DIVERSITY OF EXTENDED-SPECTRUM BETA-LACTAMASE GENES IN AVIAN PATHOGENIC *ESCHERICHIA COLI* IN SCAVENGING LOCAL CHICKEN IN MOROGORO MUNICIPALITY, TANZANIA

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ABSTRACT

The poultry industry, especially chicken production has in recent times faced a major set-back due to devastating effects of Extended Spectrum Beta-Lactases (ESBL) producing organisms.This research aimed at investigating the diversity of ESBL genes in avian pathogenic *Escherichia coli*(APEC) among scavenging local chickens. A total of 400 cloacal and oropharyngeal swabs were obtained, out of which 192 *Escherichia coli* were isolated. By use of virulence factor profiling, these 192 samples were screened for the presence of 16 virulence factors by multiplex PCR. All 192 samples harbored at least one of the 16 virulence genes and 19 of them carried at least four, making them APEC. The virulence traits *ibeA, iss, traT and chuA* were observed to lead the chart with percentages of 84.21, 78.95, 63.16 and and 52.63respectively. In the pathogenesis of APEC, Iron acquisition, serum resistance, toxins and invasins were found to be very significant ($P<0.05$). The antimicrobial profiles of these APEC strains were determined by Kirby-Bauerdisc diffusion test using 10 antimicrobials. These include:augmentin (30µg), imipenem (10µg), cephalothin (30µg), cefotaxime (30µg), ceftazadime (30µg), ceftriaxone (30µg), nalidixic acid (30µg), ciprofloxacin (5µg), gentamycin (10µg) and trimethroprim-sulfamethoxazole(25µg). All APEC strains were found to be resistant to at least one of these drugs, with 10.52% of them being multi-drug resistant. By double-disc synergy test, eight of the APEC isolates were found to be ESBL producers. They were screened for the presence of beta-lactamase genes and the following were present: *bla*TEM-100%, *bla*OXA-1 -75.0%, *bla*CMY-2 62.5%, CTX-M group III (CTX-M-8)-50%, CTX-Mgroup IV (CTX-M -9)-37.5%, CTX-M group I(CTX-M -1,and-15) -12.5% and *bla*SHV-12.5%. Occurrence of virulence strains of APEC and ESBLs genes are also alarming.

DECLARATION

I, Emmanuel OdarteiArmah, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor concurrently submitted in any other institution.

…… ……..

Emmanuel OdarteiArmah Date

(MSc. Molecular Biology and Biotechnology)

The declaration is hereby confirmed by;

…… ……..

Dr. HurumaNelwikeTuntufye Date

(Supervisor)

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DEDICATION

To Henrietta AkweleyArmah, my mother.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
1GC	First generation cephalosporin
2GC	second generation cephalosporin
3GC	Third generation cephalosporin
APEC	Avian pathogenic *Escherichia coli*
AUG	Augmentin
bp	Base pair
CAZ	Ceftazadime
chuA	*E. coli*haem utilization-A
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycinase
CN	Gentamicin
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaxime-Munich
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene Diamine Tetra –acetic Acid
ESBL	Extended Spectrum Beta-Lactamase
gimB	Genetic island associated with newborn meningitis
hr	Hour
ibeA	Invasion of brain endothelium
IMI	Imipenem

irp	Iron-repressible protein
iss	Increased serum survival
iucD	Aerobactin synthesis
KF	Cephalothin
km	Kilometer
MDR	Multi-Drug Resistance
MH	Mueller-Hinton
mm	Millimeter
NA	Nalidixic acid
NC	Negative control
ºC	Degree celcious
ompA	Outer membrane protein-A
OXA	Oxacillin
papC	Pilus associated with pyelonephritis-C
sec	Seconds
SHV	*Sulfhydryl* variant
sitchr	Salmonella iron transport-chromosomal
sitepi	Salmonella iron transport-episomal
sitDChr	Salmonella iron transport system gene- chromosomal
sitDepi	Salmonella iron transport system gene-episomal
SLC	Scavenging local chicken
STX	Trimethroprim-Sulfamethoxazole
TAE	Tris Acetate EDTA
TEM	Temoniera
traT	Transfer protein T
tsh	Temperature-sensitive haemagglutinin

vat	Vacuolatingautotransporter toxin
µl	Microlitre
µM	Micromole
µm	Micrometer

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Extended spectrum beta-Lactamase (ESBL)-producing organisms pose major health and economic threats to livestock production, especially in the poultry industry.Selective pressure exerted by antimicrobials leads to the spread of multidrug resistance among avian *Escherichia coli*(Johnson *et al*., 2009).ESBLs have been widely identified in *Escherichia coli*(*E. coli*) from both healthy and diseased animals and hence considered epidemiologically important(Leigue *et al*., 2013). Lima-Filho*et al*. (2013) reported that apparently-healthy poultry could harbor multidrug resistanceof extra-intestinal *E. coli*. This presents a health risk to the main consumers, the human populace. Needless is to say, the increasing incidences of ESBL-infections among humans is attributable to the contamination of retail chicken by bacteria that carry them (Cohen-Stuart *et al.*, 2012).

Scavenging local chickens makes over 70% of the entire chicken population in Tanzania(Minga *et al*., 2001).Ironically, studies on ESBLs have shunned away from these birds, although they stand a high chance of being the main source of transfer to humans than any poultry. Sambo*et al*. (2015) observed that majority of these birds are keptmainly as free-ranged. A few of them, however,are housed as semi-intensive and a far lesser percentage of them are kept entirely intensive. This is hypothesized to have effect on the control of disease amongst them(Fotsa, 2011).

The aim of this research is toaccess the diversity of ESBL genes in scavenging local chickens in selected areas of Morogoro Municipality, Tanzania.

As an indicator, the avian pathogenic *Escherichia coli* (APEC)was used to monitor the pattern of antimicrobial resistance of the isolates.APEC is known to be the main cause of *collibacillosis,*which is responsible for the annual million-dollar loss in the poultry industry worldwide (Khaton *et al.*, 2008).APEC strains infect poultry and other avian species through fecal dust via the respiratory tract(Sun *et al.,* 2015). It also causes *cellulitis* which is the second most leading cause of chicken morbidity and mortality(Barbieri *et al*., 2013).

APEC and ExpEC strains from human hosts are also known to share similarities in contents of virulence genes and capacities to cause disease. This is because they both are known to encounter similar challenges in establishing infection in extra-intestinal locations (Johnson *et al*., 2008).

1.2 Problem Statement and StudyJustification

1.2.1 Problem statement

The current crises of ESBL-mediated infections have extended its malaise beyond the humans to animals and the ecosystem as large (Geser*et al*., 2012). This could be blamed on low level of utilizable knowledge regarding the genetic diversity of ESBLs isolates among companion and domestic animals (Seni*et al*., 2016). Researches on ESBLs in Africa are obscure, especially when it has to do with scavenging local chicken (Aarestrup *et al.,*2008). The very few researches on ESBLs in Tanzania included those by Seni*et al*.(2016) and Katakweba*et al*. (2012). None of these focused on scavenging local chicken, which is mainly consumed in Tanzania.Since ESBL-related illness among humans has been attributed to the consumption of chicken(Blaak *et al*., 2014), there would be a need to engage in an investigation to reveal how diverse ESBL genes are in these animals.

1.2.2 Justification of the Study

Scavenging local chickens is heavily consumed in Tanzania and other developing countries leading to an increase in their demand(Katakweba *et al*., 2012). The need to engage in surveillance of ESBLs among scavenging local chickenin the country therefore arises; since it is virtually non-existent.With its focus of the ESBLs, this study aims to add its bid to the battle against antimicrobials resistance. Revealing the mechanisms and strategy employed by ESBLs among chicken would aid devise a strategy to deal with them.

1.3Objectives of the Study

1.3.1 Main objective

To determine the diversity of Extended-Spectrum Beta-Lactamase genesinavian pathogenic*Escherichia coli* in scavenging local chicken inselected areas of Morogoro municipality,Tanzania.

1.3.2Specific objectives

i. To detect APECstrainsamong scavenging local chicken circulating in selected areas of Morogoro Municipality.
ii. To determine the antimicrobial susceptibility patterns of these APEC strains.
iii. To detect ESBL-producing APEC strains among scavenging local chickens circulating in selected areas ofMorogoro Municipality.
iv. To determine the occurrence and frequency of selected ESBL genes in APEC among the scavenging local chicken.

1.3.3 Research questions

i. What are the APEC strains among scavenging local chicken circulating in selected areas of Morogoro Municipality?

ii. What is the antimicrobial susceptibility pattern of these APEC strains?

iii. What are the ESBL-producing APEC stains among scavenging local chickens circulating in selected areas ofMorogoro Municipality?

iv. What is the occurrence and frequency of selected ESBL genes in ESBL- producing APEC among the scavenging local chickens?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1Background Information

E. coli organisms are either commensal or pathogenic. Pathogenic *E. coli* can also be categorized into two broad types based on the site of infection: intestinal pathogenic *E. coli* (IPEC) causes infections in the gastrointestinal system whiles extra-intestinal pathogenic *E. coli* (ExPEC) affects the extra intestinal system (Johnson *et al*., 2006). Depending on phylogeny and virulence factors, ExPEC can be classified as avian pathogenic *E.coli* (APEC), newborn meningitis *E.coli*(NMEC) oruropathogenic*E. coli* (UPEC) (Johnson *et al*., 2008). Although its genotype is not clearly defined, APEC is considered an ExPEC that causes collibacillosis(Kwon *et al*., 2008)

APECis an extra-pathogenic *Escherichia coli* (ExPEC) which causes mainly extra intestinal diseases in chickens, turkeys and other avian species and is found in all stages of poultry production chain (Cunha *et al*., 2014).Diseases caused by APEC include; *aerosacculitis, polyserositis* and *septicemia* which is collectively referred to as *collibacillosis*(Dziva and Stevens, 2008).Virulence traits expressed by APEC can be categorized as adhesions, iron acquisition,invasins, serum resistance or protectins and toxins (Dozois *et al*., 2000). By exhibiting these factors, previously known intestinal commensal*E.coli*organisms become APEC and infect extra-intestinal sites(Ewers *et al.*, 2004).Different APEC strains harbour different combinations of virulence factors that perform similar functions in disease establishment as reported by Barbieri*et al.*(2013).

