivers of AMR, as mentioned in Chapter 4. In addition, scientists and policy makers may need to be educated about the different methods for interpretation of results from AST, and their impact on reported AMR prevalence estimates. AMR is recognised as a One Health problem, and the fact that resistance occurs in poultry even though some antimicrobials are not used in poultry indicates that transfer between humans and animals is likely to occur, possibly via shared water sources or environments. Due to the human, animal and environment dimension of AMR, it is important to use a One Health approach when addressing the problem. This includes preserving the effectiveness of the existing antimicrobials by eliminating their inappropriate use and limiting the spread of infection in humans and animals. Additionally, standardised methods are required in screening human and animal isolates, however, it is important to recognise as this effort is being made that there is not one approach for everything.

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