OCCURRENCE OF ANTIBACTERIAL RESISTANT-*ESCHERICHIA COLI* BEARING QUINOLONE RESISTANCE AND EXTENDED SPECTRUM β-LACTAMASES GENES FROM COMMERCIAL POULTRY IN ZARIA, KADUNA STATE, NIGERIA.

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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE,
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ZARIA, NIGERIA

MARCH, 2016

DECLARATION

I declare that the work in this Dissertationtitled**"Occurrence of Antibacterial Resistant-*Escherichia coli* bearing Quinolone Resistance and Extended Spectrum β-lactamases Genes from Commercial Poultry in Zaria, Kaduna State, Nigeria"** has been carried out by me in the Department of Veterinary Public and Preventive Medicine, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Jibril Yakubu JIBRIL _____ _____

Signature Date

CERTIFICATION

This Dissertationtitled**, "Occurrence of Antibacterial Resistant-*Escherichia coli* bearing Quinolone Resistance and Extended Spectrum β-lactamases Genes from Commercial Poultry in Zaria, Kaduna State, Nigeria"** by Jibril Yakubu JIBRIL, meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Fluoroquinolones and Extended Spectrum Beta-Lactam (ESBL) antibiotics represent important classes of antibiotics in both veterinary and clinical practice. Given their efficacy in treating infections resistant to other drugs, these drugs have enjoyed widespread and often indiscriminate use. This study was aimed at determining the occurrence of antibacterial resistant *E. coli* from poultry and their potential for disseminating plasmid-borne *qnr*and ESBL gene variants. Twenty-five (25%) of farms that are registered with the poultry clinic of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria within the year 2014 were selected using simple random sampling technique. 30% *E. coli*isolation rate was recorded. The highest resistance to fluoroquinolone was seen with enrofloxacin (49.3%) followed by ofloxacin and ciprofloxacin with 33.3 and 22.7% respectively, while 58.7% were resistant to nalidixic acid. About 93.3, 80 and 76% were respectively resistant to amoxicillin/clavulanic acid, streptomycin and sulphamethoxazole/trimethoprim. Resistance to chloramphenicol, tetracycline and gentamicin also respectively accounted for 68, 65.3 and 52%. Least resistance was however seen with doxycycline (10.7%), nitrofurantoin (6.7%), ceftriaxone (6.7%), imipenem (2.7%) and cefepime (1.3%). Multidrug resistance was seen in 92% of the isolates tested,all of which had a MAR index of ≥0.20. *qnrS* and *bla TEM* were found in eighteen (24%) of the *E. coli* isolates. This study however, showed no significant association between management type and feed type with fluoroquinolone and ESBL resistance. This study has demonstrated a high prevalence of multidrug resistance among *E. coli* isolates from poultry in Zaria. It further revealed the existence of *qnrS* and *bla TEM*,an ESBL resistance encoding gene. It is therefore recommended that poultry farmers use antibiotics as therapeutic only when necessary in accordance with good drug use and management practices under the prescription of a certified veterinarian.

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ABBREVIATIONS

AMR= Antimicrobial resistance

DNA= Deoxyribonucleic acid

E.coli= Escherichia coli

EMB= Eosin methylene blue agar

FAO= Food and Agriculture Organization

IMVIC= Indole, methylred, Voges Proskaeur, citrate

JETACAR= Joint Expert Technical Committee on Antibiotic Resistance

LST= Lauryl sulfate tryptose broth

MAR= Multipleantibiotic resistance index

MIC= Minimum inhibitory concentration

MR= Methyl red

MUG= 4-Methylumbelliferyl-β-D-glucuronide

CLSI= Clinical and Laboratory Standards Institute

PCR= Polymerase chain reaction

SIM= Sulphate, indole, motility test

SPSS= Statistical package for social science

TSI= Tripple sugar iron test

USFDA= United States Food and Drug Administration

VP= Voges Proskauer

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Antibacterial drugs revolutionalized the control of bacterial infections. The success in infection control is being threatened by the emergence of resistance. Resistant infections have been estimated to costbetween $18,588 to $29,069 per patient in the US with an annual economic burden of $20 billion in health care cost and another $35 billion in lost productivity (Golkar *et al*., 2014). A similar analysis (Smith *et al*., 2005) estimated a loss of 0.4 - 1.6% in real GDP to antimicrobial resistance in the UK.

One of the major sources of resistant pathogens is agriculture where these drugs are used for chemoprophylaxis, chemotherapy and metaphylaxis. This use of antibiotics as growth supplements in livestock is common to both developed and developing economies of the world (Bartlett *et al*., 2013). An estimated 80% of antibiotics use in the US is said to occur in the livestock industry either for growth promotion or prevention of infection (Spellberg and Gilbert, 2014).Van Boeckel *et al*. (2015) estimated that the global average annual consumption ofantimicrobials per kilogram of animal produced was 45 mg/kg,148 mg/kg, and 172 mg·kg−1 for cattle, chickens, and pigs, respectively. They also estimated that between 2010and 2030, the global consumption of antimicrobials will increaseby 67%, from 63,151 ± 1,560 tons to 105,596 ± 3,605 tons.

Emergence of resistance in pathogens of public health significance is as old as the use of antibiotics in animals itself. Empirical evidences have associated the use of growth promoting antibiotics(GPAs) to contamination of flocks and food products by antibiotic resistant pathogens, including *Campylobacter*, *Salmonella*, *Enterococcus* and *Escherichia coli* (Cohen & Tauxe, 1986; Feinman, 1979; Van den Bogaard and Stobberingh, 2000; Randall *et al*., 2003; Johnson *et*

al., 2007) resulting in the transfer of resistance genes to humans (Greko, 2001; De Leener, 2005; Nsofor *et al*., 2013).

Beta-lactam antibiotics and fluoroquinolones represent important groups of antibiotics in both clinical and veterinary practice. While the beta-lactam antibioticswhose mechanism of action is inhibition of the last stage of bacterial cell wall synthesis, constitute one of the oldest and most popular classes of antibiotics in current clinical practice(Suarez and Gudiol, 2009; Page, 2012), fluoroquinolones (FQs) are the only class of antimicrobial agents in clinical use that directly inhibits bacterial DNA synthesis (Dalhoff, 2012).FQs have become first- and second-line antibacterials ofchoice for acute respiratory, enteric and urinary tractinfections as well as serious systemic infections such asbacteremia (Thielman and Guerrant, 2004; Jamison, 2006). They are also employed incombination with other antimicrobials to treat multidrugresistant tuberculosis (Mondero & Caminero, 2009).As *E.coli* readily acquires resistance either by way of mutation to FQs or through mobile genetic elements to cephalosporins, a class of beta-lactam antibiotic, development of resistance to carbapenem, another beta-lactam considered to be the last resort for treating many complicated bacterial infections may be easily predicted (Kunduru *et al*., 2016). The useand misuse of these drugs in the informal sector by unsanctioned providers and through self medication is commonplace inNigeria and in many other parts of Africa (Van den Boogard *et al.*, 2010; Paolo *et al.*, 2008) raising serious concern for public health.

1.2 Statement of the Problem

Poultry and poultry products are a major source of animal protein to the large and increasing Nigerian population. In the recent past, Nigeria's poultry industry has witnessed a remarkable growth;becoming the largest egg producer and having the second largest chicken population after South Africa on the African continent. This market is estimated at about ₦80 billion ($600

million) (Sahel Capital Reports, 2015). This growth however, has taken place without a reciprocal commitment to standards and quality control on the part of regulatory authorities. Biosecurity is therefore often neglected or poorly implemented (Paolo *et al.*, 2008) by most farmers thus ultimately making the use of antibiotics inevitable and routine. It is a common practice to prophylactically administer antibiotics; readily available over the counter without prescription (Paolo *et al.,* 2008), to day-old birds. Consequently, widespread misuse and/or abuse of almost every available antibiotic have become rampant. The resultant selection pressure on the gastrointestinal microflora of poultry generates a pool of resistant microorganisms for dissemination to other population of birds and humans.

Already a number of reports have demonstrated increased emergence of resistance among bacteria from poultry and human populations in Nigeria (Kabir, 2007; Kayode *et al*., 2010; Ajayi *et al*., 2012; Enabuelele *et al*., 2006; Aibinu *et al.,* 2008; Lamikanra *et al*., 2011; Ojo *et al.,* 2012), with some showing very high levels of multidrug resistance (Ogbolu *et al*., 2011). Emergence of resistance has been shown to be associated with the use of antibiotics in both humans and animals either prophylactically or therapeutically (Lamikanra *et al*., 2011; Aibinu, *et al.*, 2004; Kwaga, 2012).

The problem of antimicrobial resistance (AMR) especially infection with multidrug resistant strains colloquially referred to as "superbugs" has emerged as a significant public health issue of both human and veterinary concern, and AMR fostered by inappropriate use of antibiotics in animal husbandry is an underlying contributor to the emergence and spread of antibiotic-resistant germs. The global nature and severity of the problem is summarized in a World Health Organization report of April, 2014 which states, "this serious threat is no longer a prediction for the future; it is happening right now in every region of the world and has the potential to affect

anyone, of any age, in any country" (WHO, 2014). This therefore raises the need to continuously monitor the status of AMR among bacterial organisms in animals and humans globally and especially in a developing country like Nigeria where the problem of misuse of antibacterial agents is more prevalent.

1.3Justification

Escherichia coli is an important zoonotic pathogen (Yngvild, 2001) and infections caused by this organism in poultry are common and most often intractable to treatment. The disease results in economic losses in Nigeria. While poultry has become a stable source of animal protein for most Nigerians, this species has been shown to be a reservoir of extra intestinal pathogenic *E. coli* in humans (Racicot Bergeron*et al*., 2012). *E. coli* is also a major pathogen of commercial poultry causing colibacillosis with manifestations such as airsacculitis, pericarditis, septicemia, and death of the birds (Biswas *et al.*, 2006).Clinically,*E. coli* is the causative agent of some important diseases. Enterotoxigenic *E. coli* (ETEC) is the most common cause of food and water-borne human diarrhea worldwide (Zinnah *et al*., 2007). In developing countries, the incidence of enteric diseases due to ETEC is estimated at about 650 million cases per year, resulting in 800,000 deaths, primarily in children of below five years old (Turner *et al.*, 2006).

Fluoroquinolones especially enrofloxacin is a common drug within the Nigerian poultry industry and has been indiscriminately used (Oluwasile *et al.*, 2014). Fluoroquinolone resistance in poultry is therefore of great public health significance. Antibiotic resistance also constitutes an economic burden on health care system as infections due to resistant bacteria do not only cost more to treat, but also can prolong health care use.

Although plasmid mediated fluoroquinolone resistance in bacteria of human and animal origin has been documented especially from southern Nigeria, such information is grossly scanty from

various livestock and poultry production systems across the country (Ajayi *et al*., 2012; Enabulele *et al*., 2006; Ogbolu *et al*., 2011).

1.4 Aim

This study was aimedat determining the occurrence of antibacterial resistant *E. coli*from poultry and their potential for disseminating plasmid-borne *qnr*and ESBL gene variants.

1.5 Objectives

The objectives of this study were:

1. To isolate and identify *E. coli* from commercial poultry in Zariausing conventional biochemical tests and Microbact E12 test kit.
2. To determine the antibacterial sensitivity patterns of the *E. coli* isolates to fluoroquinolones and other antibiotics.
3. To determine if there is association between*qnr* and ESBL gene carriage by the isolates.

1.6 Research Questions

1. Are *E. coli*isolates present in commercial poultry in Zaria?

2. Are *E. coli* isolates of poultry origin in Zaria resistant to fluoroquinolones?

3. Do these isolates carry *qnr* genes?

4. Is there association between *qnr* and ESBL genes carriage in these isolates?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The organism *Escherichia coli*

Bacterium coli commune,as it was known was first isolated and described from the feces of newborns in 1885 (Bettelheim *et al*., 1974). It was later renamed *Escherichia coli*, after Theodor Escherich, the German Paediatrician and bacteriologist who first isolated and described it. For many years the bacterium was simply considered to be a commensal organism of the large intestine until 1935 when a strain of *E. coli* was shown to be the cause of an outbreak of diarrhoea among infants. Although some strains of *E. coli* are harmful, most are harmless and constitute a part of the normal flora of the gut benefiting their hosts by producing vitamin K2(Bentley & Meganathan*et al*., 1982) and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al*., 2001; Reid *et al*., 2001).

E. coli is a member of the family *Enterobacteriaceae* which consists of many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. It is a Gram-negative, facultatively anaerobic, rod-shapedbacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded animals. The bacterium is about 2.0μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3 (Kubitschek, 1990). It has the ability to live on a wide variety of substrates and growth can be driven by aerobic or anaerobic respiration. *E. coli* uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon dioxide (Vuoristo *et al*., 2015). Optimal growth occurs at 37 °C (98.6 °F) but some laboratory strains can multiply at temperatures of up to 49 °C (120 °F) (Fotadar *et al*., 2005). The robustness of this organism which makes it easy and relatively cheap to grow in a laboratory setting has made it the most widely studied prokaryoticmodel organism, and an

important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA.