2.2 Virulence Factors of APEC

2.2.1Adhesins

Adhesion to the epithelium tissue by the bacteria is an important step for the establishment of *E. coli* infection, and it's a characteristic of both commensal and pathogenic strains

(Amabile de Campos *et al*., 2005). It permits the bacterial linkage and its maintenance in close contact to the host epithelial tissues. The two significant adhesive properties of APEC include the pilus adhesions and the non-pilus adhesions.Fimbraietype1 (F1) and P-piliconstitute thepili adhesions whiles the non-pilus adhesions are the temperature-sensitive hemaglutination*(tsh)* and curli(Lynne *et al.*, 2007). The pilus associated with *pyelonephritis* (*pap*)gene adhere to internal organs and occurs at later stages of infection while the*temperature-sensitive haemaglutinin (tsh)* gene causes colonization of the air sacs (Ewers *et al*., 2004).

2.2.2 Iron acquisition

APEC has multiple iron-acquisition systems: heme and siderophores systems that enable it to survive in iron-deficient environments. The heme system is involved in the direct uptake of iron while the siderophores system in is involved in the indirect uptake of iron(Gao *et al*., 2012). In the direct uptake, theheme molecules are transferred from the hemiproteins when specific outer membrane receptors *Hma* and *ChuA*(*E. coli*haem utilization) bind to them. These receptors are then internalized into the periplasm and then transferred into the cytoplasm by means of an ATP-binding cassette (Stojiljkovic and Perkins-Balding, 2002).The siderophoresare small molecule compounds that are employed in a shuttle mechanism in indirect uptake of iron (Bauer*etal.*, 2002). They constitute a group of high-affinity ferric-iron chelators,essential amongst them is the *salmochelin* which is considered very critical in the pathogenesis of *E.coli* and *Salmonella*. Amongst ExPEC strains, *salmochelins*' biosynthesis and transport are borne by genes located on the *ColV* or *ColBM* virulence plasmids as well as pathogenecity associated islands, PAI (Fischbach *et al.*, 2006).

2.2.3Protectins/serum resistance

APEC strains are associated with bacterial resistance to the complement. This is known to be mediated by bacterial surface structures such as lipopolysaccharide (LPS), capsule, *col V*colicin, and outer membrane proteins. Some of these structures have been found in mammalian *E. coli* isolates as well. This fueled speculation that both avian and mammalian *E. coli* strains may have similar serum resistance mechanisms. Mellata*et al.* (2003)investigated and revealed that serum resistance is one of the mechanisms employed by APEC strains in pathogenecity.

The genes involved in serum resistance include increase serum survival (*iss),* structural genes of colicin-V operon *(cvi/cva),*outer membrane protein (*ompA*) and transfer protein *(traT)*(Ideses *et al*., 2005).

2.2.4 Toxins

The two toxins associated with APEC that were investigated in this research include; vacuolatingautotransporter toxin(*vat*) gene and heat stableenteroaggregative toxin (*astA*)gene. The *vat* gene encodes a vacuolating toxin expressed by an APEC strain. It belongs to a pathogenecity island which is exhibited by other APEC strains. It is highly conserved and tightly regulated in urosepsis associated virulence factor (Nichols *et al*.,2016). The *astA*gene is a heat stable toxin that is produced by strains of *enteroaggregativeE.coli* strains (EAEC). It is also present in other categories of *diarrheagenicE.coli* and could play a role in the pathogenecity of other enteropathogensas well. It was first detected in the stool of a child and has been shown to induce diarrhea in humans, although it's been associated with piglets and calves as well (Ménard and Dubreuil, 2002).

2.2.5 Invasins

Two very vital invasions involved in the pathogenesis of APEC are the *ibeA*(invasion of the brain epithelium)gene *and gimB*(genetic island associated with newborn meningitis)gene.The *ibeA*gene is involved in the pathogenesis of both APEC and new meningitis *E. coli* (NMEC) and is located on the genomic island *gimA*(Cortes *et al*., 2008). It contains four operons, which are known to poses metabolic potential. The *ibeA* gene, together with other proteins contribute to the invasiveness of brain microvascularepithelialcells (BMEC) in both APEC and newborn meningitis *E.coli* (NMEC)(Wang *et al.*, 2011).Investigation by Wang *et al.*(2011) revealed that the loss of *ibeA* ledto decrement of the colonization capacities of APEC in the brain during system infection.

The *gimB*gene was first found by hybridizationof NMEC. It is typical of ExPEC and APEC, and consist of a sequence approximately 5,200 with six Open reading frame(Matter *et al.*, 2015).

2.3 Beta-lactamase

Beta-Lactamase may be chromosomal or plasmid mediated and their classification is generally based on two schemes; Molecular classification and Functional classification(Bush and Jacoby, 2010).The molecular classification was proposed by Ambler, in 1980, and it's based on the amino acid sequence(Rao, 2012). It divides beta-lactamase into four(4) major classes: A, B, C and D. Classes A,C and D make use of serine for the hydrolysis of the beta-lactam ring while class B uses metalloenzymes. These metalloenzymes require divalent zinc ions for substrate analysis(Drawz and Bonomo, 2010).Bush *et al.*(1995) proposed the functional classification scheme. This scheme is based on substrate and inhibitor profiles (Bush and Jacoby, 2010). Three major groups are

presented bythis classification. Group one is made up of cephalosporinases. These are not very much inhibited by clavulanic acid. They correspond to class C of the molecular classification (Mayers, 2009).

Group two comprises penicillinases, cephalosporinsases and broad-spectrum beta-lactamase. These are generally inhibited by active site-directed beta-lactamase inhibitors and correspond to classes A and D of the molecular classification(Thirapanmethee, 2012). Group three are the metallo-betalactamase that hydrolyzepenicillins, cephalosporins and carbapenems. They are poorly inhibited by almost all beta-lactam molecules (Bush *et al*., 1995).

2.4Extended-Spectrum Beta-lactamases (ESBLs)

2.4.1 Background information

ESBLs belong to group 2 of the functional classification and classes A and D of the molecular classification. Increase in ESBL identification over the last two decades has made the group the largest amongst the beta-lactamases (Bush and Jacoby, 2010).

In the functional group system, ESBLs are further identified with subgroup 2b, with a general characteristic that they easily hydrolyze penicillins and early cephalosporins such as cephaloridine and cephalothin(Ahmed and Shimamoto, 2008). They are however strongly inhibited by clavulanic acid and tazobactam. These beta-lactamase include *bla* TEM-1, *bla*TEM-2 and *bla*SHV-1 and are the commonest plasmid-mediated beta-latamases identified.

2.4.2TEM ESBLs

TEM-1 was the first isolated plasmid-mediated beta-lactamase discovered in gram negative bacteria in 1960s in Greece(Patterson, 2000). Later, a related enzyme, TEM-2 was discovered. Compared to the original beta-lactamase, TEM-2 had a single amino acid substitution and hence a shift in its iso-electric point, although the substrate profile remained unchanged (Blazquez *et al*., 2000). In 1989, TEM-3 was reported as the first TEM-type beta-lactamase that displayed ESBL phenotype. Subsequent years have seen the discovery of several derivatives of TEM, majority of which are ESBLs and a few, inhibitor-resistant enzymes, according to Bradford (2001).TEM-1 has become the progenitor of over 90 additional derivatives of TEM. However, there have been recent reports on TEM-AQ, a naturally occurring TEM-like enzyme. Unlike the other TEM enzymes, TEM-AQ has a number of amino acid substitutions and one amino acid deletion. It is the belief that it was the result of selective pressure offered by several beta-lactams, and not a single agent(Blazquez *et al*., 2000).

2.4.3SHV ESBLs

The name comes from *Sulfhydryl* variant, the amino acids in the enzyme that cross-link with other molecules (Viscount and Mahlen, 2010).The SHV beta-lactamases has a SHV-progenitor and twenty-three variants, majority of which exhibit extended-spectrum activity on recent broad-spectrum cephalosporins(Shaikh *et al.*, 2015). They are usually encoded by self-transmissible multi-resistant plasmid and are highly mobile, as noted by Bradford (2001).Although majority of the SHV-type have the ESBL phenotype, one variant, SHV-10 has been observed to poses an inhibitor -resistant phenotype. SHV-10 is known to be derived from SHV-5 and has one additional amino acid substitution of glycerin for serine 130.Majority of SHV-type ESBLs are found in strains of *K. pneumoniae*. They are also present in *E. coli*, *Citobacterdiversus* and *P. aeruginosa*.

2.4.4CTX-MESBLs

This type of plasmid-mediated ESBLs are found among the family *Enterobacteriaceae,* especially strains of *Salmonella enterica* and serovar*Typhimurium* and *E.coli*(Jorgensen *et al*.,2010). They arose by the initial transfer of chromosomal beta-lactamase gene from *Kluveraspp* to conjugate plasmids. Mutations in these genes gave rise to enzymes CTX-M1 through CTX-M91 and over 30 variants have been discovered in 2008 and 2009(Bush and Jacoby, 2015). They are known to hydrolyze cefotaxime preferentially. Phylogenetically, the CTX-M family of beta-lactamase are of four major types; CTX-M-1, CTX-M-2, Toho-1 and CTX-M-8. CTX-M enzymes are mildly related to SHV and TEM beta-lactamase with an identity of 40%(Naas *et al*., 2007).

2.4.5OXAESBLs

The OXA type beta-lactamasebelongs to molecular class D and functional group 2 and resist ampicillin and cephalothin. They have high hydrolytic activity against oxacillin and cloxacillin and are poorly inhibited by clavulanic acid(Bou *et al.*, 2000).In *E. coli*, these enzymes confer weak resistance to oxyimino-cephalosporins, but when cloned in *P. aeruginosa*transconjugants, the resistance offered is strong(Yan *et al*., 2006).

Amongst the OXA-type ESBLs, the OXA-17 beta-lactamase confers resistance to cefotamine and ceftriaxone but give marginal protection against ceftazidime(Nordmann *et al*., 2000). OXA beta-lactamasewere originally characterized by their resistance to clavulanic acid but OXA-18 beta-lactamase were later reported to be inhibited by this compound. Other OXA derivatives that are not ESBLs were described later (Afzal-Shah *et al*., 2001).

2.5Scavenging Local Chickens

Scavenging local chickens are predominant among other poultry in African villages, owing to their relatively low input requirement in their rearing(Pousga, 2007). Production system in keeping them is mainly scavenging (free ranged). In terms of popularity, the semi-intensive system is second to the scavenging andfew farmers engage in. Absolute-intensive systems of keeping them is relatively obscure (Sambo *et al.*, 2015). The free-range system has been practiced for years and can be found in most rural areas, where they serve as the basis of food production and economic enhancement.In traditional African settings, birds reared under this system have no regular health control program, may or may not have shelter and scavenge for most of their nutritional requirement. They thus serve as means of converting low-quality feed into high protein. They also have short life cycles and quick turnover(Carvalho *et al*., 2015).

With the current population of about 45 million and a land area of 945000km^2, about 80% of Tanzanians live in rural areas where scavenging local chicken is mostly distributed (Minga *et al*., 2001). Among the east African community, Tanzania is relatively rich is wildlife and livestock production. Livestock accounts for 18% of the country's gross domestic product (GDP) and 30% of agricultural GDP.

Increased incidences of ESBL infection in human is attributable to ESBL-producing bacteria in poultry, especially chicken (Beninati *et al*., 2015), there would be a need to engage in an investigation to assess the diversity of ESBL genes in scavenging local chicken.

2.6ESBLs in Chicken

Geser*et al.* (2012) investigated the impact of food animals as a possible reservoir for ESBL producing *Enterobacteriaceae.* They assessed fecal samples from pigs, cattle,

chicken and sheep in Switzerland. Remarkable among their findings was the fact that fecal samples of chicken recorded the highest ESBL producers among the animals: 63.4% as against porcine (15.3%), bovine (13.7%) and sheep (8.6%). They thereby concluded that the high rate of ESBL producers in food animals, especially chicken is alarming.

In a more current research, Beninati *et al.*(2015) investigated the contribution of ESBL producing bacteria in food-producing animals to the increased incidences in the infection in humans in Italy, in 2015. They observed that 68.4% of the total poultry products analyzed proved to be positive for *E .coli* and phenotypical detection of ESBL. They also noted that 33 out of 38 (86.84%) of *E. coli* isolates from poultry products showed resistance to at least one of the antimicrobials tested.

Few of these researchers also occurred within the African continent. Schaumberg *et al.,* (2014) assessed the risk of importing ESBL producing *Enterobacteriaceae* and *S. aureus* through chicken meat in Gabon. They observed that the contamination rate per chicken part with ESBL is 23%. This figure was far higher than the contamination rate of *S. aureus*(3%). Based on their findings, they made a similar conclusion; there is a high risk to import ESBL *E.coli* through chicken.

Within the East African sub-region, Chisimba*et al*. (2016) detected the presence of ESBL producing in *E. coli* in poultry in Zambia. They also reported that 20.1% of the samples harbored ESBL genes.

In Tanzania, the jurisdiction of this study, earlier researches on ESBLs focused on humans. The few recent ones that employed animals included one bySeni*et al*.(2016). They determined the carriage of ESBLs among domestic and companion animals;

including dogs, sheep, goat, chicken, pig and cattle. They reported a general prevalence of 21.7%. Since scavenging local chicken are heavily consumed in Tanzania, an ESBL research with its sole focus of local chickens becomes necessary.

With the huge knowledge gap created on the effect of ESBLs in chicken, especially scavenging local chickens in Tanzania and Africa as a whole, this research aims at investing and bringing to light, the diversity of ESBL genes in E. coli isolated from these birds.

CHAPTERTHREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area chosen for this research is the Morogoro Municipality. It has a total area of about 531.6 km^2 and a population growth rate of 4.7% per annum. The percentage of the populace thatis engaged in livestock keeping and subsistence farmingis 33% (National Berau of statistics, 2011). The Municipal is subdivided into 29 administrative wards, out of which six vicinities were randomly selected. These are: Vibandani, Mazimbu, Kidididimo, Misufini, Magadu and the farm at the department of Animal Science and Production at the Sokoine University of Agriculture.

3.2 Study Design and Sample Collection

$N= Z^2 P (1-P) /\Sigma^2$. N is sample size, Z is constant (1.96), P is prevalence and Σ is error margin (0.05). Due to the absence of epidemiological data on ESBLs in Tanzania, an assumed prevalence of 50 percent was used in the calculation, which gave us a sample size, N=384 for each. However due to financial and time constraint, 200 samples were collected. This comprised 100 cloacae and 100 oral-pharynges swabs from each housing system; giving a total of 400 samples. This was a cross-sectional study; the samples were collected only once. The birds were not exposed to any pre-defined conditions.

Swabs from semi-intensive birds were collected from the following wards: Mazimbu, Magado and the animal science farm(SUA) whiles those from extensive birds were from Vibandani, Mesophini and Kidimo.The swabs were collected and kept in transport media and transferred into the laboratory on ice.

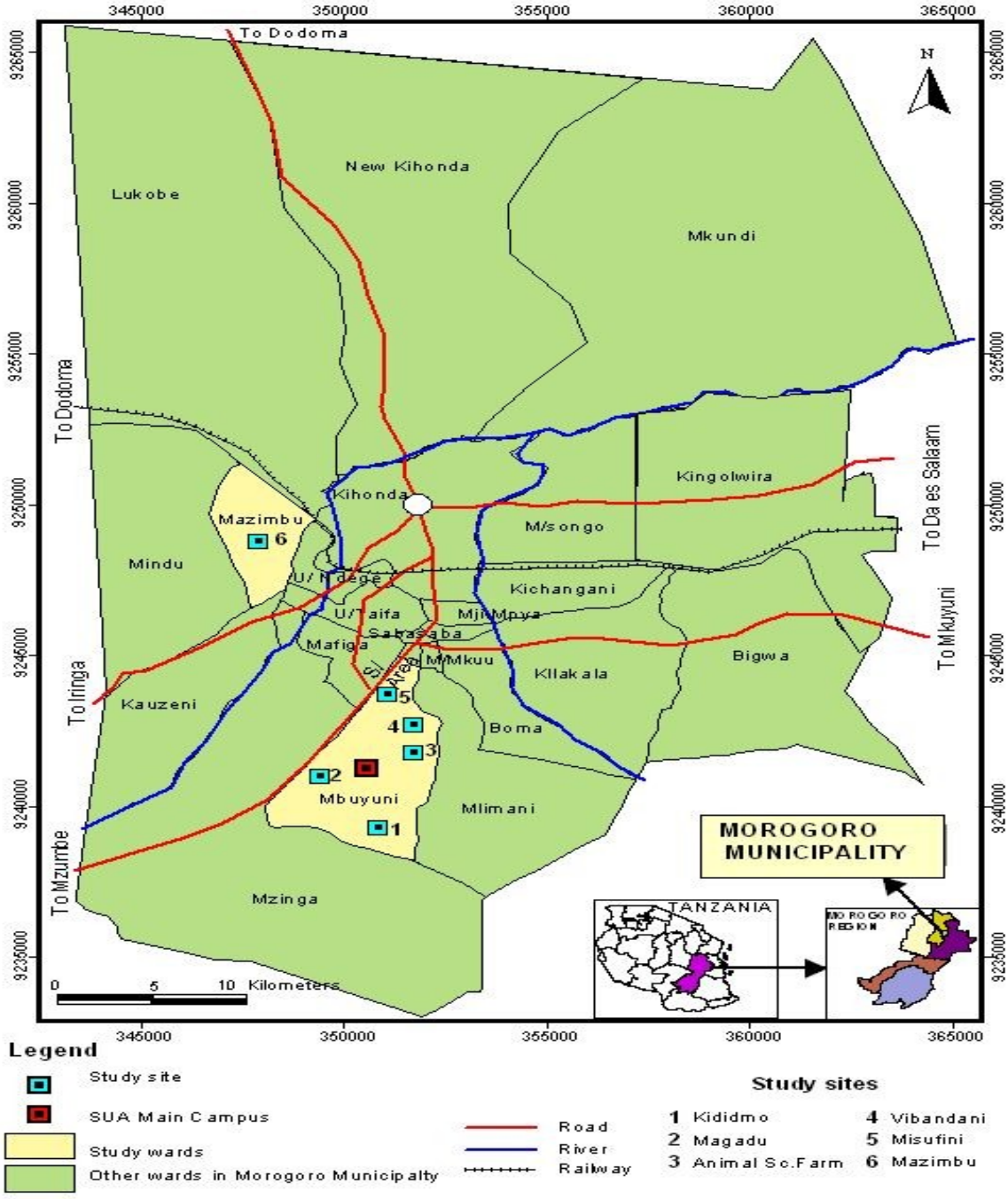

Figure 1: Map of Morogoro Municipality showing wards(map constructed using Arc view GIS)

3.3 Isolation of *Escherichia coli*

Procedures used were as described in the Bacterial Analytical Manual(BAM 2007). The organisms were grown on MacConkey and Blood Agar(OXOID, Hampshire, England) for primary isolation. The Blood agar base was supplemented with 10% horse blood.A loop full of each sample from the transport media was introduced on the media plate and was streaked appropriately with a sterile inoculating loop.

The media plates were appropriately labeled and incubated at 37ºC for 24 hours. Based on morphological appearance, the suspected positive isolates were further cultured on MacConkey agar. The colonies were selected and stored in nutrient broth and refrigerated at 4ºC to await further analysis. The following biochemical tests were performed to confirm the suspected isolates: Indole,Methyl Red, Voges-Paskeur,Citrate, Triple Sugar Iron and Motility test.

3.4 Biochemical Tests

3.4.1 Indole test

The organisms were grown on nutrient agar and cultured overnight to obtain pure isolates. Peptone water suspension was prepared according to the manufacturer's protocol; and three to five isolates were cultured in them and grown overnight. Two to three drops of Kovac's reagent were added to the suspension and the bottle was shaken a little. The formation of a pink-colored ring that rises to the surface indicated a positive for *Escherichia coli* (BAM, 2007).

3.4.2 Methyl Red and Vogues-Paskeur (MR-VP) test

The following reagents and broth were prepared according to BAM 2007 and manufacturer's protocol: Methyl red reagent: 0.1g of methyl red was dissolved in 300ml

of 95% ethyl alcohol and 200ml of distilled water was added.Voges-Proskauer reagent A (VPa) was made by dissolving 5g of alpha-naphthol in 200ml of absolute ethyl alcohol. The volume of the alcohol was brought to a 100ml mark.Voges-Proskauer reagent B (VPb) was made by dissolving 40g of potassium hydroxide pellets in 200ml of distilled water. The volume of the distilled water was brought to the 100ml mark.The MR-VP broth was prepared according to the manufacturer's protocol. It was distributed into universal bottles and autoclaved at 121ºC for 15 minutes. Pure isolates of the organisms on nutrient agar were inoculated into the broth and grown for 48 hours. The broth was then divided into two separate universal bottles; one to the MR test and the other for the VP test. In performing the MR test, 5 drops of the MR reagent were added to the broth. A positive test result is when the color of the broth remains red.

In the VP test, 6 drops of the VPa reagent were added to the broth, followed by 2 drops of VPb. The bottle was shaken a little and allowed to sit for a few seconds. Formation of a pink to red color indicated a positive resultfor *Escherichia coli* (BAM, 2007).

3.4.3 Citrate test

The Simon's citrate agar was prepared according to the manufacturer's protocol. Pure isolates of the organisms on nutrient agar were inoculated into the agar and incubated for 24 hrs. The citrate agar is green before inoculation. A positive result is when the color changes to blue, meaning that the citrate is utilized, a characteristic of *Escherichia coli* (BAM, 2007).