2.2 Isolation and Identification

While a number of methods are available for the isolation of *E. coli*, culture remains a basic step in this process irrespective of sample source. Use of enrichment broth before selective/differential plating has been shown to enhance the growth of this organism in both starved and unstarved states. Some, e.g. buffered peptone water containing 0.5% sodium thioglycolate (STG-BPW), have the ability to suppress the growth of non-carbohydrate-fermenting obligate aerobes whose colonial morphology on sorbitol MacConkey agar containing cefixime and tellurite (CT-SMAC) could resemble that of *E. coli* (Sata*et al*., 2003). Other broths with similar purpose include modified tryptic soy broth (TSB) or modified *E. coli* broth (EC broth) supplemented with antibiotics, bile salts, and, in some instances, elevated incubation temperatures (Grant, 2004). *E. coli* can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions. *E. coli* in stool are most often recovered on MacConkey or eosin methylene-blue agar (Ahmed *et al*., 2014; Virpari *et al*., 2013), which selectively grow members of the *Enterobacteriaceae* and permit differentiation of enteric organisms on the basis of morphology (Nataro and Kaper, 1998). Light microscopy following Gram staining is also very useful in exposing the cellular morphologic characteristics of *E. coli*(Reshes *et al*., 2008).

The IMViC (Indole, Methyl Red, Voges-Proskauer and Citrate) biochemical tests have been used to identify *E. coli* (Kudva*et al*., 1997). These tests can be performed in individual culture tubes or by using test “strips” which are commercially available with either method producing satisfactory results.

Serologically *E. coli* can be identified as serogroups based on major surface antigens. Kauffman in 1944 proposed a scheme for the serologic classification of *E. coli* which is still used, however in modified form (Nataro and Kaper, 1998). According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles where a specific combination of O and H antigen defines the serotype of an isolate (Nataro and Kaper, 1998) example O157:H7. *E. coli* has more than 80 K antigens which in combination with O and H antigens leads to about 50 000–100 000 or more serotypes (Kunduru *et al*., 2016).Although *E. coli* of specific serogroups have been associated reproducibly with certain clinical syndromes, these serologic antigens themselves do not confer virulence. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whittam *et al*., 1993). Despite its successes in identifying pathogenic strains of *E. coli*, this protocol has the disadvantage of limited sensitivity and specificity, it is tedious and expensive and only few laboratories have this capacity (Nataro and Kaper, 1998; Lisa *et al*., 2005). Given these limitations *E. coli* identification has focused increasingly on the identification of characteristics which themselves determine the virulence of these organisms. This may include in vitro phenotypic assays which correlate with the presence of specific virulence traits or detection of the genes encoding these traits. Lisa *et al*. (2005) used molecular serotyping to identify *E. coli* O26:H11. This protocol has the advantage of genetically targeting the same somatic and flagellar antigens of *E. coli* O26:H11 used in traditional serotyping. It also correctly serotypes strains untypeable by traditional methods, affording primary laboratories serotyping capabilities. Other molecular techniques that have been used include nucleic acid probes and polymerase chain reaction (PCR). Molecular diagnostic

techniques have so far proved highly reliable in differentiating pathogenic from non pathogenic strains of *E. coli* (Li *et al*., 2016; Dou *et al*., 2016).

2.3 Virulence Mechanisms

Escherichia coli is considered as the normal bowel flora of different species of mammals and birds but some strains of *E. coli* possess pathogenic character due to the acquisition of virulence factors (Zinnah *et al*., 2007). Like most mucosal pathogens, *E. coli* can be said to follow a requisite strategy of infection: (i) colonization of a mucosal site, (ii) evasion of host defenses, (iii) multiplication, and (iv) host damage (Nataro and Kaper, 1998). Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrhoeal disease (Nataro and Kaper, 1998). The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence-related plasmids and chromosomal pathogenicity islands (Nataro and Kaper, 1998). Chromosomal virulence genes of some pathogenic *E. coli* (e.g. EPEC and EHEC) are organized as a cluster referred to as a pathogenicity island (McDaniel and Kaper, 1997). Such islands have been described for uropathogenic *E. coli* strains (Donnenberg and Welch, 1996) and systemic *E. coli* strains (Bloch and Rode, 1996) as well and may represent a common way in which the genomes of pathogenic and nonpathogenic *E. coli* strains diverge genetically. Plasmids and pathogenicity islands carry clusters of virulence traits, yet individual traits may be transposon encoded (such as ST) or phage encoded (such as Shiga toxin) (So and McCarthy, 1980).

E. coli enteritis may be caused by at least six distinct *E. coli* pathotypes: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), diffusely adherent (DAEC) and enteroaggregative *E. coli* (EAEC)(Kaper, 2004). These pathotypes exhibit

distinct clinical, epidemiologic and pathogenic profiles. Strains within each pathotype are characterized by shared virulence traits and can typically be further distinguished by O (lipopolysaccharide) and H (flagellar) antigens (Kaper , 2004).

2.4 *E. coli* in the environment

Although some *E. coli* are found naturally in the environment (Caplenas *et al*., 1984), a number of pathogenic strains find their way into the environment through faecal contamination.In 1892, Shardinger proposed the use of *E. coli* as an indicator of faecal contamination based on the premise that *E. coli* is abundant in human and animal faeces and not usually found in other niches (Feng *et al*., 2002). Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of frank pathogens. This concept, although sound, was however, complicated due to the presence of other enteric bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35°C (Feng *et al*., 2002).

The association of coliforms with faecal contamination became questionable as some coliforms are found naturally in environmental samples (Caplenas *et al*., 1984). The term “faecal coliforms” was thus introduced. Faecal coliform, first defined based on the works of Eijkman is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature,

hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C (American Public Health Association, 1970; 1998; Neufield, 1984). The faecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms. The inclusion of *Klebsiella* spp in the working definition of faecal coliforms diminished the correlation of this group with faecal contamination. *E. coli* however, has reemerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*(Feng *et al.*, 2002).

2.5 *E. coli* in Humans

Escherichia coli, albeit a normal inhabitant of the gastrointestinal tract of animals and humans, has become highly adapted to cause diarrhoea and a range of extra-intestinal diseases (OIE, 2008). Pathogenic *E. coli* are transmitted through contaminated water or food, or through contact with animals or persons (Scallan *et al*., 2011). Thesestrains are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis) (Nataro and Kaper, 1998).

ETEC is the most common bacterial cause of diarrhoea worldwide (Walker *et al*., 2007). In developing countries, the incidence of enteric diseases due to ETEC is estimated at about 650 million cases per year, resulting in 800,000 deaths, primarily in children of below five years old (Turner *et al.*, 2006).

E. coli O157:H7, a serotype of EHEC, was first recognized in 1982 as a human pathogen with cattle, although not susceptible, identified as a major source for human infection (Elder *et al.*, 2000). This strain, considered the dominant and most virulent (OIE, 2008) is transmitted from

bovine reservoirs via many food vehicles including cooked meat, such as hamburger (CDC, 1993) and salami (CDC, 1995), and raw vegetables, such as lettuce (CDC, 2006) and spinach (CDC, 2006). In North America, *E. coli* O157:H7 is the most common EHEC serotype contributing to about 75,000 human infections (Meads *et al*., 1999) and 17 outbreaks (Rangel *et al*, 2005) per year. The significance of this strain as a major food-borne pathogen causing outbreaks involving haemorrhagic colitis and haemolytic-uraemic syndrome has been on the increase (Sata*et al*., 2002; OIE, 2008; Manning*et al*., 2008). Although some serotypes have been predominantly associated with certain clinical syndromes; some, however, overlap depending on virulence factors. The serotype *E. coli* O26 which can either be enterohemorrhagic (EHEC) or Enteropathogenic (EPEC) is the most frequently isolated non-O157 Shiga-toxigenic *E. coli* (STEC) associated with human clinical illness (Bettelheim, 2003), and *E. coli* O26:H11 is the clinically most important and epidemiologically most predominant EPEC and EHEC O26 serotype (Anjum *et al*., 2003). This serotype, first reported to cause paediatric EPEC diarrhoea has been isolated from EHEC outbreaks in Europe, South America, and Japan; from sporadic cases in Canada, Australia, and the United States; and from sick and healthy livestock (Lisa *et al*., 2005).

Despite advances in antimicrobial chemotherapy and supportive care, neonatal meningitis remains a significant problem (Yao*et al.*, 2006). Mortality rates range between 5 and 50% with majority of surviving infants (30 to 50%) manifesting neurological sequelae (Sata*et al*., 2003). *E. coli* has been demonstrated as the most common culprit with strains possessing the K1 capsular polysaccharide as predominant (Sarff *et al*., 1975).

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is the leading cause of community-acquired urinary tract infections (UTIs) in humans, accounting for >85% of UTIs (Zhang *et al*., 2003).

Each year, 6–8 million UTIs are diagnosed in the United States alone and 130–175 million are diagnosed worldwide. Estimated direct health care costs related to uncomplicated UTIs in the United States are $1–$2 billion per year (Zhang *et al*., 2003; Russo and Johnson, 2003). UTIs also can lead to more severe illnesses, such as pyelonephritis, bacteremia, and sepsis (Smith *et al.,* 2007).

2.6 *E. coli* in Poultry

Avian pathogenic *E. coli* (APEC) causes colibacillosis, an acute and mostly systemic disease resulting in significant economic losses in poultry industry worldwide. Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis being most typical (Ewers *et al*., 2003). APEC isolates commonly belonged to certain scrogroups, Ol, O2 and O78, and to a restricted number of clones with serogroups O2 and O78 considered preferentially associated with colibacillosis outbreaks in poultry representing 80% of disease cases worldwide (Dho-MoulinandFairbrother, 1999; Dziva and Stevens, 2008) but recently other APEC serogroups with minor importance in the poultry health context, which could, nonetheless, have some impact on public health have been described. These include O6, O8, O21, O46, O88, O106, O111, and O143 (Knöbl*et al*., 2012). Virulence vary profoundly according to isolates, however, known virulence factors of APEC include adhesins (F1- and P-fimbriae), iron acquisition systems (aerobactin), hemolysins (hemolysinE and temperature sensitive hemagglutinin), resistance to the bactericidal effects of serum and phagocytosis (outer membrane protein, ISS protein, lipopolysaccharide, K/1-capsule and colicin production) as well as toxins and cytotoxins (heat stable toxin, cyto-/verotoxin and flagella toxin). Experimentally, the respiratory tract, principally the gas-exchange region of the

lung and the interstitium of the air sacs have been shown to be the most important sites of entry for avian pathogenic *E. coli* (Ewers *et al*., 2003).

Colibacillosis occurs with marked variation in severity. Problems range from severe acute infections with sudden and high mortality to mild infections of a chronic nature with low morbidity and mortality. Infections may result in a respiratory disease from air sac infection, a septicemic disease from generalized infections, enteritis from intestinal infection or a combination of any or all of these conditions. Poultry has also been shown to serve as natural reservoir for EPEC and ExPEC (Racicot Bergeron*et al*., 2012).

2.7 Antibiotic Use in Poultry and the Emergence of Resistance

Antibiotics have been used in poultry to treat bacterial infections and for growth promotion. The poultry industry witnessed the earliest use of antibiotics on livestock when in the 1940s farmers noticed an improved growth in animals fed dried mycelia of *Streptomyces aerofaciens* containing chlortetracycline residues (Castanon, 2007). This development led to various countries granting approvals for use of antibiotics as growth promoters. While the United States licensed the use of antibiotics as animal additive without veterinary prescriptions in 1951, various European states approved their own regulations concerning use of antibiotics in animal feeds in the 50s and 60s (Jones and Ricke, 2003; Castanon, 2007).

Increase in global population means an increase in protein demand. A shift therefore from the traditional system of poultry production to one with a high turnover rate within a short time period became necessary if the animal protein demand is to be met. Thus, the poultry industry has emerged as one of the world's fastest growing sources of meat, representing nearly one-fourth of all the meat produced during the year 2000 (Apata, 2009). The modern production unit

can produce market ready broiler chickens in less than six weeks. This development arose from genetic selection, improved feeding and health management practices involving use of antibiotics at therapeutic levels to treat bacterial diseases in intensive farming systems and also at prophylactic levels in the water of healthy birds or as growth promoters at sub-therapeutic concentrations in feed (Apata, 2009). It has been reported that 80 percent of all antibiotics sold in the United States are for use on livestock and poultry, not humans, with majority (90%) of this given for growth promotion and prophylaxis (Spellberg and Gilbert, 2014).

The use of Growth Promoting Antibiotics (GPAs) is loosely defined as antibiotics provided to healthy animals at concentrations below 200g per ton of feed for more than 14 days. This is distinguished from therapeutic and prophylactic antibiotic use, which is delivered at higher doses and generally administered in water. Bacitracin, chlortetracycline, tylosin, avoparcin, neomycin, oxytetracycline, virginiamycin and others are used for these purposes(Graham *et al*., 2007). These drugs, given at low doses in animal feeds, are considered to improve the quality of the product, with a lower percentage of fat and higher protein content in the meat. Other benefits of the use of antibiotic growth-promoters include control of zoonotic pathogens such as *Salmonella*, *Campylobacter*, *Escherichia coli* and Enterococci (Hughes and Heritage, 2004). The economic and health advantages of using antibiotics have thus revolutionized intensive poultry and livestock production (Apata, 2009). These small amounts of antibiotics though useful, often result in subtherapeutic doses, a condition which is particularly conducive to selection for resistance in bacteria (Graham *et al*., 2007).