3.4.4 Triple sugar iron (TSI) test

TSI agar was prepared according to the manufacturer's protocol. It was distributed in test tubes and autoclaved for 121ºC for 15mins. The tubes were then slanted on the working

bench and allowed to solidify for 24 hrs. The tip of a well isolated colony was picked with an inoculation needle. The center of the agar was stabbed with the needle and then the surface of the slant was streaked. It was then incubated at 37ºC for 24hours. A yellow butt and slant and the presence of gas (bubbles) indicated that the organism is positive for *Escherichia coli* (BAM, 2007).

3.5 DNA Extraction

The positive *Escherichiacoli* genomic DNAs were extracted by boiling. The frozen cultures were stored at -45ºC in 15% glycerol nutrient broth. They were then thawed, homogenized and sub cultured on nutrient agar for 24hrs at 37ºC.

3.6 Polymerase Chain Reaction

3.6.1 Reagents and chemicals used

Nuclease free water for all PCR reactions was purchased at Ambion limited, United States of America.Taq polymerase standard buffer, 2x concentrated together with 100bp DNA ladder were manufactured byBiolabs incorporated, New England. The loading dye used was 6X loading dye. Agarose gel was prepared by diluting 1.5% of agarose in100mlof X1 TBE (Tris Borate EDTA)buffer and stained with 3µl of gel red stain. The gel was run at 100Volts for 40 minutes and gel pictures taken with a gel documentation machine. The gel electrophoresis machine as well as the PCR thermal cycler weremanufactured by Takara in Japan.Gel Documentation machine was manufactured by Biorad limited.

3.6.2 16SrDNA sequencing

The 16S rDNA genes in the *Escherichia coli*isolates were amplified by PCR and sequenced. The protocol for the PCR was as follows: The procedures were performed in 20µl reaction mixture. This included: 10 µl of Taq polymerase (Dream Taq PCR Master

mix, (Thermofischerthermoscientificltd), 1 of each 100Mm dNTP, 0.1µl (100pmol) oligonucleotide primer pair, 5 µl of nuclease-free water and 3µl of template DNA. Primer concentration was 0.5 M. Conditions of the reaction mixtures included: 5mins at 95ºC initial denaturation, 95ºC of denaturation for 30s, annealing at 56ºC for 30s, elongation at72ºC for 45seconds at 25 cycles, a final elongation at 72ºC for 10 minutes and a hold at 4ºC.The PCR products were then sequenced by the Sanger method of sequencing with the BigDye terminator v3.1 sequencing kit. Primers used are listed in Appendix 3 and they were purchased from Macrogen Inc., Seoul, Korea.

3.6.3 Virulence factor profiling to detect APEC strains

The positive *E. coli*strains, 192 in number, were investigated for various virulence genes by multiplex PCR, with protocol adopted was that ofEwers *et al.*(2007). The procedures were performed in 25µlreaction mixtures. This included: 12.5 µl ofTaq polymerase (Dream Taq PCR Master mix, ThermofischerthermoscientificLtd), 0.5 µl of each 100Mm dNTP, 0.1µl(100pmol) oligonucleotide primer pair, 6.9 µl of nuclease-free water and 4µl of template DNA. Primer concentration was 0.4 M. Conditions of the reaction mixtures included: 5mins at 95ºC initial denaturation,94ºC of denaturation for 30s, annealing at 56ºC for 30s, elongation at72ºC for 3minutes at 25 cycles, a final elongation at 72ºC for 10 minutes and a hold at 4ºC. A List of primers used is shown in appendix 3.

3.7 AntimicrobialSusceptibility Test of APEC Strains

3.7.1 Kirby-Bauer disc diffusion test

The Kirby-Bauer antimicrobial sensitivity test method was used to determine the isolates that were susceptible to cephalosporins and beta-lactams. Ten antimicrobial drugs were used. These included;augmentinin (30µg), imipenem (10µg), cephalothin (30µg), cefotaxime (30µg), ceftazadime (30µg), ceftriaxone (30µg), Nalidixic acid (30 µg),

ciprofloxacin (5µg), Gentamycin (10µg) and Trimethoprim-sulphamethiozole (25µg). The drugs were manufactured by Liofilchem limited, Italy.

Mueller-Hinton agar was prepared according to the manufacturer's protocol. The organisms were cultured on nutrient agar. Between 4 and 5 isolated colonies of the organisms were suspended into about 2ml of sterile saline by use of inoculating loop. The saline tube was vortexed to create smooth suspension. The turbidity of the suspension was adjusted to a 0.5 McFarland standard. Exactly 200ml of the suspension was introduced unto the Mueller-Hinton agar plate. Sterile glass spreader was used to spread the organisms on the plate. The surface of the plate was allowed to dry for 5minutes before the antimicrobial discs were placed on them.

A pair of sterile forceps was used to remove the antimicrobial discs from the dispensers. After placing the discs on the agar, each disc was gently touched with the inoculating loop to ensure their contact with the agar surface.

3.7.2 Double disc synergy test: detection of ESBL producers

As described by Ravi *et al.*(2011), the double disc synergy was performed to confirm the ESBL producers. The discs of ceftazadime (30µg), ceftriaxone (30µg) and cefotaxime (30µg) were placed around an augmentin disc (30µg), 20mm apart, on a Mueller-Hinton Agar plate swabbed with the test isolate. Enhancement of the inhibition zone of the cephalosporin toward the augmentin disc was interpreted as positive for ESBL production.

3.8 Screening of ESBL genes

Isolates that were positive for ESBL were screened to know the types of beta-lactamase(*bla*) genes they harbored.A total of eight samples were screened for the

presence of 12 *bla* genes. The protocol adopted was that of Kiiru*et al*.(2012).Thereactions were carried out in a 25 µl reaction volume. This consisted of 12.5 µl of taq polymerase, 1 µl each of the primer sequence, 5.5 µl of the Nuclease free water and 5 µl of the DNA template.The primer concentration was 0.4M.The PCR reactions included the following:5 minutes of initial denaturation at 95ºC,94ºC of denaturation for 30 seconds, annealing for 30 seconds, elongation at72ºC for 30 seconds at 30 cycles, a final elongation at 72ºC for 10 minutes and a hold at 4ºC.The annealing temperatures were different for the different primers. The primers used for this and the corresponding primers sequences, as well as the annealing temperatures have been listed in Appendix 3.

3.9 Analysis of Results

Biodata and laboratory data were analyzed using Epidemiological Package for Information (EPI Info) version 7 statistical software (CDC, Atlanta, GA, USA). The threshold for statistical significance was indicated in the table with a $P < 0.05$ reflected statistical significance.In biological analysis; the following software were employed: Molecular Evolutionary for Genetic Analysis (MEGA) 7. Sequencing products were analyzed on the National Committee for Biotechnology Information (NCBI) using the basic alignment search tool (BLAST)

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *E. coli*

After primary culture of samples on Macconkey and blood agar; 192, out of 400 samples were positive for *Escherichia coli.*

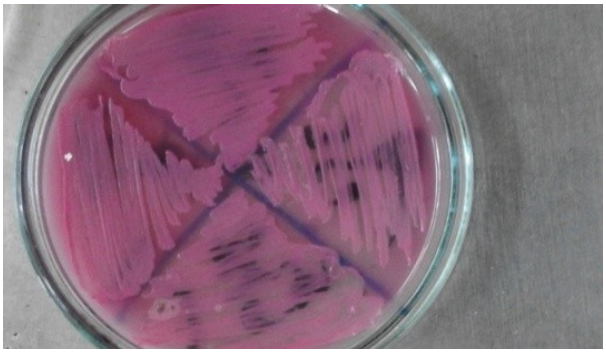

Figure 2:Pure isolates of *Escherichia coli* on Blood Agar plate

4.2 Biochemical Tests

The suspected positive *Escherichia coli* isolates were all confirmed in the biochemical tests. All suspected isolates, 192, were confirmed to be positive for*E. coli.*

Figure 3: Biochemical tests. From left: indole, citrate, methyl red,Voges-Paskeur and Triple Iron Sugar.

Table 1: Occurrence of *E. coli* isolates from scavenging local chickens in selected wards in Morogoro Municipality

	Wards	Total Samples collected(N=400)	*E. coli* isolated (N=192)	
			Number	Percent
Semi	SUA	120	94	78.33
Intensive	Magado	52	3	5.77
	Mazimbu	34	0	0.00
Extensive	Vibandani	110	51	46.36
	Mesophini	42	14	33.33
	Kididimo	60	32	53.33

Note: *E.coli*isolated from chickens in the extensive housing systemwere more than the semi-intensive system.

4.3 Confirmation of *Escherichia coli*

4.3.1 *Escherichia coli*confirmation by 16SrDNA gene sequencing

The *Escherichia coli*isolates were analyzed by PCR for the presence of 16S ribosomal DNA gene. All of them detected this gene as shown below. The PCR products were then sequenced

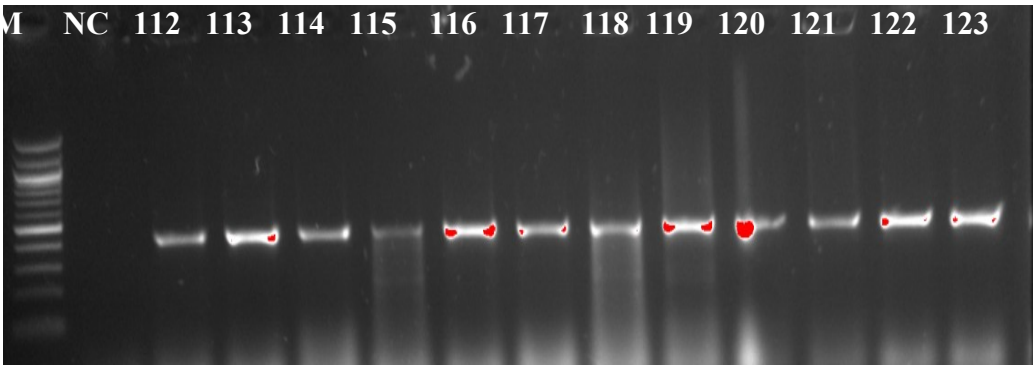

Figure 4: PCR detection of 16SrDNA gene(401bp). PCR products visualized under gel documentation. Lanes 112,113,114,116,117,118,119,120,121,122 and 123 were all positive. NC is negative control. M is marker (100bp).

4.3.2 APEC: virulence factor profiling and antimicrobial sensitivity test

Virulence factor profiling to detect APEC strains

PCR amplification to detect the virulence genes showed that 19 out of 192 samples, (9.8%) were APEC positive. Thus, these isolate had at least 4 virulence factors.

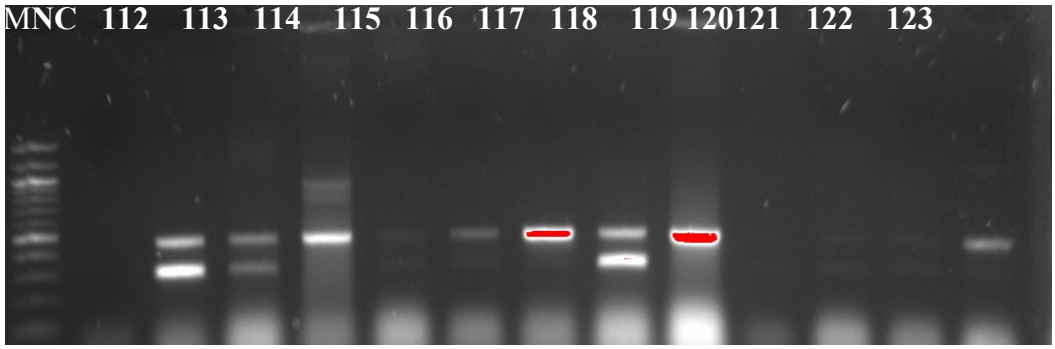

Figure 5: PCR detection of virulence of APEC(*traT and chuA*). MultiplexPCR visualized under gel documentation. Lanes 112,113,114,116,117,118,119 and 123 are positive for gene *traT*(430bp)while lane 112,113 and 118 are positive for gene *chuA*(278bp). NC is negative control. M is marker (100bp).

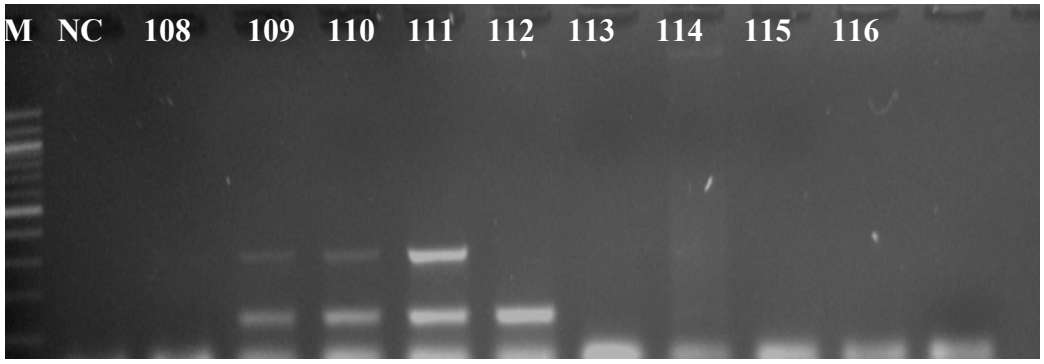

Figure 6: PCR detection for virulence of APEC(*iss and astA*), MultiplexPCR products visualized under gel documentation. Lanes 109,110 and 111 are positive for gene *iss*(309bp)and *astA*(116bp) while lane112 is positive for gene *astA*. With a 100bp DNA marker. NC is negative control. M is marker (100bp).

Table 2: Prevalence of virulence genes among APEC strains as detected by multiplex PCR

Virulence factor	Number of APEC isolates that harbored gene (N=19)	Percentage	P-value	Percentage
Iron Acquisition				
chu A	10	52.63		52.63
iro N	4	21.05		21.05
irp 2	2	10.53		10.53
iucD	5	26.32		26.32
sit Chr	1	5.26		5.26
sit ep	4	21.05	0.016	21.05
Serum resistance				
cvi/cva	1	5.26		5.26
iss	15	78.95		78.95
omp A	4	21.05		21.05
tra T	12	63.16	0.030	63.16
Adhesins				
pap C	2	10.53		10.53
tsh	2	10.53	0.602	10.53
Toxins				
ast A	9	47.37		47.37
vat	2	10.53	0.005	10.53
Invasins				
gimB	1	5.26		5.26
ibe A	16	84.21	0.00001	84.21

Note: The virulence traits *ibeA, iss, traT and chuA* were observed to lead the chart with percentages of 84.21, 78.95, 63.16 and and 52.63 respectively.Iron acquisition, serum resistance toxins and invasins were found to be very significant ($P<0.05$)

4.4 Antimicrobial Susceptibility Test of APEC Isolates

4.4.1Kirby-Bauer disc diffusion test

The antimicrobial phenotypes of APEC isolates were investigated using Kirby-Bauer disc diffusion method. The zones of inhibition were measured and the resistance was recorded based on Clinical and Laboratory Standards Institute (CLSI).

Table 3: Proportion of antimicrobial resistance profile of APEC

	Susceptible isolates		Intermediate isolates		Resistant isolates	
Antimicrobial	**Number**	**Percent**	**Number**	**Percent**	**Number**	**Percent**
CRO(30µg)	14	73.68	3	15.79	2	11.78
CTX(30µg)	12	63.16	4	21.05	3	17.65
CAZ(30µg)	15	78.95	3	15.79	1	5.88
CN(10µg)	19	100	0	0	0	0
STX(25µg)	8	42.11	0	0	11	64.71
AUG(30µg)	16	84.21	0	0	3	17.65
NA(30µg)	12	63.16	0	0	7	41.18
CIP(5µg)	10	52.63	9	47.37	0	0
KF(30µg)	3	15.79	2	10.53	14	82.35
IMI(10µg)	19	100	0	0	0	0

Note: Antimicrobial resistance profiles of APEC. CRO: ceftriaxone, CTX: cefotaxime, CAZ: ceftazadime, CN: gentamycin, STX: Trimethroprim-Sulfamethoxazole, AUG: augmentin, NA: nalidixic acid, CIP: ciprofloxacin KF: cephalothin, IMI: imipenem.Resistance of isolates were high to cephalothin and sulphonamide drugs while more isolates were susceptible to imipenem, augmentin, ceftriaxone and ceftazidime drugs.

4.5 ESBL Producers (Double DiscSynergy Test)

After the disc diffusion test, 10 isolates appeared to be potential ESBL producers. These isolates had the following zone of inhibition: Ceftriaxone (CRO) ≤ 25mm, cefotaxime(CTX) ≤27mm and ceftazadime(CAZ) ≤ 22mm.Double-disc synergy test was

carried out on these isolates for confirmation. After the synergy test, isolates that showed enhancement of the inhibition zone were considered ESBL producers(Ravi *et al*.,2011). A total of eight isolates qualified as ESBL producers.

Table 4: Double-disc synergy test

APEC isolates that showed an enhancement of inhibition zone after DDST (n=19)		
Oxyimino-Cephalosporins	**Number**	**Percent**
CRO	8	42.11
CTX	8	42.11
CAZ	5	26.32

Note: A total of 8 isolates showed enhancement of zone of inhibition to ceftriaxone and cefotaxime while 5 isolates demonstrated enhancement of zone of inhibition to cefotaxime.

4.6ESBL Genes Detection and Diversity

Screening for beta-lactamase genes

Thephenotypically positive ESBL isolates (8) were screened for the presence of twelve beta-latamase genes, out of which sevengenes were found to be present in various percentages.

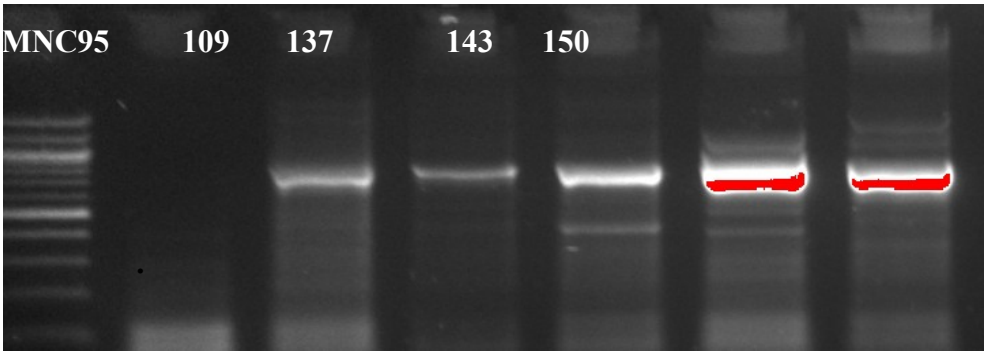

Figure 7: PCR detection of *bla*CMY-2 gene. Five samples (95, 109, 137, 143 and 150) were positive for the *bla*CMY-2(758bp); samples.NC is negative control. M is marker (100bp).

Table 5: Prevalence of beta-lactamase genes amongst ESBL producing isolates

Primer name	Target gene	Number (n=8)	Percentage
TEM	*bla* TEM	8	100
CTXM 1	CTX-M group I	1	12.50
CTXM 914	CTX-M group IV	3	37.50
CTXM 825	CTX-M group III	4	50.00
CMY 2	*bla* CMY-2 group	5	62.50
OXA 1	*bla* OXA-1	6	75.00
SHV	*bla* SHV	1	12.50

Note: Among the beta-lactamase genes, the highest recorded was *bla* TEM, followed by OXA-and CMY-2 while SHV was the least.

Table 6: Occurrence of beta-lactamase genes among housing systems of scavenging local chicken

ESBL gene	Extensive		Semi-Intensive	
	Number	Percent	Number	Percent
*bla*TEM	3	100	5	100
CTX-M group I	0	0	1	20
CTX-M group IV	2	33.33	2	40
CTX-M group III	1	66.67	2	40
*bla*CMY- 2	3	100	2	40
*bla*OXA1	3	100	3	60
*bla*SHV	0	0	1	20

Note: Isolates from the semi-intensive system were observed to be more widely distributed and diverse than those from the extensive system. The former harbored CTX-M group I and *bla* SHV genes which were absent in the later.

Table 7: Prevalence of *E. coli*, APEC and ESBL in characteristics of scavenging local chicken

Characteristics		*E.coli* n=192	P-value	APEC n=19	P-value	ESBL (n=8)	producers P-value
HOUSING SYSTEM	Extensive	95	0.918	13	0.051	3	0.619
	Semi-Intensive	97		6		5	
SEX	Hen	108	0.018	8	0.517	1	0.010
	Cock	84		11		7	
SITEOF SAMPLE	Cloacae	107	0.032	13	0.050	4	1.000
	Oral-pharynges	85		6		4	

Note: The sex of the chicken is statistically significant to the absence or present to *E. coli* and ESBL isolates (P<0.05). The site of sample is also statistically significant to the absence or presence of *E.coli.* For all other characteristics studied, there was no statistical significance to the presence of E. coli, APEC or ESBL isolates (P>0.05)

Table 8: Prevalence of beta-lactamase genes among characteristics of scavenging local chicken

Characteristic	Number (n=32)	Percentage	P-value
HOUSING SYSTEM			
Semi-intensive	17	53.13	0.802
Extensive	15	46.87	
SEX			
Cock	27	84.38	$4.5X10^{-8}$
Hen	5	15.62	
SITE OF SAMPLE			
Cloacae	13	40.63	0.211
Oro-pharynges	19	59.37	

Note: Beta-lactamase genes were harbored by cocks as compared to hens, and this proved to be statistically significant (P<0.05). Housing system and site of sample wasn't statistically significant on the harboring of beta-lactamase genes (P>0.05).