2.7.1 Emergence of Resistance

Shortly after the discovery of the beneficial effects of antibiotics in livestock; there began to arise concerns for development of resistance owing to the heavy use of these antibiotics. Starr and Reynolds (1951) reported one of the first cases of resistance in food animals after experimental feeding of streptomycin in turkeys. Other researchers (Barnes, 1958; Elliott & Barnes, 1959) also reported an association of resistance to tetracycline when growth-promoting levels of antibiotic are fed to chickens. Three years after the United Kingdom government (in 1969) found that a potential risk to humans exists from the use of antibiotics in animals and that the overuse of these drugs in livestock was increasing the amount of drug-resistant bacteria,similar concerns led the US Food and Drug Administration (FDA) to conclude that the use of antibiotics is causing resistant bacteria and requires manufacturers to show the effectiveness and safety of these drugs, aposition buttressed by a study conducted by Tufts University's Stuart Levy (1976) who found out that farmers who fed chickens the antibiotic, tetracycline, had resistant bacteria present in them. This resulted in the frightening conclusion that the increase in resistance of bacteria was due to the use of antibiotics.

Despite empirical evidences, growth promoting antibiotics still remain integral part of poultry production even in some developed countries. There is therefore increasing public health concern over the use of antibiotics for growth promotion in food animal production related to the contribution of this use to increasing rates of antibiotic resistance. Earlier studies have shown that the use of GPAs contribute to contamination of flocks and food products by antibiotic resistant pathogens, including *Campylobacter*, *Salmonella*, *Enterococcus* and *Escherichia coli* and thereby increasing risks of human infections by these and other resistant pathogens (Feinman, 1979; Cohen and Tauxe, 1986). For example, worried by the amount of antibiotics

used for growth promotion in poultry (90%) and pigs in Denmark, Frank Arestrup and his colleagues decided to study the incidence of antibiotic-resistance among bacteria in the feces of healthy chickens and pigs and they discovered a clear relationship between the use of the antibiotic avoparcin; a glycopeptide with chemical structure similar to vancomycin, a treatment of last resort in human patients with life-threatening infections (Giguere, *et al*., 2013), and the widespread occurrence of resistant bacteria (Aarestrup, 2000). This prompted the Danish government in 1995 to ban all uses of avoparcin becoming the first European country to do that.This resulted in a marked decline in levels of vancomycin resistant enterococci in pigs since the 1995 avoparcin ban (Agerso *et al*., 2011).

Potential transfer of resistant bacteria from poultry products to human population may occur through consumption or handling meat contaminated with the pathogens (Van den Bogaard and Stobberingh, 2000). Once acquired, the resistant bacteria can colonize the human intestine and the genes coding resistance to antibiotics can be transferred to other bacteria belonging to the endogenous flora of humans, thereby jeopardizing effective treatment of bacterial infections (De Leener, 2005). Indeed, increasing evidence exist that antibiotic resistance genes can be and are transmitted from animal to human microbiota (Greko, 2001). For example, the use of flouroquinolone antibiotic in broiler chickens has caused an emergence of resistant *Campylobacter* in poultry which was linked to 10 per cent of human *Campylobacter* diseases found in the area (Randall *et al*., 2003). Administration of avilamycin as a growth promoter resulted in an occurrence of avilamycin-resistant *Enterococcus faecium* in broiler farms (Aarestrup *et al*., 2000). In turkeys fed vancomycin, there was a transfer of vancomycin resistance genes between turkey *Enterococci* strains, from turkey to turkey and from turkey to humans (Stobbering *et al*., 1999). Johnson *et al*. (2007) in a study to assess the hypothesis

that poultry products, may transmit antimicrobial drug–resistant *Escherichia coli* to humans demonstrated that many human-source drug-resistant faecal *E. coli* isolates more likely originated in poultry than in humans, whereas drug-resistant poultry isolates likely derive from drug-susceptible poultry isolates. In a related study, Nsofor *et al*. (2013) reported the circulation of identical clones of resistant *E. coli* strains among human and animal population in Nigeria.

2.7.2 Public Health Significance of Bacterial Resistance

Antibiotics kill or inhibit susceptible bacteria. Bacteria that are intrinsically resistant or that can acquire resistance will survive and replace the drug-susceptible bacteria. Thus, any antibiotic use will provide a selective pressure that perpetuates resistant bacteria. The more that antibiotics are used, the greater the selective pressure. Antibiotics are the most important tool we have to control many life-threatening bacterial diseases once infection has occurred, yet increasing levels of resistance are compromising the effectiveness of these antibiotics (Frieden, 2010). Development of resistance in bacteria therefore, poses a grave danger as organisms are developing resistance faster than the development of newer antibiotics. The world could therefore be faced with an inevitable and unceremonious return to the pre-antibiotic era. Resistant bacteria cause increased suffering through extended hospitalization. Resistant bacterial strains will adversely affect the efficacy of antibiotic chemotherapy for those that acquired the new strains of infectious disease (Anon, 2006; Chastre, 2008). Furthermore, it encourages the need for more expensive and toxic medications. Some resistant infections can cause death. Moreover, resistance is not just a problem for the patient who is infected. When an infection is not effectively treated because of resistance, the microorganisms will persist and potentially spread to others, further extending the resistance problem (Frieden, 2010). Resistant bacteria

thwart antibiotic treatment by interfering with their mode of action via a range of effector mechanisms including synthesis of inactivating enzymes, alteration of the specific configuration of target sites and inhibition or changes in membrane transport systems to remove the antibiotic (Cetinkaya *et al*., 2000).

Consequently some developed nations have taken measures to curb this ugly trend. The European Union, in 1999, banned the use of most antibiotics for growth promotion to preserve the effectiveness of important human drugs (Casewell *et al*., 2003). The U.S. has not adopted this broad policy, but in 2004 the U.S. Food and Drug Administration banned enrofloxacin (a fluoroquinolone applied for therapeutic uses, not growth promotion) in food animals on the grounds that its use contributed to fluoroquinolone resistance in human pathogens (Graham *et al*., 2007). In Australia, the Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) was established in April 1998. The terms of reference for the committee were broadly to review the scientific evidence on the link between the use of antibiotics in food-producing animals, the emergence and selection of antibiotic-resistant bacteria and their spread to humans; and to develop evidence-based recommendations for the appropriate future management of antibiotic use in food-producing animals. The report of this committee was tabled in October 1999. In its overall conclusion following meticulous groundwork, the committee agreed that antibiotic use in animal production can affect human health, but that decisions relating to antimicrobial drugs should be based on sound science and risk analysis. They recommended the development of an antibiotic-resistance management programme that will focus simultaneously on human and animal use of antibiotics. The Australian government responded favorably to the report in October 2000 and decided to establish an inter-departmental JETACAR implementation group to oversee and coordinate the antibiotics strategy (OIE, 2001).

World Health Organization (WHO, 1997) published a report on the medical impact of the use of antimicrobials in food animals suggesting a link between the two on an epidemiological basis. This organization (WHO, 2000), therefore recommended on precautionary grounds, that national governments adopt a proactive approach to reduce the need for antimicrobial use in animals and establish surveillance of antimicrobial usage and resistance. With respect to the use of antimicrobial growth promoters, it also suggested that use of antimicrobial growth promoters that are in classes also used in humans be terminated or rapidly phased out, by legislation if necessary, unless and until risk assessments are carried out (WHO, 2000). The organization also suggested that animal health management should be routinely practiced so as to avoid the prophylactic use of antimicrobials, and antimicrobial availability should be limited to therapeutic use by prescription. The recommendations are precautionary, based on the potential for a reservoir in food animals of an antibiotic resistant bacterial population that could be transferred to humans.

2.8 Fluoroquinolones

Fluoroquinolones are a class of compounds that comprise a large and expanding group of synthetic antimicrobial agents (Martinez M. *et al*., 2006).Structurally all fluoroquinolones contain a fluorine molecule at the 6-position of the basic quinolone nucleus. The parent of the group is nalidixic acid, discovered in 1962 by Lescher and colleagues. Since then, research on 4-quinolone-3-carboxylates has led to the discovery of a family of 6-fluoro-7-piperazinyl-4-quinolones active against gram-negative and gram-positive bacteria *in vitro* (Wolson & Hooper, 1985) as well as intracellular pathogens (Fitzgeorge *et al*., 1988) and trimethoprim/sulfonamide resistant microbes (Preheim *et al*., 1987); in addition these antimicrobials are also active against

mycoplasmas. Collectively, these compounds are called fluoroquinolones. Although dozens of fluoroquinolones have been synthesized and reported, enrofloxacin was the first fluoroquinolone introduced into veterinary medicine. Notable ones being developed, or used, in veterinary medicine worldwide include amifloxacin, benofloxacin, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, norfloxacin and norfloxacin nicotinate, ofloxacin, orbifloxacin and sarafloxacin. Other major fluoroquinolones in human medicine include enoxacin, ofloxacin, sparfloxacin, temafloxacin, and tosufloxacin (Sarkozy, 2001). The first fluoroquinolones were widely used because they were the only orally administered agents available for the treatment of serious infections caused by gram-negative organisms, including *Pseudomonas* species. The newer fluoroquinolones have a wider clinical use and a broader spectrum of antibacterial activity including gram-positive and gram-negative aerobic and anaerobic organisms (Soni, 2012).

2.8.1 Mechanism of Action of Fluoroquinolones

Fluoroquinolones are the only class of antimicrobial agents in clinical use that directly inhibits bacterial DNA synthesis (Dalhoff, 2012).This is achieved through interaction of the drug with complexes composed of DNA and either of the two target enzymes, DNA gyrase and topoisomerase IV. These enzymes are structurally related to each other, both being tetrameric with pairs of two different subunits. The GyrA and GyrB subunits of DNA gyrase are respectively homologous with the ParC and ParE subunits of topoisomerase IV (Hooper, 2001). For many gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many gram-positive bacteria. Both enzymes are type 2 topoisomerases, which act by breaking both strands of a segment of DNA, passing another segment through the break, and then resealing the break. For DNA gyrase, this topoisomerization reaction results in introduction

(or removal) of DNA supercoils, thus affecting the negative supercoiling of DNA necessary to initiate DNA replication and remove positive supercoils that accumulate before an advancing replication fork. For topoisomerase IV, the topoisomerization reaction results in separation of the interlocking of daughter DNA strands that develop during replication; this facilitates the segregation of daughter DNA molecules into daughter cells. In both cases, fluoroquinolones appear to trap the enzyme on DNA during the topoisomerization reaction, forming a physical barrier to the movement of the replication fork, RNA polymerase, and DNA helicase (Hooper, 2001). The collision of the replication fork with these trapped complexes triggers other poorly defined events within the cell that ultimately result in cell death.

2.8.2 Mechanisms of Bacterial Resistance to Fluoroquinolones

Broad use of fluoroquinolones has been followed by emergence of resistance. Resistance to quinolones occurs by mutation in chromosomal genes that encode the subunits of DNA-gyrase and topoisomerase IV (altered target mechanism), and that regulate the expression of cytoplasmic membrane efflux pumps or proteins that constitute outer membrane diffusion channels (altered permeation mechanism) (Dalhoff, 2012).Although mutations in the quinolone target genes are required to achieve a clinical level of resistance, several other mechanisms including decreased uptake of the drug due to the loss of a membrane-bound porin; drug extrusion via efflux pumps, some of which may have a broad substrate specificity; or one of the more recently described plasmid-mediated quinolone resistance (PMQR) mechanisms may also contribute to quinolone/fluoroquinolone resistance, (Hopkins *et al*., 2005; Strahilevitz *et al*., 2009). The genetic information for target site or efflux resistance mechanisms is commonly chromosomally encoded. However, the emergence of plasmid-mediated and thus transferable

fluoroquinolone resistance has also been reported.Several mechanisms are known: 1. Qnr (target protection), 2. Aminoglycoside acetyltransferase AAC(6_)-Ib-cr (enzymatic drug modification), and 3. OqxAB, QepA (efflux pumps)(Tran and Jacoby, 2002; Poirel *et al*., 2008; Robicsek *et al*., 2006; Strahilevitz *et al*., 2009; Poirel *et al*., 2012; Rodr´ıguez-Mart´ınez *et al*., 2011).

Qnr interacts with DNA gyrase and topoisomerase IV to prevent quinolone inhibition (Tran & Jacoby, 2002;Nordmann and Poirel, 2005). This protein causes nalidixic acid resistance and reduced susceptibility to or low-level fluoroquinolone resistance (Nordmann and Poirel, 2005). The occurrence of *qnr*-genes in ciprofloxacin-susceptible isolates as well as quinolone resistant isolates suggests that their presence promotes higher level resistance due to chromosomal mutation, as has been shown in the laboratory. Therefore, the presence of *qnr* genes in clinically relevant species of both Gram-positive and Gram-negative bacteria may foster quinolone resistance development (Dalhoff, 2012). Furthermore, *qnrA* and *qnrB* genes are usually integrated into integrons which harbor other antibiotic resistance genes such as *β*-lactamases or aminoglycoside inactivating enzymes. Qnr S and bla TEM are also known to be associated with transposons (Hernandez *et al*., 2011).

The second plasmid-encoded quinolone resistance determinant identified was a variant of the *aac(6_)Ib* gene encoding an aminoglycoside acetyltransferase. The bifunctional aminoglycoside and fluoroquinolone active variant AAC(6_)-Ib-cr catalyzes acetylation of both drug classes (Vetting *et al*., 2008). The variant enzyme has acquired the ability to acetylate ciprofloxacin and norfloxacin and reduces ciprofloxacin's activity fourfold (Ruiz *et al*., 2012; Frasson *et al*., 2011). Moxifloxacin and levofloxacin are not acetylated due to the absence of a piperazinyl substituent at position C-7. AAC(6_)-Ib-cr may be more widespread thanQnr-determinants. Both, Qnr- and AAC(6_)-Ib-cr-production are associated with the ESBL production, thus, representing a second

mechanism of co-selection of drug-resistance due to exposure to chemically unrelated agents (Dalhoff, 2012).