PCR products of the APEC isolates were sequenced using the Sanger sequencing method. The sequencing products were blasted on the NCBI website and various samples revealed identities to various genes at different identities as shown below.

Table 9: Description of beta-lactamase genes on basic local alignment sequence tool (BLAST)

Gene	Housing system	Description gene	Identity	Accession
*bla*OXA 1	Semi-Intensive	OXA-1	99%	NG 049392.1
	Extensive	OXA-1	99%	NG 049392.1
*bla*TEM	Semi-Intensive	TEM 171	99%	NG 050214.1
	Extensive	TEM 171	99%	NG 050214.1
*bla*CTX-M9	Semi-Intensive	CTX-M-15	92%	FJ997866.1
	Extensive	OXY-1-6	92%	NG 049845.1
*bla*CMY-2	Semi-Intensive	CMY-71	98%	NG 048859.1
	Extensive	CMY-71	98%	NG 048859.1
*bla*SHV	Semi-Intensive	LEN-2	92%	NG 049274.1

Note: When blasted the *bla*SHV gene detected a LEN-2 gene with an identity of 92% and the bla CTX-M 9 gene detected a CTX-M-15 gene with an identity of 92%.

CHAPTER FIVE

5.0 DISCUSSION

A total of 400 samples from cloacae and oral-pharynges of scavenging local chicken were taken in selected areas in the Municipality of Morogoro, Tanzania. Out of these, 192 (48.0%) of them were positive for *E. coli*.These were also confirmed by 16SrDNA sequencing (Figure 4). *Escherichia coli is* known to be the most prevalent commensally enteric bacteria in animals, as such food contamination of them is closely associated with fecal contamination (Odwar *et al.*, 2014). In line with this, cloaca swabs have become the target for *E. coli* isolation in chicken.With regards to statistics, Khaton *et al*. (2008)isolated *E. coli* from 83% of apparently healthy chicken that they employed in their research on *collibacillosis* in chicken. This research recorded a higher number of *E.coli* in cloaca swabs than the oral-pharynges swabs. Cloaca swabs accounted for more than half (55.23%) of the total number of *E. coli* encountered(Table7). *E.coli* is one of the pathogenic bacteria that are picked up by chicken during scavenging. Since these types of chicken scavenge mainly for their nutritional requirement, they have a tendency of harboring *E.coli*(Hamisi *et al*., 2014).

APEC is known to cause extra-intestinal infections in chickens, turkeys and other avian species. The infection often begins at the respiratory tract and air sacs. All single virulence genes that are found in any APEC strains are present in all non-pathogenic strains as well. This suggests that different virulence mechanisms are employed by different putative pathotypes as observed by Dziva and Stevens (2008). Samples were collected from apparently healthy chickens. However, at least one virulence factor was present in all 192 *E. coli* isolates. This supports the notion that commensal *E.coli* in the intestines of healthy birds may carry an array of virulence factors of APEC that makes them potentially

pathogenic (Collingwood*et al*., 2014). Virulence factors known to be expressed by APEC strains include; adhesions, invasions, toxins, iron acquisition and serum resistance. Out of the 16 virulence factors detected in this study, 12.5% of them were invasions (*ibeA* and *gimB*), 12.5% were adhesions (*papC* and *tsh*), 12.5% were toxins(*EAST-1* and *vat*), 37.5% were for iron acquisition (*chuA*, *OmpA*, *sit ep*, *iucD*, *iron* and *sit chr*) and the remaining 25% were for serum resistance (*iss*, *tra T*, and *cvi/cva*), (Table 2). Based on the criteria used by Dziva*et al*. (2008), isolates that harbored at least four of the 16 virulence factors were considered APEC. Out of 192 *E.coli*, 19 (9.5%) were found to be APEC. The four most prevalent virulence genes that were present in more than 50% of the isolates were *ibeA*, *iss*, *traT*and*chuA*in the percentages; 84.21, 78.95 63.16 and 52.63 respectively (Table 2).

Iron acquisition, serum resistance, toxins and invasion were seen to be statistically significant to the pathogenesis of APEC, with P-values < 0.05 (Table 2). The most widely distributed virulence gene is *ibeA*(invasion of the brain epithelium),which was harbored by 84.21 % of the isolates. Germon (2005)investigated and established its association with the pathogenecityof avianExPEC strains for chicken. It is located on the *GimA* island, encodes a 50-kDa protein and known to increase in early stage of chicken infection (Cortes *et al*., 2008). With the background that the expression of *ibeA*is known to increase in early stage of chicken infection, we could infer that majority of these chicken may be at risk of any opportunistic disease that may break.Wang *et al*.(2011) investigated the effects of the *ibeA* gene on virulence and biofilm formation of APEC. They realized that the *ibeA* gene enhances invasion capacity and biofilm formation of chicken, which increases the ability to cause disease. Flechard *et al.* (2012)recently demonstrated a new role for the gene in oxidative stress.In investigating this role, they observed that the gene by itself is

able to confer increased H_2O_2 resistance to *E.coli*. The *gimB* gene, which is also an invasion, was present in only 5.25% of the isolates.

The second most prevalent factor noted is the*iss* (increased serum survival) gene which was harbored by78.95% of the isolates (Figure 6). The significant feature about this gene is that it is a trait that distinguishes avian ExPEC from that of human (Johnson,2008). In investigating the immune response of recombinant *E. coliiss* protein in poultry, Lynn *et al.* (2006) found out that the presence of *iss* gene is strongly associated to APEC than commensal *E. coli*. It then became a target of *colibacillosis* control procedure.Findings byRocha *et al*. (2008)supported the knowledge on increased prevalence of the *iss* gene. They probed a total of 61 *E. coli* isolates from chicken flocks with respiratory symptomatology for the presence of seven virulence genes. The *iss* gene was harbored by 73.8% of the chicken, the highest amongst the seven. Although their results correspond to one observed in this study, we took samples from apparently healthy chicken while they isolated samples from diseased birds.This gives an indication that apparently healthy chickens could be a repository of virulence genes, especially *iss*.

The *traT* gene was the third most prevalent virulence factor recorded (figure 5). It is a surface protein that is encoded by conjugative plasmids. Binns *et al.*(1982)showed that the gene was responsible for the complement resistance of the R100 plasmid. Its significance to this work lies in the fact that, together with the *iss* gene, the two mediate virulence by serum resistance. That is to say, that serum resistance is critical in the pathogenicity of APEC. Other virulence genes of serum resistance were also observed.*Cvi/Cva*and *OmpA*genesrepresented only 5.26% and 21.05% respectively.

In reference to iron acquisition virulence factors, the *chuA* gene was the highest (Table 2). Sequestration of iron by host compound results in reduced iron concentration, an environment that hampers the growth of bacteria. By been associated with this phenomenon, iron acquisition systems have been linked to bacterial virulence (Li *et al*.,2005). The *chuA* gene, specifically, encodes an outer-membrane protein that is involved in heme uptake in *E .coli*. It's been known to be a very significant iron acquisition factor(Stocki*et al*.,2002). It was harbored by 52.63% of the isolates. The rest *iroN, irp2, iucD*, sit *chr*and *sit epi* were present in 21.10%, 10.53%, 26.32%, 5.26% and 21.05% of the samples respectively (Table 2).

Toxins of APEC were amongst the least recorded. The *vat* and *astA*genes were harbored by 10.53% and 47.37% of the APEC isolates respectively. In a longitudinal study to explore the carriage of virulence-associated genes by chicken, Kemmet*et al.* (2013) made similar observations. They realized that toxin encoding genes of APECwere the least frequently detected. In their study, the *vat* and *astA*genes averaged 2.11% and 1.12% prevalence respectively. On their part, Priost*et al.*(2013) noted that the *astA* gene product is an enteroaggregative heat-stable toxin called EAST 1. They further added, that the EAST 1 is mainly related to diarrheagenic-enteroaggregative strains which are associated with persistent watery diarrhea in human. They also recorded low prevalence of these APEC toxins in comparison with other virulence genes when they assessed the genetic similarity between APEC and *E. coli* strains in healthy birds.

Adhesins are being reported to have low incidence in avian pathogenic isolates, as such they are not reliable tools for diagnosis (Stordeur*et al*.,2002). It is therefore not surprising that this study observes them as amongst the least virulence factors;*Pap C* and *Tsh*genes each present in 10.53% of the isolates.

The antimicrobial phenotypes of APEC isolates were investigated using Kirby-Bauer disc diffusion method. As high as 94.7% of them showed resistance to at least one of the antimicrobial tested against (Table 3).The first study to investigate antimicrobial resistance phenotypes in *E. coli* isolates from scavenging local chicken in Tanzania was by Hamisi*et al.* (2014). In their findings, 66 out of 77(87.01%) of the birds showed resistance to at least one of the drugs, with 84.37% being multi-drug resistant. Similar result, after two years gives the indication that antimicrobial resistance amongst local scavenging chickens is on the increase.

Among the APEC isolates, resistance to drugs was observed more in isolates from the extensively kept chickens than those from the semi-intensively kept ones. The abuse of antimicrobials has been associated with high prevalence of their resistance in bacterial isolates from chickens(Byarugaba, 2009). One would therefore expect that for animals that are not exposed to antimicrobials, resistance amongst their isolates should be minimal. This however is not the case. When the same observation was made by Amadi*et al.*(2015), they concluded that the environment could be a plausible source of thesegenes. They investigated the antimicrobial resistance profiles of *E. coli* from feces of healthy free-range chickens and realized that the resistance is on the increase.

There have been reports of multi-drug resistance and zoonotic potential of APEC. An isolate is said to be multi-drug resistant (MDR) if it resists drugs from three or more categories. Research by Cunha *et al*. (2014) showed that the MDR of APEC has been on the ascendency. They characterized APEC strains isolated from poultry and realized that 92% of the strains presented MDR with the highest resistance to sulfamethoxazole. In this study, 10.52% of APEC isolates were MDR, hence calling for concern. Also

sulfamethoxazole was the second most in-actice drug, with 64.71% of the isolates being resistant to it.