Most recently, a third type of plasmid-mediated quinolone resistance has been identified: the quinolone efflux pumps OqxAB and Qep. The OqxABand QepA-proteins confer resistance to hydrophilic fluoroquinolones like norfloxacin, ciprofloxacin, and enrofloxacin, causing a 32- to 64-fold increase in MICs. QepA extrudes in addition to quinolones a narrow range of agents such as erythromycin, ethidium bromide, and acriflavine;OqxAB exports a wider range of agents like ethidium bromide, tetracyclines, chloramphenicol, trimethoprim, olaquindox, and disinfectants like triclosan. Extrusion of chemically unrelated agents by efflux-pumps represents a third mechanism of cross-resistance. In conclusion, fluoroquinolone resistance can emerge even in the absence of exposure to this drug class as several co-selection mechanisms favour the emergence of quinolone resistance (Dalhoff, 2012).

In *Escherichia coli*, mutational alterations in the fluoroquinolone target enzymes are recognized to be the major mechanisms through which resistance develops. In fluoroquinolone-resistant isolates, mutational hot spots are localized in defined regions known as the quinolone resistance-determining regions (QRDRs). Inisolates displaying resistance, DNA gyrase, the primary target in Gram-negative bacteria, commonly presents substitutions at amino acid position Ser83 and/or Asp87 of the GyrA subunit, while substitutions at residues Ser80 and Glu84 are commonly identified alterations in the ParC subunit of the topoisomerase IV (Heisig, 1996; Yoshida *et al*., 1991).

A major drug efflux system of *E. coli*, AcrAB-TolC, has been shown to contribute to the resistance to fluoroquinolones and other classes of antimicrobials (Mazzariol*et al*., 2000; Oethinger*et al*., 2000; Yang *et al*., 2003). AcrAB-TolC belongs to the resistance-nodulation-

division (RND) family of membrane transporters and is dependent on the proton motive force to energize the extrusion of structurally different compounds (Amaral *et al*., 2011; Zgurskaya and Nikaido, 1999). The expression of this broadsubstrate-range efflux pump is usually at a low constitutive level due to the negative repression imposed by AcrR. AcrABTolC is also subject to regulation at the global level by transcriptional factors, such as MarA, involved in the induction of resistance to structurally diverse antimicrobial compounds and organic solvents; SoxS, a regulator of the superoxide stress response; and Rob, known to be involved in a wide range of functions, including resistance to bile salts (Alekshun and Levy, 1997;Kern *et al*., 2000; Rosenberg *et al*.,2003). Isolates expressing increased levels of the AcrAB-TolC efflux pump have been shown on a number of occasions to display a socalled Mar phenotype that is linked with the activation of a global regulatory gene(s). Such mutants can display deletions and/or amino acid sequence substitutions within relevant local or global loci. Activation of the global regulatory *mar* locus has been linked with up-regulation of genes involved in efflux and posttranscriptional down-regulation of the major porin in *E. coli*, which can lead to a multiple-antibiotic-resistant (*mar*) phenotype (Alekshun and Levy, 1997; Kern *et al*., 2000). Decreased expression of OmpF, the major porin of *E. coli*, has been shown to contribute to resistance to several antimicrobials, including quinolones. Karczmarczyk *et al*.(2011) demonstrated mutations in DNA gyrase as being the principal factor contributing to high-level fluoroquinolone resistance. They also identified enhanced efflux activity and decreased production of OmpF in some of the isolates. This however, could not be linkedwith overexpression of MarA, Rob, or SoxS, despite several point mutations identified in relevant regulatory loci suggesting that regulatory networks other thanthose already recognized remain to be described.

2.8.3 Methods of Detecting Antimicrobial Resistance

Resistance to fluoroquinolones has emerged as a serious challenge to the fight against microbial infections. The favourable characteristics of this drug class i.e. excellent oral bioavailability and tissue penetration, broad antimicrobial spectrum,well characterized and predictableconcentration-effectrelationships(relative low incidence of development of microbial resistance) (Goldman and Kearns, 2011) underscores the need for accurate detection of resistance to guide therapy. Consequently, both phenotypic and genetic methods have been developed to achieve this goal.

Traditionally, clinical microbiology laboratories have relied on phenotypic methods to determine the antibiotic susceptibility profiles of pathogens (Tang and Stratton, 2006). Although these methods remain useful and have advantages such as low costs as well as being easy to perform and having established interpretation criteria, they however, lack the ability to generate timely susceptibility results and hence delaying initiation of treatment (van Belkum*et al*., 2013). Phenotypically a number of methods including broth microdilution, agar dilution, disk diffusion, MicroScan conventional panels, and Vitek cards have been used. Steward *et al*.(1999)compared the susceptibility of one hundred and ninety-five isolates of the family *Enterobacteriaceae* for resistance to ciprofloxacin and ofloxacin using these methods. While disk diffusion method measures zone of inhibition, the other methods determine Minimum Inhibitory Concentrations (MICs) as a measure of susceptibility/resistance. A modification to the disk diffusion is the Etest strip method. During the 1950s, Hans Ericsson developed a method to standardize the disc diffusion method and to improve its reproducibility and reliability for clinical susceptibility predictions. The inhibition zone sizes from disc test results were compared to Minimum Inhibitory Concentration (MIC) values based on the reference agar dilution procedure. The

correlation between zone sizes and MIC values was then assessed using regression analysis and regression lines were used for extrapolating zone of interpretive limits that corresponded to the MIC breakpoint values that defined susceptible, intermediate and resistant categorical results. The Etest is thus a quantitative technique for determining the antimicrobial susceptibility (AST) and MIC (in µg/mL) of Gram-negative and Gram-positive bacteria.

In response to the limitations of phenotypic methods and the desires to improve patient management and curb the spread of antimicrobial resistance, rapid antimicrobial susceptibility testing methods are continuously developed. These methods have been found to identify a pathogen and its antimicrobial susceptibility profile within a short period of time. There are basically five different ways to accelerate susceptibility testing in clinical diagnostics: (I) bypassing conventional culture by direct detection of the pathogen or resistance mechanism in the primary sample; (II) bypassing plate or broth culture dependent susceptibility testing (secondary culture); (III) avoiding time consuming work steps/methods; (IV) increasing the sensitivity to the detection of the infectious agent; that means detecting the infectious agent in earlier disease stages at lower viral or microbial loads; and (V) earlier detection of an evolving drug resistance during treatment in spreading less susceptible quasispecies (Frickmann *et al*., 2014). Agglutination assays have been developed as rapid culture-associated options. They are based on a suspension of microparticles coated with specific antibodies, leading to agglutination in contact with their specific antigens. Such procedures are useful for a preliminary resistance screening from pure bacterial colonies if the resistance mechanism of interest is associated with a single antigen only, which is expressed on the surface of the pathogen. Accordingly, agglutination assays are unfeasible for the screening for complex resistance patterns, which may be associated with multiple structurally different families of enzymes as in the case of extended

spectrum beta-lactamases (ESBL) or carbapenemases in Gram-negative rod-shaped bacteria. Other more robust rapid methods are based on genetic principles.

The usage of genotypic methods in the rapid detection of antimicrobial resistance genes is gradually shifting from academic research laboratories to diagnostic laboratories and point-of-care testing (Frickmann *et al*., 2014). Thesemethods are gaining more acceptance in determination of antimicrobial resistance for two main reasons: firstly, their capability to generate results within a short time as compared to phenotypic methods; secondly, their capability to detect antimicrobial genes directlyfrom the patient sample without necessarily waiting for culture results (Woodford and Sundsfjord, 2005). These two attributes aid clinicians in prescribing appropriate treatment to patients at the opportune time, hence making a positive contribution to antimicrobial stewardship programs (Doron and Davidson, 2011). However, genotypic tools for the detection of antimicrobial resistance may generate false negative results due to (i) their inability to detect new resistance mechanisms or (ii) false-positive results, because they may detect inactive or incomplete resistance genes in a specimen, which have not inferred resistance to the antimicrobial drug under test (Fournier *et al*., 2013). Current genotypic methods that are used for the rapid detection of antimicrobial resistance genes include (i) nucleic acid amplification methods, particularly real-time quantitative PCR (qPCR); (ii) DNA hybridization based methods,particularly DNA microarrays; (iii) Luminex xMAP technology; and (iv), next generation sequencing methods (Frickmann *et al*., 2014). Most of these methods are not without limitations. For example, most molecular assays such as qPCR and pyrosequencing lack the capability to simultaneously detect co-occurring single nucleotide polymorphism (SNP) mutations in different genes in a given specimen (Taylor and Taylor, 2004). However, this challenge has been overcome by Luminex xMAP Technology, a

multiplexing technology, which allows for simultaneous detection of multiple nucleic acid sequences in a single reaction (Dunbar, 2006). This technology has been used to simultaneously detect 11 mutations in *gyrA*, *gyrB,* and *parE* of *Salmonella* Typhi and *Salmonella* Paratyphi A(Song *et al*., 2010). Further, it has been used to simultaneously detect mutations in *gyrA* of *C. jejuni* and *C. coli* (Barco *et al*., 2010). In comparison to sequencing andmicroarray technology, LuminexxMAPTechnology has been found to be flexible, rapid, and cost effective (Dunbar, 2006; Song *et al*., 2010; Barco *et al*., 2010). Other rapid methods include Fluorescence In Situ Hybridization (FISH), direct fluorescent imaging of resistance determinants by Fluorescence Resonance Energy Transfer (FRET) and mass spectrometric approaches.

2.8.4 Epidemiology of Fluoroquinolone Resistance

The first quinolone used clinically, which is, nalidixic acid, was classified as a "urinary antiseptic.Previous nonfluorinated quinolones were almost exclusively used for treatment of lower urinary tract infections (UTIs). The fluorinated quinolones are characterized by more marked antibacterial activity against uropathogens, so that ciprofloxacin resistant *E. coli* strains isolated from female outpatients were almost nonexistent (<1%) till the mid-1990s; resistance to ciprofloxacin increased slowly from 1.2% in 1998 to 2.5% in 2001 (Karlowsky *et al*., 2002). The same holds true for uropathogenic *E. coli* isolated from male and inpatients, respectively, with a trend towards higher resistance rates among elderly patients (Karlowsky *et al*., 2001; Gupta *et al*., 1999). However, the North American Urinary Tract Infection Collaborative Alliance (NAUTICA) study revealed that ciprofloxacin resistance increased to 5.5% in 2004 (Zhanel *et al*., 2006).

The first plasmid-mediated quinolone resistance was reported in 1998 from a clinical strain of *Klebsiella pneumoniae* isolated in Alabama (Martinez-Martinez *et al*., 1998). This strain carried plasmid pMG252, which harboured the gene encoding quinolone resistance, later named *qnr*A1(Martinez-Martinez *et al,* 2009). This plasmid has been shown to increase quinolone MICs between eight-fold and sixty four-fold even in an *E. coli* strain with intact porins. Although this increase from baseline was not to the level designated as representing clinical resistance, the plasmid also facilitated selection of higher-level quinolone resistance (Ari *et al*., 2006). Wild-type *E. coli* carrying pMG252 plated on agar containing nalidixic acid or ciprofloxacin was 100 times more likely to give rise to spontaneous resistant mutants than a plasmid-free strain (Martinez-Martinez *et al,* 2009). Since this discovery, plasmid-mediated quinolone resistance has been described in different species from different parts of the world. Hata *et al*. (2005) described another *qnr* gene from a *Shigella flexneri* 2b isolate which was isolated during an outbreak of food poisoning in Japan. This strain contained a plasmid, designated pAH0376, containing a gene with high similarity (59%) to *qnr*, and was designated *qnrS.* This gene encoded a 218-amino acid protein that conferred low level resistance to fluoroquinolones. A third gene encoding quinolone resistance, *qnrB*, was described by Jacoby and colleagues in 2006. This gene was first found in a *Klebsiella pneumoniae* isolate from India and encoded a 214- amino-acid protein of the pentapeptide repeat family, which had 41% amino acid identity with *qnrA* and 39% amino acid identity with *qnrS* (Jacoby *et al*., 2006). A variant of this gene, *qnr*B2 was described by Nevashan *et al.* (2009)in an *S*. Typhimurium isolate collected from an HIV patient in South Africa in 2006. Wang and colleagues described another *qnr* gene, *qnrC*, which was found in *Proteus mirabilis* and was found to encode a 221-amino-acid protein (Wang *et al*., 2008) with different amino acid identities from *qnrD,* which encoded a 214-amino-acid pentapeptide repeat

protein. This wasdescribed by Cavaco *et al*. (2009) which has been found to cause reduced susceptibility to fluoroquinolones in isolates of *Salmonella enterica* serovar Bovismorbificans and Kentucky strains isolated from humans in the Henan province of China. *E. coli* was the first organism shown to be capable of carrying the *qnr* gene following its discovery in *Klebsiella pneumoniae* by Martinez-Martinez*et al*. (1998) where it was used as a recipient. Since then *qnr* carrying *E. coli* has been reported from almost all the continents of the world (Ari *et al.,* 2006; Schink *et al.*, 2012).

In Nigeria, while Kayode *et al*. (2010) demonstrated high frequency (87%) of decreased susceptibility to ciprofloxacin and high frequency (81%) of resistance to nalidixic acid among *Salmonella* isolates of chicken origin in Ibadan, Ajayi *et al.* (2012) described *qnr A* and *qnr B* genesin *E. coli* from cattle in Ado Ekiti.Clinically, *qnr* transfer genes were found in *S.* Typhi from faecal samples of HIV patients in Benin City by Enabulele *et al.* (2006) and *qnrA, qnrB, aac(6′)-Ib-cr , qepA and qnrD* alleles were described in gram negative isolates by Ogbolu *et al.* (2011). Aibinu *et al.* (2008) also reported a 21.4% prevalence of fluoroquinolone resistant gram negative isolates from cancer patients from Lagos university Teaching Hospital. Lamikanra *et al*. (2011) reported that by 2009, 35 (34.5%) *E.coli* isolates from undergraduate students of Obafemi Awolowo University, Ile-Ife were resistant to nalidixic acid of which 11 were fluoroquinolone resistant with 6 isolates carrying *qnr S* gene. Prevalence of 40.0%, 23.2%, and 41.1% to ciprofloxacin,enrofloxacin, and norfloxacin, respectively among *E. coli* isolates from chicken carcasses in Abeokuta was also reported by Ojo *et al*. (2012).

2.8.5 Economic Implications of Antibiotic Resistance

Antibiotic resistance is not always, but usually, associated with significant morbidity, longer hospitalization, excess costs and mortality. Excess costs associated with resistant microorganisms may be due to: obligation to use more expensive antibiotics, longer hospital stay, higher mortality, delayed appropriate antibiotic therapy or a necessity to perform surgery (Sipahi, 2008). Antibiotic resistance thus constitutes an economic burden on the healthcare system. Infections caused by resistant agents not only cost more to treat, but also can prolong healthcare use. In a 2008 study of attributable medical costs for antibiotic resistant infections, it was estimated that infections in 188 patients from a single healthcare institution cost between $13.35 and $18.75 million dollars (Roberts *et al*., 2009). Unfortunately, infections caused by antibiotic resistant bacteria are an everyday occurrence in healthcare settings.A recent review led by economist Jim O'Neill(2014) concluded that drug-resistant superbugs could cost the global economy as much as $100 trillion between now and 2050, a threat that warrants as much attention as climate change.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1Materials and Equipment

Sampling bottles, Latex hand gloves, Sterile swab sticks, Test tubes, inoculating loop, Coleman flask, Bijou bottles, Cotton Wool, Petri dishes, Test tube rack, Pipette, Petri dishes, Conical flasks, Masking tape, Permanent marker pen, Stuart medium, Lauryl Sulfate Tryptose Broth(LST), Mueller Hinton Agar, Eosin Methylene Blue (EMB), Nutrient Agar, Biochemical substrates (Simmons citrate, Urea, Tripple Sugar Iron (TSI), Sulfate Indole Motility (SIM), Methyl Red (MR), Voges-Proskauer (VP), Antibiotic discs, Agarose, Eppendorf tubes, Spin columns, Ethidium bromide, DNA Ladder (100bp), Lysis Buffer, Wash Buffer 1and 2, Elution Buffer, Microbact 12E kit, Antibiotic disc Dispenser, Incubator, Autoclave, Hot Air Oven, Hood, Refrigerator, Freezer, Centrifuge, GPS Reader, Camera.

3.2 Study Area

Zaria is a major city in Kaduna State and consists of two Local Government Areas namely Zaria and Sabon Gari (Figure 3.1). Zaria (Zazzau) was one of the original seven Hausakingdoms. The city is located within latitudes 11°04′N 7°42′E and longitudes 11.067°N 7.700°E with an altitude of 675 meters above sea level and a total land area of 300 km^2 (100 sq mi). It also has a density of 1,400/km^2 and a population of 975,228according to the 2006 Census.

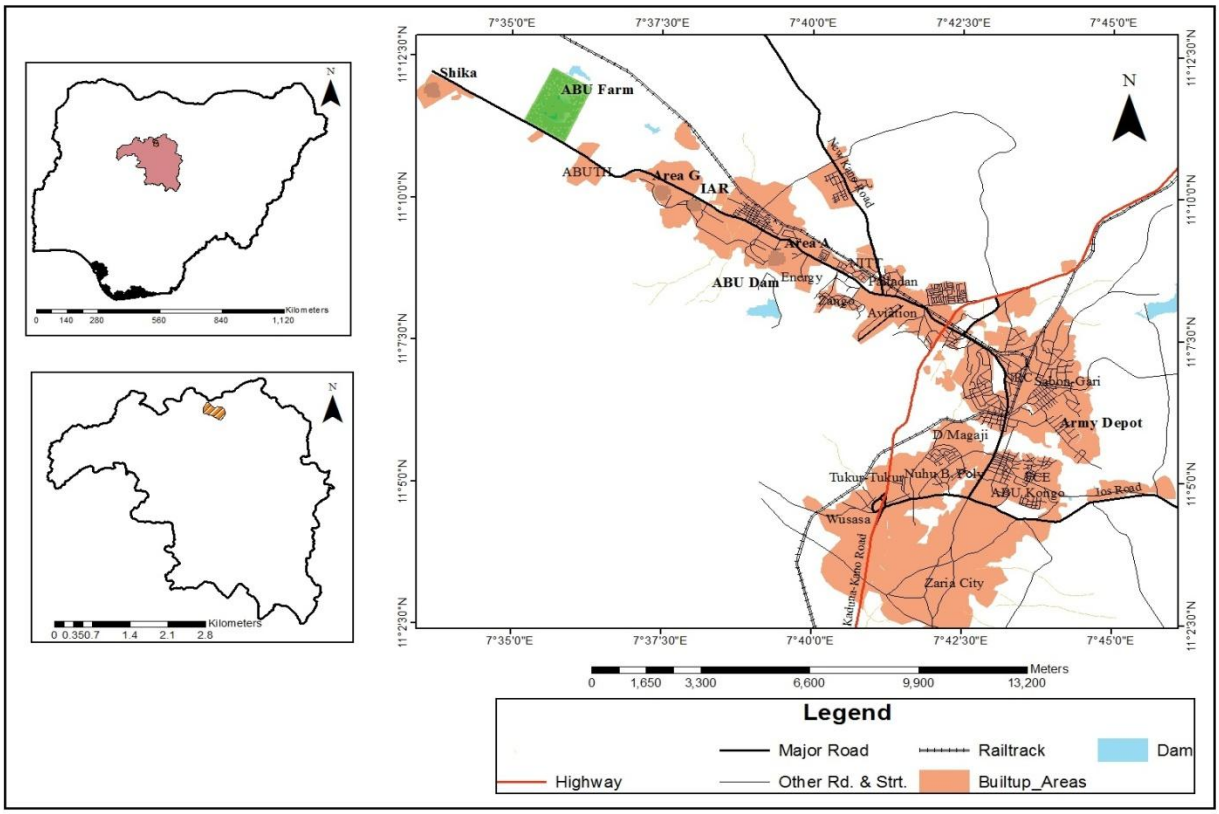


Figure 3.1: Map of Nigeria showing Zaria and Kaduna State.

3.3 Sample Size Determination

Win Episcope 2.0 was used to determine a sample size (215) for a cross sectional study based on a quinolone resistance prevalence rate of 23.2% (Ojo *et al*., 2012) at 95% confidence interval and 5% acceptable error.

3.4 Study Design

A cross sectional studyinvolving commercial poultry was conducted. Sampling was carried out between October 2014 and January 2015. The poultry clinic register of the Veterinary Teaching Hospital, Ahmadu Bello University Zaria for the year 2014 was used to construct a sampling frame according to the study's inclusion criteria. From this, 25% (20 farms) were selected.

3.5 Inclusion criteria

1- Poultry farms that visited the poultry clinic of the Veterinary Teaching Hospital, Ahmadu Bello University Zaria for veterinary medical services.
2- Poultry farms registered in the clinic with a minimum of 250 birds.
3- Samples were collected only with the farmer's consent and willingness to participate in the research.

3.6 Collection of Samples

Ten cloacal swabs perbatch/house depending on the number of batches or houses in a given farm were randomly collected. A total of 250 cloacal swabs were thus collected.Each swab tip was aseptically cut into a sample bottle of 5ml Stuart medium using sterile scissors. These were then transferred into a Coleman flask with ice pack and immediately transported to the Bacterial

Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, where they were analysed within 4-6h of collection.

3.7 Laboratory Analyses

3.7.1 Isolation and identification of *E. coli*.

3.7.1.1 Enrichment of sample

Lauryl sulfate tryptose (LST) broth containing MUG (4-methylumbelliferyl-B-D-glucuronide) was used for the enrichment of samples. The Stuart medium containing the swab was thoroughly but gently mixed and then one milliliter (1ml) was added to nine (9) mls of the broth (LST) in test tubes containing inverted Durham's vials and incubated for 24h at 37°C and observed for fermentation/gas formation. This was evidenced by gas trapped at the top of the invertedDurham's vials.

3.7.1.2 Plating

A loopfull of the enriched sample in broth was streaked on to Eosin Methylene Blue (EMB) agar, with a sterile inoculating loop and incubated at 37°C for 24h. Colonies that were raised, moist withgreenish metallic sheen, suggestive of *E. coli*, werethen inoculated on nutrient slants for further biochemical tests.

3.7.1.3 Conventional biochemical test.

Five biochemical tests were used to screen suspected *E.coli* isolates pickedfrom EMB. These tests includedTripple Iron Sugar (TSI), Simmons citrate, Urea, Sulfate-Indole-Motility (SIM) and Methyl Red (MR), Voges Proskauer (VP). Various reactions of the tests such as colour change, motility and gas formation were used to interpret results as either positive or negative

after 24h incubation. These tests were carried out as described in the methodology of Khandaghi *et al*. (2010).

3.7.1.3.1 Triple sugar iron agar test (TSI)

Triple Sugar Iron Agar was prepared according to manufacturer's instruction.This was then inoculated with an isolate by stabbing with a sterile Pasteur loop right down to the butt followed by streaking on the slant surface. This was then incubated at 37°C for 24 hours. Following this, the tube was then observed for hydrogen sulfide production as indicated by formation of a black precipitate and carbohydrate fermentation which is indicated by gas production and colour change.

3.7.1.3.2 Sulphur- indole-motility tests (SIM)

Following preparation of this medium,suspected isolates from EMB were inoculated into the medium by stabbing.The tube was then incubated at 37^{o}C for 24h. Hydrogen sulphide (H_2S) production wasindicated by a black discolouration while motility was indicated by migratory movementsaway from the line of stabbing giving a test tube brush-like appearance. Indole positive reaction was indicated by the appearance of a red coloured ring on the surface of the medium within few minutes following the addition of three drops of Kovac's indole reagent.

3.7.1.3.3 Methyl Red-Voges Proskauer test

Pure suspected isolates from EMB plates were inoculated into 5ml of MR-VP broth and incubated for 48hrs at 37^{0}C. After incubation, about 2.5ml of the broth was transferred to a second sterile tube and 2-3drops of methyl red was added. Appearance of a red coloration was indicative of a positive methyl red test. 5 drops of 40% potassium hydroxide (KOH) was added

to the remaining broth in the original tube followed by another 5drops of 5% of alcoholic (ethanol) alpha-Naphthol and shaken. The development of a red color starting from the liquid-air interface within 1hour indicated a positive test.

3.7.1.3.4 Citrate utilization test

An isolated colony from a pure culture was picked with a sterile Pasteur loop and then lightly streaked on the surface of the Simmons citrate agar slant. This medium contains Bromothymol blueas pH indicator. Following 24hincubation at 37^0C,citrate utilization was indicated by a change of colour to blue while a negative test retained the original green colour.

3.7.1.3.5 Urease test

Following purification, an isolated colony was picked with a sterile Pasteur loop and streaked lightly on the surface of a sterile urea agar slant in a test tube. This was then incubated for 24h at 37^oC.*E. coli* is not capable of hydrolysing urea to produce ammonia and carbon dioxide. The culture medium thus remained yellowish as against a positive test indicated by a bright pink (fuchsia) colour on the slant.

3.8 Microbact 12E Biochemical Test.

Isolates tentatively shown to be *E. coli* in the conventional biochemical screening tests were subjected to further tests using commercially available biochemical test strip, Microbact GNB 12E (Oxoid, Uk), for confirmation. This kit was used according to the manufacturer's instructions.

3.8.1 Test Procedure

Each isolate was purified on a selective medium (EMB). Three colonies were picked using a sterile Pasteur loop and emulsified in 5.0 ml peptone water and incubated at 37^0 C for 4h. A sterile pipette was then used to transfer one drop of the peptone water culture into 2.5ml of sterile normal saline solution (0.85%).

The wells of each individual substrate set were exposed by gently raising the sealing strip and slowly peeled backwards. 100µl of the bacterial suspension was then added to each well using a sterile micropipette. Mineral oil was also used to overlay the substrates in wells 1, 2 and 3, using a sterile micropipette. The inoculated rows were then resealed and labeled at the end of the tag with the specimen identification number followed by incubation at 37^0 C for 24h.

After 24hof incubation, the wells were again exposed as described earlier for reading of test results.The report form provided by the manufacturer was used to record result as positive or negative by comparing the colour of each well with that of an accompanying chart which comes along with a table of reactions. The results of wells 8, 10 and 12 were however not read immediately.Whereas two (2) drops of kovacs reagent was added to well 8 (indole production), and the result recordedtwo(2) minutes later, one (1) drop of TDA reagent was added to well 12 (Tryptophan Deaminase) and the result recorded immediately, while 1 drop each of VP1 and VP2 reagents were added to well 10 (Voges-Proskauer reaction) and the result recorded within 15 to 30 minutes.