Among the APEC isolates, the leastactive drug observed was cephalothin. As high as 82.35% of the isolates resisted it (Table3). This was anticipated because it is a first generation cephalosporin and has been known for its prophalytic use in animals for years. As such, it is the weakest cephalosporin(FAO, 2011). In a retrospective study on antimicrobial resistance in *E.coli* from humans and food animals from 1950 to 2002,Tadesse*et al.*(2012)realized that cephalosporin resistance increased over time among the animal *E. coli* than the human *E.coli.* Since it's an early cephalosporin, the cephalothin becomes the first to lose its strength.

Sulfamethoxazolewas the second most in-active drug, 64.74% of the isolates resisted it (Table 3).Bacteria from farm animals are noted for their frequent resistance to sulphonamides(Kozak *et al*., 2009).Furtula *et al*.(2010) also confirmed this when they researched on the veterinary pharmaceuticals and antimicrobial resistance in *E. coli*. They reported that resistance to sulphonamides was the most prevalent among the *E. coli* isolates.In rationalizing the occurrences of this,Huovinen and Eliopoulous(2001)noted that the drug is widely used in developing countries as a first-line drug of choice for the treatment of bacterial infections, thus a huge resistance is to be expected.

All APEC isolates were sensitive to gentamycin and imipenem, an indication that these drugs are the most active antimicrobials.Aminoglycosides, particularly gentamicin are the most effective antimicrobials against isolated bacteria. These drugs are rarely used in the treatment of human or veterinary-associated illnesses due to their high cost. As such, there is no selective pressure on these drugs among the bacteria and hence bacteria are very

sensitive to them. Mwambete and Stephen(2015)reported that the most effective antimicrobial against isolated bacteria were aminoglycosides, particularly gentamicin.In a cross-sectional study, they collected fresh chicken droppings from 100 chickens in 13 localities and determined their multiple antimicrobials resistance indices. More than half of the isolates (69.3%) were sensitive to gentamicin.With regards to resistance of ESBL isolates to drugs, resistance to nalidixic acid was leading (Table 2). A total of 87.5% of the ESBL isolatesresisted it. Also, all multi-resistant ESBL isolates showed resistance to it. High levels of resistance to nalidixic acid is often considered first step in mutation to fluro-quinolones resistant strains(Smet *et al*., 2008).That is to say, resistance of isolates to nalidixic acid can be linked to their resistance inciproflaxin (flouro-quinolone) as well. In this study,all but 2 of the isolates that were resistant to nalidixic acid were also sparingly resistant (intermediate) to ciprofloxacin.This was also noted bySmet*et al*.(2008) when they investigated the diversity of ESBL and class C beta-lactamases among claocae*E.coli* isolates.

Sensitivity of the beta-lactamases genes to the antimicrobials varied from the various drugs used. All isolates were sensitive to Imipenem, a carbapenem (Table2). Carbapenems, including imipenem are very active against ESBL-producing organisms. This is because of their stability to hydrolyze ESBLs (Paterson and Bonomo, 2005).A similar observation was made when Rasheed *et al.* (2014) investigated the antimicrobial drug resistance in strains of *E. coli* isolated from food sources, including chicken. They detected a total of six ESBL isolates, all of which were sensitive to imipenem and gentamicin. In a related research, Gundogan and Avci (2013)observed the prevalence and antimicrobial resistance of ESBL-producing *E.coli* and *Klebseilla* isolated from food animals. Once again, all isolates were susceptible to imipenem and other carbapenems used and gentamicin recorded one of the least resistances (6.7%).

After the double-disc synergy test, eight samples were found to be ESBL producers (Table 4). These samples were screened for the presence of twelve beta-lactamase genes.Eight of these genes were present among isolates in varying percentages:*bla*TEM-100%, *bla*OXA-1 -75.0%, *bla*CMY-2 62.5%, *bla*CTX-M-consensus 50.0%, CTX-M group III (CTX-M-8)-50%, CTX-M group IV (CTX-M -9,-13,-14,16,-17,18,-19,-20,-21 and -27) -37.5%, CTX-M group I (CTX-M -1,-3,-10,-11,-12,-15,-22,-23,-28,-29 and -30)-12.5% and *bla*SHV-12.5% (Table 5). Although the prevalence of ESBL types greatly vary with geographical location, the TEM gene has been reported with higher frequently in most cases while in others, SHV leads the chart (Rameshkumar *et al.*, 2016).The former was observed in this study.

The *bla*TEMand CTX-M genes have been observed to be the most abundant ESBL genes as reported by other researchers and seen here. In a cross-sectional study in Mwanza, Tanzania,Seni*et al*. (2016), identified the ESBL producing *Enterobacteriaceae*from rectal and cloacae swabs of 600 companion and domestic animals. They also discovered that TEM and CTX-M (specifically CTX-M-15) genes were the most prevalent ESBL genes. The CTX-M-15 gene was harbored by all isolates whiles the *bla*TEM was harbored by 60% of the isolates. The CTX-M-15 gene was detected in this study,with an identity of 92% to the CTX-M 9(group IV) gene when blasted (Table 9).It is one of the most prevalent variants in the world and are mainly associated with the FII plasmids (Nagano *et al*., 2009). The CTX-M-15 is related to phylogenetic group B2, a virulent extra-intestinal strain (Peirano and Pitout, 2010). Its association with apparently healthy chicken is somewhat alarming.

Two years before the Tanzanian study, Schaumburg *et al*.(2014)assessed the risk of importing ESBL producing *Enterobacteriaceae* and *S. aureus* through chicken meat in

Gabon. They also realized that CTX-M-1 and CTX-M-14 were predominant in ESBL *E. coli* from chicken. In this study, the CTX-M1 and CTX-M15, together with other genes in the CTX-M group I was detected by the primer CTX-M1. On the other hand, the CTX-M-14 belonging to CTX-M group IV was detected by the CTX-M914 primer. Together, these genes were harbored by 50% of *E. coli* in this study. In effect, the CTX-M and TEM genes of ESBL are still predominant among *E. coli* in chicken.

The *bla*CMY-2 gene is the most common plasmid mediatedAmpC beta-lactamase(Shayan and Bokaeian, 2015). Its resistance is also known to be against oxyimino-cephalosporins (cefotaxime, ceftriaxone and ceftaxidime) and other drugs eg. cephamycin. A total of 5 isolates harbored this gene. All but one showed complete and intermediate resistances to one or more of the oxyimino-cephalosporins. A number of researches, although carried outside the shores of Africa shows constituency to this.Shaheen *et al*. (2011)investigated the resistance to extended-spectrum cephalosporin in clinical *E. coli* isolates from companion animals in the United States. They reported that the *bla*CMY- gene was widely distributed and exhibited resistance against oxyimino-cephalosporins. Although they employed companion animals (as against chicken in this study) and worked in the United States (as against Africa), similar results could only imply that regardless of location and kind of isolates, CMY-gene exhibits high resistance against oxyimino-cephalosporins. On the blast, the *bla*CMY-2 gene detected an OXY-1 gene with an identity of 92%(Table 9). The OXY gene was first identified with *Klebsiellaoxytoca.* It is chromosomally encoded and offers resistance to amino-penicillin and carboxy-penicillins(González-López *et al*., 2009).

The *bla*SHV- gene was one of the least detected (12.5%). Derivatives of the SHV-1 gene, unlike their projenitor, is known to confer resistance to broad-spectrum penicillinas well

asoxyimino-cephalosporins(Rawat and Nair, 2010). In line with this, the only*bla*SHV-harbored isolate was resistant to all oxyimino-cephalosporins.When blasted the *bla*SHV gene detected a LEN-2 gene with an identity of 92%(Table 9). This gene chromosomally encoded, found in *K. pneuminiae* and does not hydrolyze extended-spectrum cephalosporins(Sieb*et al*.,2005).

E. coli was observed in the hen than in the cock, but vice-versa occurred in ESBL ($P<0.05$) (Table 7). Isolates from the cloacae harbored more beta-lactamase genes than those take from the oral-parynges ($P<0.05$) (Table 8). Other characteristics observed in the chicken in relation to *E. coli*, APEC, and ESBL proved of no statistical significance.

Although not statistically significant, beta-lactamase genes were found to be widely distributed and more diverse among isolates from the semi-intensively kept birds than the extensive ones. In reviewing the characteristics of scavenging local chickens in Africa, Fotsa(2011) stated that, extensively kept chickens have adequate immuno-competence. This is because they roam extensively in the environment for their nutritional requirements and are free from vaccines and antimicrobials. Unlike the semi-intensively kept ones, vaccines and anti-microbials are obscured in their rearing of chicken in the extensive system. Semi-intensive system of poultry rearing is characterized by farm in put supplies like drugs, feed and vaccine (FAO, 1999). In supporting evidence, only 2 out of 3, (66.67%) of isolates from extensively kept birds were resistant to at least one of the antimicrobials used, whiles all isolates from the birds in the semi-intensive (100%) showed resistance at various levels. In reference to multi-drug resistance (resistance to more than two categories of drugs), none of the isolates from the extensively kept birds proved to be multi-drug resistant. On the other hand, 40% of isolates from the semi-intensively kept birds were multi-drug resistant.

The subject of biosecurity measures as opposed to administration of antimicrobials favors the extensive system more. Gary and Miles (2015) reported on a relevant factor in the development of biosecurity. According to them, down time can be used to reduce the level of disease organisms in the environment. Down time is defined as the time between successive flocks when no chicken are present on the premises. That is to say that when the animals spend more time enclosed in a particular place, as in the case of semi-intensive birds, persistence of disease amongst them is inevitable. Although unconsciously practiced, birds under the extensive system enjoy a relatively greater amount of downtime as they are readily realized in the morning and come back late at night. Thus their premises are always left free.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

*Eschericha coli*is highly prevalent among scavenging local chickens. As much as 48.0% of the samples collected harbored *E.coli*, majority being isolated from the cloacae.Commensal *E. coli* in the intestines of healthy birds may carry an array of virulence factors of APEC, which makes them potentially pathogenic.

Single virulence genes observed among APEC strains were also present among the commensal *E. coli* as well, thus APEC is now considered a primary pathogen rather than a result of respiratory or immune-suppressive viral illnesses. Clinically healthy local scavenging chickens may act as reservoirs for APEC.

Majority of the APEC strains harbored *ibeA, iss*, *traT*and *chuA* with percentages of 84.21, 78.95, 63.16 and 52.63 respectively. Also thus invasions, serum resistance and iron acquisition systems and toxins play significant roles in the pathogenecity of APEC ($P<0.05$).