3.8.2 Result Interpretation

The Microbact 12E uses an octal coding system in which every well (reaction) is assigned a value and a combination of three consecutive reactions produce a single digit of the code. From

each combination the sum of only the positive reaction(s) is taken. Each isolate therefore has a four (4)digit code. This code was then entered into a computer aided identification package and the resulting identity of the organism and its percentage probability recorded.

3.9 Determination of Antibiotic Susceptibility of *E. coli* Isolates.

Clinical and Laboratory Standards Institute (CLSI) protocol (2011**)** was used to determine the antibiotic susceptibility profiles of all isolates by disc diffusion method. Four to five (4-5) colonies of the test isolates from a purified culture were picked using sterile Pasteur loop and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to 0.5 MacFarland's standard and then 100µl of the suspension was dispensed and evenly spread on Mueller-Hinton agar plates. Antibiotic susceptibility profiles of all isolates confirmed to be *E. coli* from the Microbact 12E test was determined using a panel of fifteen antibiotic discs obtained from Oxoid (UK). These included Nitrofurantoin 300µg, Nalidixic acid 30µg, Ciprofloxacin 5µg, Ofloxacin 5µg, Enrofloxacin 5µg, Cefepime 30µg, Ceftriaxone 30µg, Doxycycline 30µg, Imipenem 10µg, Tetracycline 30µg, Streptomycin 25µg, Sulphamethoxasole/Trimethoprim 25µg, Chloramphenicol 30µg, Gentamicin 10µg, and Amoxicillin/Clavulanic acid 30µg. A disc dispenser (Oxoid UK) was used to apply the discs on the surface of each of the pre-inoculated Mueller-Hinton plates which were then incubated aerobically at 37^0C for 24h. After incubation the diameters of the zones of inhibition were measured to the nearest millimeter (mm) using a meter rule and classified as susceptible (S), intermediate resistance (I) or resistant (R) according to the CLSI (2011) criteria.

3.10 Detection of *qnr and* ESBL Genes

E. coli isolates resistant to fluoroquinolones from the disk diffusion test were subjected to a multiplex polymerase chain reaction to detect three (3)*qnr*(*qnr* A, *qnr* B, *qnr* S) and three (3) ESBL genes(*bla*-SHV, *bla* TEM, and *bla* CTX-M).

3.10.1 DNA Extraction

Following purification on EMB plates, bacterial DNA was isolated from a 24-hour culture in LB broth. This was done using ZR Fungal/Bacterial DNA MiniPrep^{TM} (from Zymo Research, USA) according to manufacturer's instructions. The isolated DNA was then ran on agarose gel electrophoresis to confirm presence of genomic DNA.

3.10.2 Gene Amplification

Extracted DNA was subjected to multiplex PCR in a 50 µl reaction mixture containing 1×PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl], 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate, 20 pmol of each of the six*qnr* primers as reported by Cattoir *et al*.(2007) and 2.5 U of Taq polymerase (Applied Biosystems, Courtaboeuf, France). Amplification was conducted using the following protocol: Initial denaturationfor 10 min at 95^0C and 35 cycles of amplification consisting of 1 min at 95^0C, 1 min at 54^0C and 1 min at 72^0C and 10 min at 72^0C for the final extension.

Multiplex PCR for detection of ESBL genes was conducted in a 25 µl mixture containing 1 µl DNA solution, Qiagen HotStarTaq Master Mix (Qiagen Nr. 203445) and 10 pmol of each gene-specific primer using the following amplification protocol: initial denaturation step at 95^0C for 15 min; 30 cycles of denaturation at 94^0C for 30 s, annealing at 60^0C for 30 s, extension at 72^0C for 2 min, followed by a final extension step at 72^0C for 10 min.

DNA fragments were analysed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1× TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate and 1 mM EDTA] containing 0.05 mg/L ethidium bromide.

3.11 Data Analyses

Data analyses were done using IBM Statistical Package for Social Sciences (SPSS version 20). Data obtained were presented in tables and charts. Chi-square and Fisher's extact test was used to test association between categorical variables (management type, feed type and resistance pattern) where values of $P<0.05$ were considered significant.

Table 3.1: Multiplex PCR primers for*qnr* and ESBL genes

Primer	Target Gene Sequence(5' 3')	Gene	Expected Amplicon Size (bp)
QnrAm-F	AGAGGATTTCTCACGCCAGG	*qnr*A1 to	580
QnrAm-R	TGCCAGGCACAGATCTTGAC	*qnr*A6	
QnrBm-F	GGMATHGAAATTCGCCACTG[C]	*qnr*B1 to	264
QnrBm-R	TTTGCYGYYCGCCAGTCGAA[C]	*qnr*B6	
QnrSm-F	GCAAGTTCATTGAACAGGGT	*qnr*S1 to	428
QnrSm-R	TCTAAACCGTCGAGTTCGGCG	*qnr*S2	
bla-SHV.SE	ATGCGTTATATTCGCCTGTG		
bla-SHV.AS	TGCTTTGTTATTCGGGCCAA	*bla*SHV	747
TEM-164.SE	TCGCCGCATACACTATTCTCAGAATGA		
TEM-165.AS	ACGCTCACCGGCTCCAGATTTAT	*bla*TEM	445
CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC		
CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG	*bla*CTX-M	593

Cattoir *et al*., 2007;Monstein *et al*., 2007

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *E. coli* from cloaca of commercial poultry in Zaria

Out of the two hundred and fifty (250) cloacal swabs collected and tested, 125 (50%) were found to be *E. coli* by conventional biochemical tests while Microbact® confirmed only 75 (30%) of these as *E. coli*.This means only 60% of the isolates suspected to be *E. coli*from conventional biochemical test were confirmed by Microbact®. This is shown in figure 4.1.

4.2 Antibiotic resistance pattern of *Escherichia coli* isolates

Out of the 75 Microbact®confirmed isolates, only 1 (1.3%) was susceptible to all antibiotics tested,while 3 isolates (4%) were sensitive to only 1 antibiotic, 2 (2.7%) others were sensitive to only 2 antibiotics of different classes. The other 69 (92%) isolates were sensitive to three or more antibiotics (Table 4.1). Highest percentage of resistance to fluoroquinolone was seen with enrofloxacin (49.3%) followed by ciprofloxacin and ofloxacin with 33.3 and 22.7% resistance respectively while 58.7% of the isolates were resistant to nalidixic acid. Up to 93.3, 80 and 76% of the isolates were resistant to amoxicillin/clavulanic acid, streptomycin and sulphamethoxasole/trimethoprim respectively. Resistance to chloramphenicol, tetracycline and gentamicin was exhibited by 68%, 65.3% and 52% of the isolates respectively. Least resistance was seen with doxycycline (10.7%), nitrofurantoin (6.7%), ceftriaxone (6.7%), imipenem (2.7%) and cefepime (1.3%)(Table 4.2). The isolates also showed 53 different patterns of resistance with AMC,S,TE,SXT,C and AMC,NA,CIP,OFX,ENR,CN,S,TE,SXT,Ccombinations having the highest frequencies with 6 isolates each (Table 4.1) followed by AMC,NA,CN,S,TE,SXT,C exhibited by 4 isolates. Overall multidrug resistance, that is resistance to more than two antibiotics, was expressed by 70 (93.3%) of the 75 isolates.

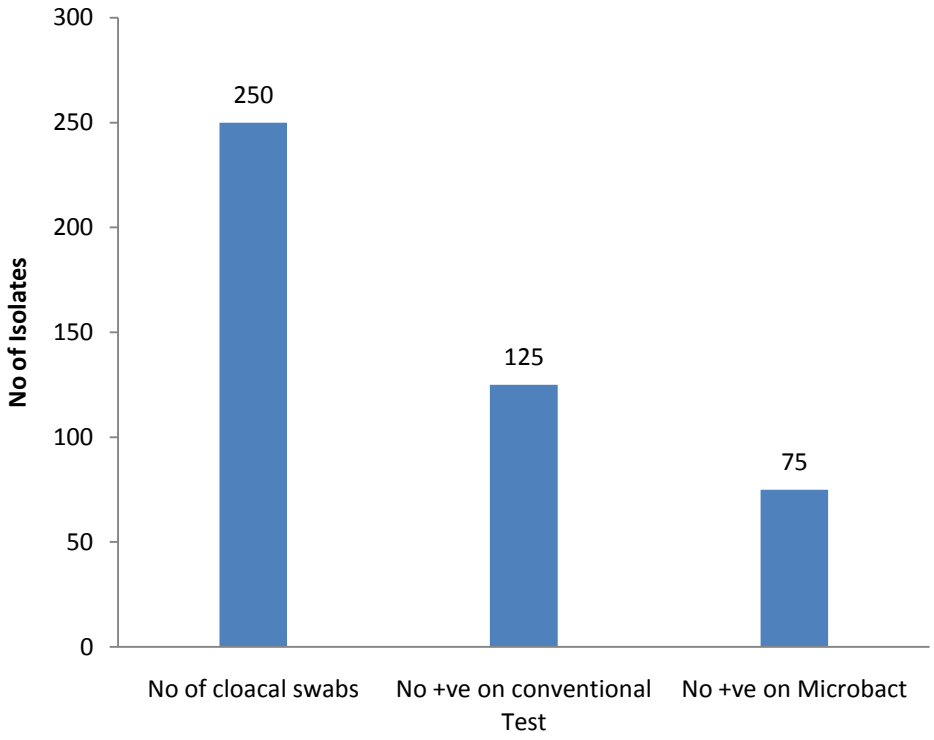


Figure 4.1: Isolation *E. coli* from cloaca of commercial poultry in Zaria.

Table 4.1: Antibiotic Resistance Patterns of *E. coli* Isolates from Cloaca of Commercial Poultry in Zaria to 15 antibiotics.

SN.	Pattern	No. of Isolates	SN.	Pattern	No. of Isolates
1	NIL	1	28	AMC,NA,CN,S,SXT,C	1
2	AMC	2	29	AMC,NA,CN,S,TE,C	1
3	ENR	1	30	AMC,NA,ENR,S,SXT,C	1
4	AMC,SXT	1	31	AMC,NA,ENR,S,TE,SXT	1
5	AMC,TE	1	32	AMC,NA,OFX,ENR,CN,TE	1
6	AMC,CN,C	1	33	AMC,CN,S,TE,DO,SXT,C	2
7	AMC,CIP,ENR,TE	1	34	AMC,NA,CIP,OFX,ENR,CN,C	1
8	AMC,CIP,S,SXT	1	35	AMC,NA,CN,S,TE,DO,SXT	1
9	AMC,CN,S,TE	1	36	AMC,NA,CN,S,TE,SXT,C	4
10	AMC,NA,ENR,C	1	37	AMC,NA,ENR,CN,S,TE,C	1
11	AMC,NA,S,SXT	1	38	AMC,CIP,OFX,ENR,CN,S,TE,C	1
12	AMC,NA,S,TE	1	39	AMC,NA,CIP,ENR,CN,S,SXT,C	2
13	AMC,S,SXT,C	2	40	AMC,NA,CIP,ENR,S,TE,SXT,C	1
14	AMC,S,TE,SXT	1	41	AMC,NA,CIP,OFX,ENR,CN,S,SXT	1
15	AMC,CN,S,TE,SXT	1	42	AMC,NA,CIP,OFX,ENR,CN,SXT,C	2
16	AMC,ENR,S,TE,SXT	1	43	AMC,NA,CN,S,TE,DO,SXT,C	1
17	AMC,NA,CIP,ENR,SXT	1	44	AMC,NA,ENR,CN,S,TE,DO,SXT	1
18	AMC,NA,ENR,S,SXT	1	45	AMC,NA,ENR,S,TE,SXT,C,F	1
19	AMC,S,TE,DO,SXT	1	46	AMC,NA,CIP,ENR,CN,S,TE,SXT,C	2
20	AMC,S,TE,SXT,C	6	47	AMC,NA,CIP,OFX,ENR,CN,S,TE,C	1
21	AMC,CN,S,TE,SXT,C	3	48	AMC,NA,CIP,OFX,ENR,S,TE,SXT,C	1
22	AMC,CRO,FEP,S,IPM,SXT	1	49	NA,CIP,OFX,ENR,S,TE,SXT,C,F	1
23	AMC,CRO,NA,S,SXT,F	1	50	AMC,NA,CIP,OFX,ENR,CN,S,TE,SXT,C	6
24	AMC,CRO,S,TE,SXT,C	1	51	AMC,NA,OFX,ENR,CN,S,TE,DO,SXT,C	1
25	AMC,ENR,S,TE,SXT,C	1	52	AMC,NA,CIP,OFX,ENR,CN,S,TE,DO,SXT,C	1
26	AMC,NA,CIP,ENR,S,C	1	53	AMC,CRO,NA,CIP,ENR,CN,S,TE,SXT,C,F	1
27	AMC,NA,CIP,ENR,TE,C	1	54	AMC,CRO,NA,ENR,CN,S,TE,DO,IPM,SXT,C,F	1
	Total	**36**			**39**

NA: Nalidixic Acid, CIP: Ciprofloxacin, OFX: Ofloxacin, ENR: Enrofloxacin, AMC: Amoxicillin/Clavulanic Acid, CRO: ceftriaxone, FEP: Cefepime, IPM: Imipenem, CN: Gentamicin, S: Streptomycin, TE: Tetracycline, DO: Doxycycline, SXT: Sulphamethoxasole/Trimethoprim, C: Chloramphenicol, F: Nitrofurantoin

Table 4.2: Percentage Resistance of *E. coli* Isolates to Various Antibiotics

S/N	Antibiotic	No. resistant	Percentage (%)
1	Nalidixic Acid	44	58.7
2	Ciprofloxcin	25	33.3
3	Ofloxacin	17	22.7
4	Enrofloxacin	37	49.3
5	Amoxicillin/Clavulanic Acid	70	93.3
6	Ceftriaxone	5	6.7
7	Cefepime	1	1.3
8	Imipenem	2	2.7
9	Gentamicin	39	52.0
10	Streptomycin	60	80.0
11	Tetracycline	49	65.3
12	Doxycycline	8	10.7
13	Sulphamethoxasole/trimethoprim	57	76.0
14	Chloramphenicol	51	68.0
15	Nitrofurantoin	5	6.7

4.3 Multiple antibiotic resistance (MAR) indicesof *E. coli* isolates

The antibiograms were used to calculate the multiple antibiotic resistance (MAR) indices. Multidrug resistance,as defined by the joint committee of the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention,was observed in69 isolates (92%) of the 75 isolates. Out of these, 5 (6.7%) isolates had MAR index of <0.20 (cut off value) while only 1 (1.3%) isolate had a value >0.80. Whereas 21 (28%) isolates had values between 0.20 and 0.39, 33 (44%) and 14 (18.7%) isolates had values between 0.40 and 0.59 and 0.60 and 0.79 respectively (Table 4.3).

4.4: Occurrence and pattern of Fluoroquinolone resistance and Beta-lactam antibiotics

4.4.1 Association between management and feed type withoccurrence of resistance to Fluoroquinolone antibiotics amongst*E. coli* isolates from chickens

There was no significant association (P>0.05) between occurrence of resistance in the *E. coli* isolatesto fluoroquinolones and management system practiced by the farmer.Similarly no association (P>0.05)was found between occurrence of resistance in the isolates with the feed type used(Tables 4.4 and 4.5).

4.4.2 Association between Management and Feed Type with Occurrence of Resistance to Beta Lactam antibiotics amongst *E. coli* isolates from Chickens

No significant association (P>0.05) was observed between occurrence of resistance to β-Lactam antibiotics and management type among the various *E. coli* isolates. A similar result was obtained when feed type was also compared with occurrence of resistance(Tables 4.6 and 4.7).

Table 4.3: Multiple Antibiotic Resistance Indices (MAR) of Various *E. coli* Isolates

S/N	MAR Index Category	No. of Isolates
1	<0.20	5
2	0.20 – 0.39	21
3	0.40 – 0.59	33
4	0.60 – 0.79	14
5	>0.80	1
	Total	**74**

Table 4.4: Association between Management and Occurrence of resistance to Fluoroquinolones among *E. coli* isolates of Chickens

Management	**Resistant**	**Sensitive**	**Total**
Deep Litter	38	32	70
Cage	1	4	5
Total	39	36	75

Fisher's exact test*P = 0.188

Table 4.5: Association between Feed Type and Occurrence of resistance to Fluoroquinolones among *E. coli* isolates of Chickens

Feed Type	**Resistant**	**Sensitive**	**Total**
Commercial	20	14	34
Self Compounded	19	22	41
Total	39	36	75

Chi Square (X^2) = 1.106, P = 0.281

Table 4.6: Association between Management and Occurrence of resistance to β-Lactam antibiotics among *E. coli* isolates of Chickens

Management	**Resistant**	**Sensitive**	**Total**
Deep Litter	67	3	70
Cage	5	0	5
Total	72	3	75

Fisher's exact test* P = 1.000

Table 4.7: Association between Feed Type and Occurrence of resistance to β-Lactam antibiotics among *E. coli* isolates of Chickens

Feed Type	**Resistant**	**Sensitive**	**Total**
Commercial	33	1	34
Self Compounded	39	2	41
Total	72	3	75

Fisher's exact test* P = 1.000

4.5: Results of Multiplex PCR for determination of plasmid-mediated fluoroquinolone resistance and ESBL genes.

Eighteen (18)fluoroquinolone resistant isolates were tested for three variants each of *qnr*(*qnrA, qnrB* and *qnrS*) and ESBL (*blaTEM, blaSHV* and *blaCTX-M*) genes using a multiplex PCR. Plates I and II show agarose gel electrophoresis pictures of the multiplex PCR. All 18 isolates tested showed amplification of genes corresponding to*qnr S* (428bp) and *blaTEM* (445bp). This indicates co-carriage of these genes inall the 18 fluoroquinolone resistant*E. coli* tested. These isolates also showed multidrug resistance.The other genes*qnr*A, *qnr*B and *bla*SHV and *bla*CTX-M were not detected in the multiplex PCR.

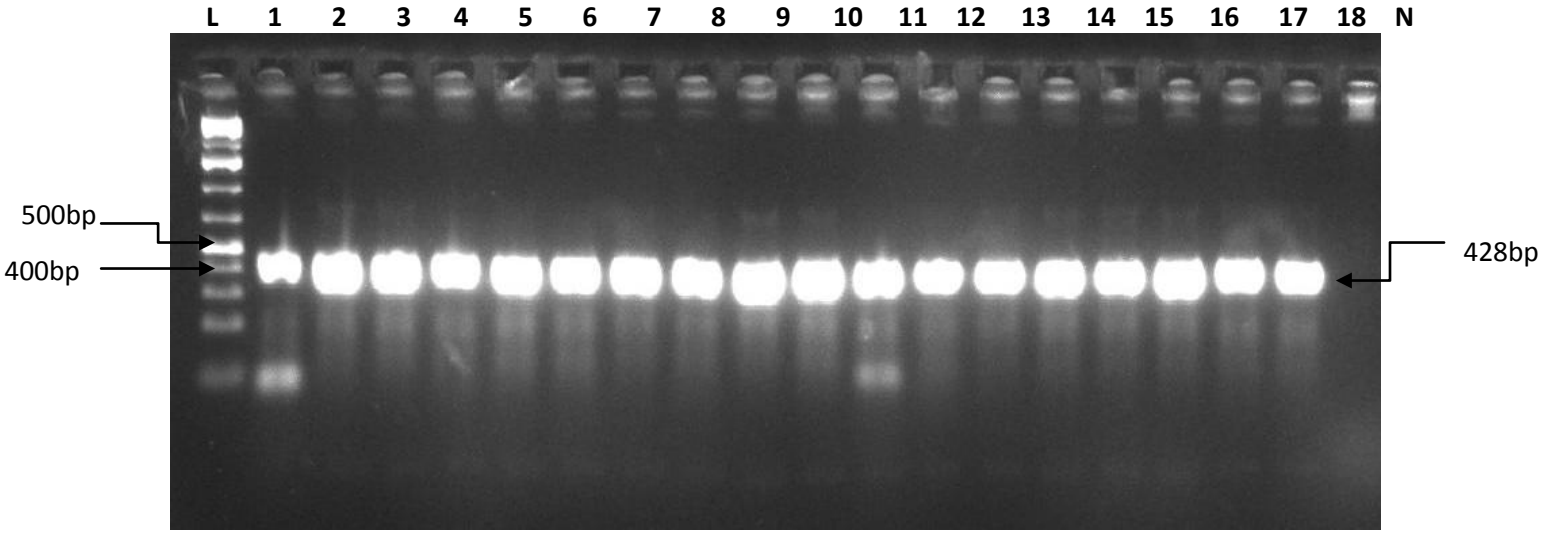


Plate I: PCR result showing *qnr* S in lanes 1-18 as follows: 1=AA/L3, 2=BF/L10, 3=GG/L2, 4=GG/L6, 5=GG/L9, 6=HH/L2, 7=IN/L5, 8=IN/L8, 9=IN/L9, 10=IN/L10, 11=JA/L7, 12=JA/L9, 13=HS/L5, 14=MA/L1, 15=MH/L2, 16=BF/L4, 17=IN/L8, 18=ST/L8 L= DNA ladder, N= Negative control.

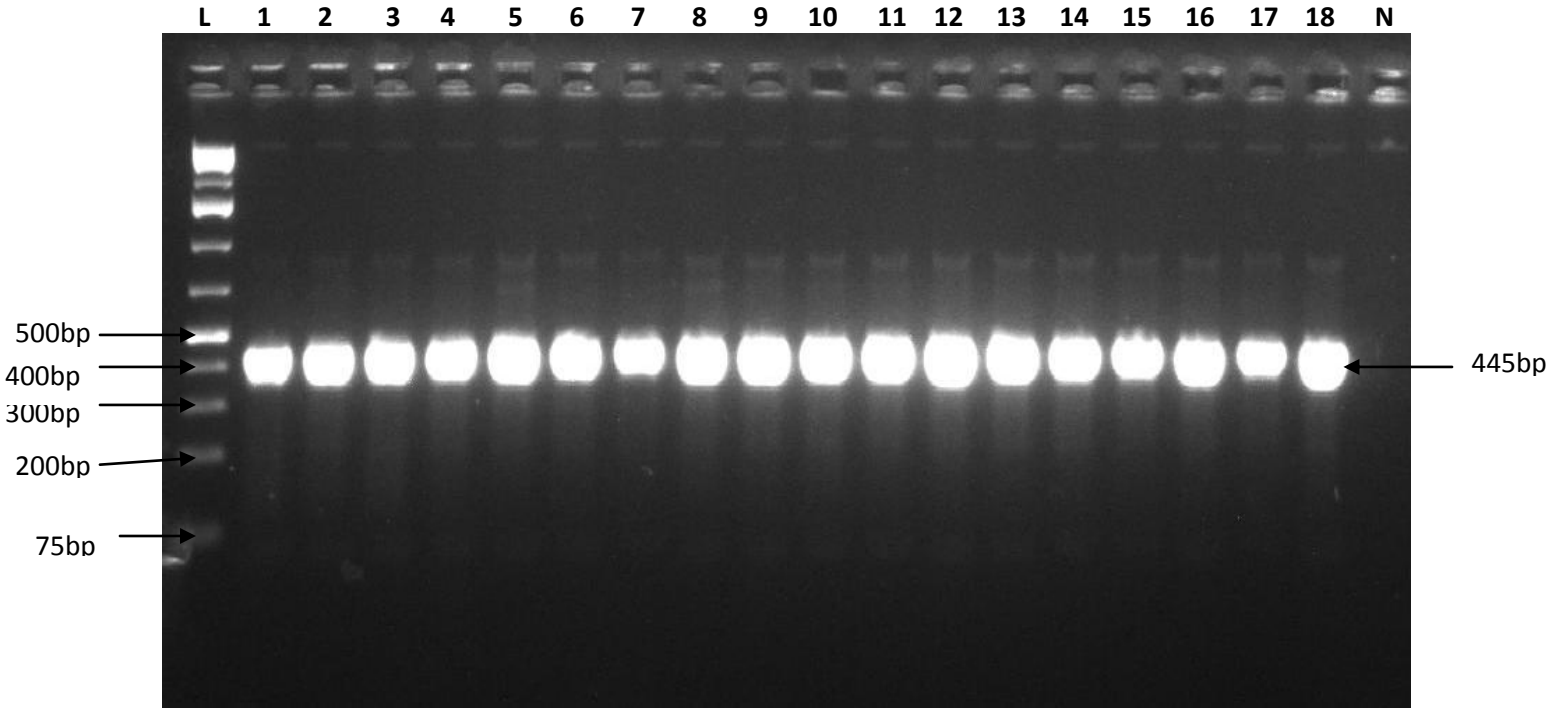


Plate II: PCR result showing *bla* TEM in lanes 1-18 as follows: 1=AA/L3, 2=BF/L10, 3=GG/L2, 4=GG/L6, 5=GG/L9, 6=HH/L2, 7=IN/L5, 8=IN/L8, 9=IN/L9, 10=IN/L10, 11=JA/L7, 12=JA/L9, 13=HS/L5, 14=MA/L1, 15=MH/L2, 16=BF/L4, 17=IN/L8, 18=ST/L8 L= DNA ladder, N= Negative control.

CHAPTER FIVE

DISCUSSION

In this study,*E. coli* was isolated from the cloaca of 75(30%) of 250 chickens sampled in Zaria for the presence of antibacterial resistant pathogens as confirmed by Microbact® 12E. Even though the bacterium is considered a part of the normal flora of the gastrointestinal tract of poultry, other factors such as survival in sample,presence of microbial inhibitors such as drugs consumed by the bird amongst other factors might have limited the isolation rate.These 75 isolates were confirmed using Microbact® 12E, a biochemical identification system developed mainly for the confirmation of human isolates which might have returned some *E.coli* from poultry as negative since the conventional test panel indicated 50% isolation rate.A similar result was reported by Tafida *et al.* (2014) in which Microbact® 12E confirmed four out of the eight isolates suspected to be *E. coli* from conventional biochemical tests.

Although high, the 30% obtained in this study is less than the 81%prevalence obtained by Adenipekun *et al*. (2015). The higher prevalence may be explained by the fact that Adenipekun *et al*. collected voided faecal samples not directly from the cloaca in which case environmental isolates may not be ruled out and also in that study, they used a different identification test kit (VITEK_2 System and the VITEK 2GNidentification card from bioMe´rieux, Durham, NC).