With regard to antimicrobial resistance, this research concludes that it is very much on the increase. As high as 94.7% of the APEC isolates showed resistance to at least one of the antimicrobials used. Resistance to cephalosporins and sulphonamide were 82.35% and 64.71% respectively and 10.5%of the APEC samples are multi-drug resistant.Amongst ESBL genes,CTX-M and *bla*TEM genes are very significant. The *bla*TEM gene was present in all isolates whiles three groups out of four of the CTX-M genes were present amongst isolates.

A fairly high percentage (48.38%) of ESBL genes were found among extensively kept chicken. Since these birds spend their day loitering in the environment, the source of ESBLs they harbor could be the environment.

6.2 Recommendations

i. Invasions,serum resistance, toxins and iron acquisition systems of APEC should be the targets of subsequent studies of APEC. Virulence factors in these systems proved very significant in the pathogenicity of APEC as revealed in this study ($P<0.05$).

ii. In the wake of high resistance to antimicrobials, the use of antimicrobials should rather be replaced with bio security measures. Since the former has been proven to be of threat to the health of the animals than the later.A biosecurity measure that allows the animals to spend more outdoor life is recommendable to reduce the prevalence of ESBLs among scavenging local chickens.

iii. Continual surveillance of ESBLs would be recommended. This would be a key to prevention of the emergence and subsequent spread of ESBL-producing *Enterobacteriaceae*.

iv. In a continent where investigations on ESBLs in food animals is rare, more research on the subject matter is highly recommendable. This is against the background that ESBL genes, especially the CTX-M genes, are very diverse while food animals, especially local chickens are heavily consumed.

v. A combination of phenotypic and molecular methods of detection of ESBLs is needed to thoroughly appreciate the mechanisms employed by these genes.

vi. Subsequent research on ESBL should focus also on the environment. It proved to be a likely source of ESBLs because 48.38% of the ESBLs gene found among birds in the extensive system of housing.

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APPENDICES

Appendix 1: The antimicrobial susceptibility chart (CLSI, 2014)

Antimicrobial Disc	**Code**	**R/≤mm**	**I/mm**	**S/≥mm**
Ceftriaxone	CRO	19	20-22	23
Cefotaxime	CTX	22	23-25	26
Ceftazadime	CAZ	17	18-20	21
Gentamycin	CN	12	13-14	15
Augmentin	AUG	13	14-17	18
Nalidixic Acid	NA	12	13-14	21
Ciprofloxacin	CIP	15	16-20	21
Cephalothin	KF	15	6-20	21
Imipenem	IMI	19	20-22	23
Trimethroprim-Sulfamethoxazole	STX	10	11-15	16

R: resistant I: Intermediate S: susceptible

Appendix 2: Classification of antimicrobial

Class	Examples
Lincosamides	Clindamycin, lincomycin
Streptogramins	Pristinamycin, quinupristin
Macrolide	Azithromycin, erythromycin, telithromycin
Aminoglycoside	Gentamicin, streptomycin, neomycin
Sulphonamide	Trimethroprim,sulfamethizole, sulfisoxazole
Monobactams	Aztreonam
first generation quinolone	Nalidixic acid, oxolinic acid, rosoxacin
second generation quinolone	Enoxacin, ciprofloxacin, ofloxacin
third generation quinolone	Baloflaxacin,galifloxacin,levofloxacin
fourth generation quinolone	Besifloxacin,clinafloxacin,gemifloxacin
Penicillin	Amoxicillin, ampicillin, bacampicillin,
Carbapenems	Imipenem, doripenem, meropenem
	Cephalothin, ceftezole, cefroxadime
First generation cephalosporin	Cefmetazole, cefotetan, cefoxitin
Second generation cephalosporin	Cefotaxime, ceftazidime, ceftriaxone
Third generation cephalosporin	

Reference: WHO (AGISAR),(2011)

Appendix 3: List of primers

Virulence factor profiling for APEC

TARGET	PRIMERSEQUENCE (5'-3'; s:sense , a:antisense)	DESCRIPTION
tsh[a]	s:ACTATTCTCTGCAGGAAGTC	Temperature-sensitive
	a:CTTCCGATGTTCTGAACGT	haemagglutinin
iuCD[a]	s:ACAAAAAGTTCTATCGCTTCC	Aerobactin synthesis
	a:CCTGATCCAGATGATGCTC	
PapC	s: TGATATCACGCAGTCAGTAGC	Pilusassociated with
	a: CCGGCCATATTCACATAAC	pyelonephritis
irp2	s: AAGGATTCGCTGTTACCGGAC	Iron-repressible protein
	a: TCGTCGGGCAGCGTTTCTTCT	
Sit ep	s: TTGAGAACGACAGCGACTTC	Salmonella iron transport
	a: CTATCGAGCAGGTGAGGA	system gene
ompA	s: AGCTATCGCGATTGCAGTG	Outer membrane protein
	a: GGTGTTGCCAGTAACCGG	
Iron	s: ATCCTCTGGTCGCTAACTG	Catecholatesiderophore
	a: CTGCACTGGAAGAACTGTTCT	(salmochelin) receptor
gimB	s: TCCAGATTGAGCATATCCC	Genetic island associated with
	a: CCTGTAACATGTTGGCTTCA	newborn meningitis
sitDchr	s: ACTCCCATACACAGGATCTG	Salmonella iron transport
	a: CTGTCTGTGTCCGGAATGA	system gene
traTa	s: GTGGTGCGATGAGCACAG	Transfer protein
	a: TAGTTCACATCTTCCACCATCG	
ibeA	s: TGGAACCCGCTCGTAATATAC	Invasion of brain endothelium
	a: CTGCCTGTTCAAGCATTGCA	
chuA	s: GACGAACCAACGGTCAGGAT	Heme receptor gene (E. coli
	a: TGCCGCCAGTACCAAAGACA	haemutilisation)
Vat	s: TCCTGGGACATAATGGCTAG	Vacuolatingautotransporter
	a: GTGTCAGAACGGAATTGTC	toxin
cvi/cva[a]	s: TCCAAGCGGACCCCTTATAG	Structural genes of colic in V
	a: CGCAGCATAGTTCCATGCT	operon(microcinColV)
iss[a]	s: ATCACATAGGATTCTGCCG	Increased serum survival
	a: CAGCGGAGTATAGATGCCA	
astA	s: TGCCATCAACACAGTATATCC	EAST1 (heat-stable cytotoxin
	a:TAGGATCCTCAGGTCGCGAGTGACGGC	associated with enteroaggregative E. coli)

Reference: Ewers *et al.*(2007)

Screening beta-lactamase genes			
Target Gene	**Primer**	**5'-3' sequence**	**Size**
*bla*TEM	TEM-F	ATGAGTATTCAACAT TTC CG	
	TEM-R	CCAATGCTTAATCAG TGA GG	840
*bla*SHV	SHV-F	TTCGCCTGTGTATTATCTCCCTG	
	SHV-R	TTAGCGTTGCCAGTGYTCG	854
*bla*CTX-M	MA1	ATGTGCAGYACCAGTAARGTKATGGC	
concensus	MA2	TGGGTRAARTARGTSACCAGAAYCAGCGG	593
CTX-M group I	CTXM1-F3	GAC GAT GTC ACT GGC TGA GC	
	CTXM1-R2	AGC CG C CGA CGC TAA TAC A	499
CTX-M group II	TOHO1-2 F	GCG ACC TGG TTA ACT ACA ATC C	
	TOHO1-1R	CGG TAG TAT TGC CCT TAA GCC	351
CTX-M group III	CTXM825F	CGC TTT GCC ATG TGC AGC ACC	
	CTXM825R	GCT CAG TAC GAT CGA GCC	307
CTX-M group IV	CTXM914F	GCT GGA GAA AAG CAG CGG AG	
	CTXM914R	GTA AGC TGA CGC AAC GTC TG	474
*bla*CMY	CF1	ATGATGAAAAAATCGTTATGC	
(consensus)	CF2	TTGCAGCTTTTCAAGAATGCGC	1200
	CMY-1 F	GTGGTGGATGCCAGCATCC	
*bla*CMY-1 group	CMY-1R	GGTCGAGCCGGTCTTGTTGAA	915
	CMY-2 F	GCACTTAGCCACCTATACGGCAG	
*bla*CMY-2 group	CMY-2R	GCTTTTCAAGAATGCGCCAGG	758
*bla*OXA-1	OXA-1 F	ATGAAAAACACAATACATATCAACTTCGC	
	OXA-1R	GTGTGTTTAGAATGGTGATCGCATT	820
	OXA-2 F	ACGATAGTTGTGGCAGACGAAC	
*bla*OXA-2	OXA-2R	ATYCTGTTTGGCGTATCRATATTC	602

Y = T or C, R = G or A, S = G or C, K = G or T

Reference: Kiiru*et al.*(2012)

Primer for 16SrDNA sequencing

Target gene	**Primer sequence**	**size**	**Location within gene**
16SrDNA	CCCCCTGGACGAAGACTGAC	401bp	1682–1701
	ACCGCTGGCAACAAAGGATA		

Reference: Filho*et al.*(2014)

Appendix 4: Permit for the Study

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR
P.O. Box 3000, MOROGORO, TANZANIA
Phone: 023-2604523/2603511-4; Fax: 023-2604651

Our Ref. SUA/ADM/R.1/8/ Date: 7th October 2015

The Municipal Director
Morogoro Municipal Council
P.O. Box 166
MOROGORO

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by Universities Act No.7 of 2005 and SUA Charter of 2007 which became operational on 1st January 2007 repealing Act No.6 of 1984. One of the mission objectives of the University is to generate and apply knowledge through research. For this reason the staff, students and researchers undertake research activities from time to time.

To facilitate the research function, the Vice-Chancellor of the Sokoine University of Agriculture (SUA) is empowered under the provisions of SUA Charter to issue research clearance to both, staff, students and researchers of SUA.

The purpose of this letter is to introduce to you **Mr. Emmanuel Odartei Armah** a bonafide **MSc. (MOLECULAR BIOL. & BIOT.)** student with registration number **MSB/E/2015/0001** of SUA. By this letter **Mr. Emmanuel** has been granted clearance to conduct research in the country. The title of the research in question is **"Effect of poultry housing systems on the diversity of Entended-spectrum beta-lactamaces genes in Avian pathogenic escherichia-coli"**

The period for which this permission has been granted is from **October 2015 to February 2016.** The research will be conducted in **Morogoro Municipal Council.**

Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/institutions/offices which could be visited. In case you may require further information on the researcher please contact me.

We thank you in advance for your cooperation and facilitation of this research activity.

Yours sincerely,

Prof. Gerald C. Monela
VICE-CHANCELLOR

Copy to: Student – **Mr. Emmanuel Odartei Armah**

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