A high prevalence (92%) of multidrug resistance was also recorded in this study.This finding is in good agreement with high rates reported by Ogunleye *et al*.(2008) and Melton(2014)who recorded a multidrug resistance prevalence of 100% among *E. coli* isolated from poultry in Abeokuta and Yola respectively.Fifteen (15) antibiotics were used in this study,while Ogunleye *et al*. (2008) and Melton (2014) used twelve (12) and eight (8) antibiotics respectively. There is a wide variation in types and panels of antibacterials used for susceptibility testing of *E. coli* for

resistance surveillance and monitoring, which is bound to yield different outcomes. Similarly there is variation in the definition of multidrug resistance.The harmonised version of the Joint Committee of the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention, which defined multidrug resistance asacquired non-susceptibility to at least one agent inthree or more antimicrobial categories, was adopted for this study (Magiorakos *et al*., 2012).

The antibacterial drugs used in an area provide suitable conditions for the emergence of multidrug resistant bacteria. All farms sampled in this study consulted the Veterinary Teaching Hospital of Ahmadu Bello University, Zaria.It is a common practice among poultry farmers particularly small scale farmers and live bird vendors to seek veterinary advice after several failed attempts with several antibiotics. Melton (2014) showed that 54% of drugs used by poultry vendors were obtained without prescription from open markets and 68% of these vendors used their experience in determining drug dosages.

Also, cocktails of antibiotics of different classes in a single formulation are commonlyavailable in Nigerian markets. Therefore poultry are frequently exposed to different classes of antibiotics at the same time thereby priming their microbial flora for multidrug resistance.This could explain why only one isolate was found to be sensitive to all antibiotics tested, while five others were sensitive either to only one or two antibiotics. This finding also almost correlates with that of Jahantigh and Dizaji (2015) in Iran, a country where poor drug use conditions are also said to prevail, who found that no*E. coli* isolate was sensitive to all antibiotics tested.

Fifty three (53) different resistance patterns ranging from resistance to a single class to a combination of seven (7) different classes of antibiotics were observed in this study. Olarinmoye *et al*.(2013) also reported thirty nine different resistance patterns with a multidrug resistance

prevalence of 84.68%among *E. coli* from chichens in South Western Nigeria. Thisindicates the simultaneous involvement of various resistance mechanisms within some of these isolates.Multidrug resistance could be due to efflux pumps known to have broad substrate specificity (Hopkins *et al*., 2005). For example, the AcrB protein of *E. coli*, in conjunction with other proteins such as TolC and AcrA, form a conduit which extrudes not only cellular metabolites but a diverse group of antibiotics (Soparkar *et al*., 2015). In addition, certain plasmid-mediated resistance genes including *qnr*A and *qnr*B have been shown to be integrated into integrons which harbour other antibiotic resistance genes such as β-lactamases or aminoglycoside inactivating enzymes, while *qnr*S and *bla*TEM are known to be associated with transposons (Hernandez *et al*., 2011).The involvement of mobile genetic elements such as plasmids, integrons and transposons in the spread of antibacterial resistance has emerged as a complicating factor in the effort to control resistance. These elements which are easily transmissible facilitate horizontal gene transfer thus promoting the evolution and adaptation of microbial communities (Smalla and Sobecky, 2002). Such developments serve to potentiate resistance in organisms with chromosomal resistance mechanisms to certain drugs such as fluoroquinolone resistance in *Enterobacteriaceae*.

Allmultidrug resistant isolates in this study had a multiple antibacterial resistance index (MAR) of ≥0.20. Thismay be evidence ofthe widespread use of antibiotics among poultry farmers in Zaria. This study further revealed no significant association between both management system (i.e. deep litter versus cage and feed type: commercial feed versus self compounded feed)withthe resistance displayed by the isolates. This therefore suggests that the occurrence and the degree of resistance may be similar across small and large farms in Zaria.

All eighteen (18) isolates representing 24% of *E. coli* isolatessubjected to PCRwere shown to simultaneously carry *qnrS* and *blaTEM* genes.Although all these isolates were multidrug resistant, they were however, all sensitive to cefepime, ceftriaxone and imipenem. Furthermore, fifteen (15) of the eighteen (18) isolates were all resistant to all FQs tested except three (3) isolates which were sensitive to ciprofloxacin, ofloxacin and nalidixic acid. This pattern could result from the fact that cefepime, ceftriaxone and imipenem which are all β-lactams are not commonly used in poultry and even clinically they are reserved for the most resistant organisms whereas FQs are commonly used. Although *qnr* genes are known to be plasmid encoded, the absence of other *qnr* and ESBL genes in these isolates and the simultaneous occurrence of*qnr*S and *bla*TEM in all 18 isolates points more towards involvement of transposons or carriage of both genes on plasmids and transposons rather than integrons since *qnr*S is not known to be associated with integrons as reported by Hernandez *et al*.(2011). Both *qnr*S and *bla*TEMgenes responsible for resistance to FQs and β-lactam antibiotics respectively, are very significant, given that FQs are important first and second line drugs for treating clinically important infections (Thielman and Guerrant, 2004; Jamison, 2006), while certain β-lactam antibiotics such as carbapenem are drugs of last resort (Kunduru *et al*., 2016). Fortini *et al*. (2011) reported occurrence of genes involved in plasmid-mediated quinolone resistance (PMQR),*qnrS* inclusive and *blaTEM* in *E. coli* isolates from healthy chickens and pigs at slaughter in Ibadan. Sumrall *et al*. (2014) also reported the mapping of *qnrS*1 gene from a Nigerian *E. coli* isolate to a 43.5 kb IncX2 plasmid (pEBG1). Variations indistribution of *qnr* genes has been suggested to be attributed todifferences in geographic area (Oktem *et al*., 2008)or selection criteria (Wang *et al*., 2008). For example while *qnrS* was shown to be the most prevalent among members of *Enterobacteriaceae* family in studies in Egypt and China, the same gene was not detected in a

US study (Robicsek, 2006; Wang *et al*., 2008; El-Mahdy, 2015).Similarly, *qnrS* was identified as the most prevalent PMQR gene among *E. coli* isolates in a study involving wild birds and chickens in South Korea(Jae-Young *et al*., 2016).

Consequently, the association of genes encoding for quinolone resistance and resistance to other drug classes like *β*-lactams and aminoglycosides favour the selection and dissemination of fluoroquinolone resistant strains by chemically unrelated drug classes, and vice versa, of *β*-lactam or aminoglycoside resistant strains by fluoroquinolones (Dalhoff, 2012) thereby complicating the issue of antibacterial resistance. This suggests that resistance to a single drug may subsequently lead to resistance to many other drug classes.

Resistance to fluoroquinolones and extended spectrum *β*-lactam antibiotics in *E. coli* of poultry origin in Zaria may have emerged as a public health threat with potentials for grave economic consequences.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

From this study therefore, it is concluded that there is high prevalence (92%) of multidrug resistance in *E. coli* isolates inpoultry in Zaria. The management system and feed types were not associated with isolation of resistant *E. coli* and to occurrence of FQ resistance. Qnr-based FQ resistance may have emerged in *E. coli* in poultry, with *qnr*S and *bla*TEM genes beingwidespread among FQ resistant *E. coli* tested in Zaria.Therefore, poultry is a potential source of multidrug resistant and *qnr*-bearing *E. coli*with potentials for spread of resistance genes to other bacteria in the environment and human populations in Nigeria.

6.2 Recommendations

1. Poultry farmers should adhere strictly to biosecurity measures on their farms to reduce the need for antibiotic use.
2. Where necessary, antibiotics should be administered in accordance with good drug use and management practicesunder the prescription of a certified veterinarian.
3. Public health authorities should encourage safe handling and use of poultry manure to avoid the dissemination of resistant genes from poultry to the environment.
4. There is need for hygiene in poultry handling and preparation by consumers to reduce the risk of transmission of resistant zoonotic bacteria such as *E. coli*.
5. Further studies should be conducted to determine the spread of *qnr* genes in other bacteria in animal hosts.

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APPENDIX

Appendix I: Antibiotic resistance patterns of *E. coli* isolates from cloacal swabs of chickens in Zaria

S/N	Resistance Profile
1	S, SXT,C,AMCs
2	CIP,ENR,TE,AMC
3	ENR,TE,S,SXT,AMP
4	OFX,CIP,ENR,DO,TE,S,SXT,NA,C,CN,AMC
5	OFX,CIP,ENR,TE,S,SXT,NA,C,AMC
6	TE,S,SXT,C,AMP
7	OFX,CIP,ENR,NA,C,CNAMC
8	CIP,ENR,S,SXT,NA,C,CN,AMC
9	TE,AMC
10	S,SXT,C,AMC
11	FEP,CRO,IPM,S,SXT,AMC
12	CIP,ENR,TE,S,SXT,NA,C,AMC
13	AMC
14	SXT,AMC
15	TE,S,SXT,C,AMC
16	ENR,TE,S,SXT,C,AMC
17	ENR,NA,C,AMC
18	AMC
19	CIP,ENR,SXT,NA,AMC

20	F,OFX,CIP,ENR,TE,S,SXT,NA,C
21	CRO,TE,S,SXT,C,AMC
22	CIP,ENR,TE,NA,C,AMC
23	TE,S,SXT,C,CN,AMC
24	F,CRO,ENR,DO,IPM,TE,S,SXT,NA,C,CN,AMC
25	ENR,S,SXT,NA,AMC
26	DO,TE,S,SXT,NA,CN,AMC
27	F,CRO,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
28	TE,S,SXT,CN,AMC
29	ENR,TE,S,SXT,NA,AMC
30	S,SXT,NA,C,CN,AMC
31	TE,S,SXT,C,AMC
32	TE,S,SXT,C,CN,AMC
33	TE,S,SXT,NA,C,CN,AMC
34	TE,S,SXT,NA,C,CN,AMC
35	CIP,ENR,S,NA,C,AMC
36	OFX,CIP,ENR,TE,S,C,CN,AMC
37	F,ENR,TE,S,SXT,NA,C,AMC
38	OFX,ENR,TE,NA,CN,AMC
39	ENR,DO,TE,S,SXT,NA,CN,AMC
40	TE,S,SXT,C,CN,AMC
41	TE,S,NA,AMC
42	TE,S,SXT,C,AMC

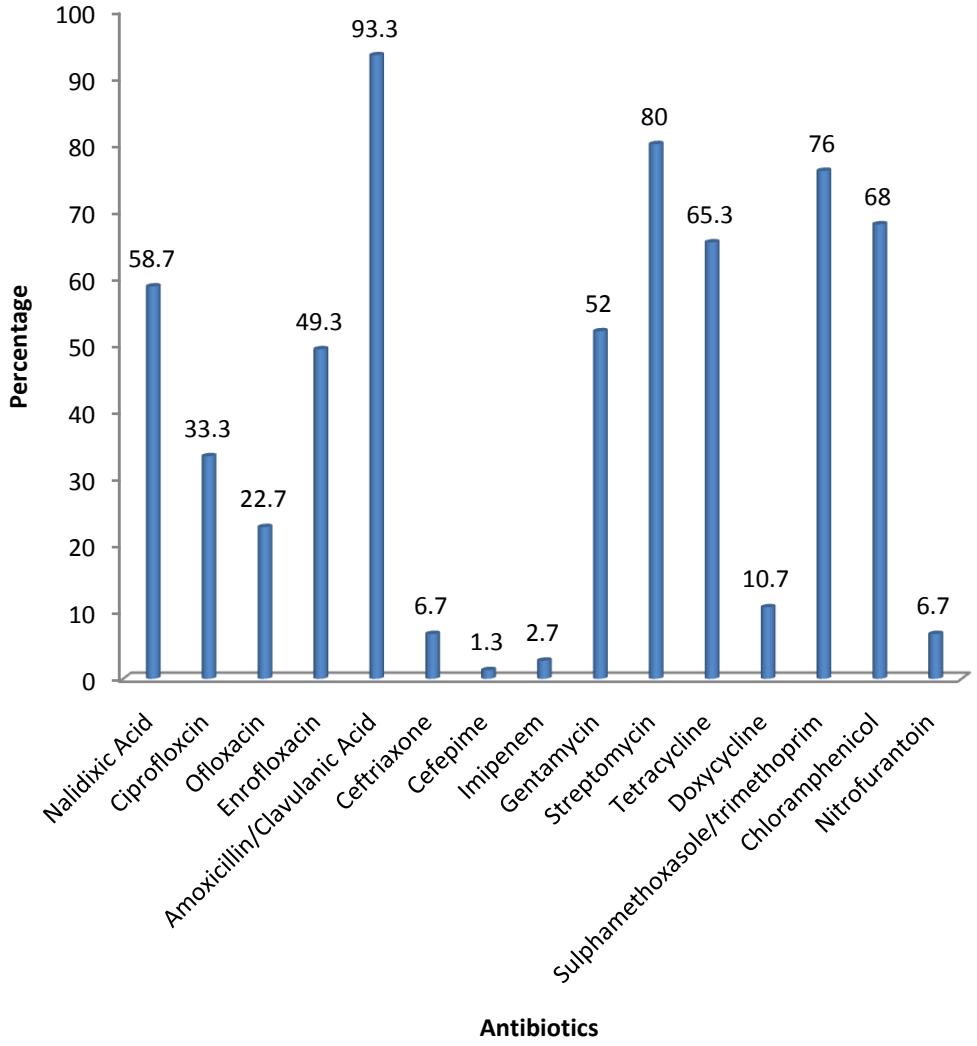
43	CIP,ENR,TE,S,SXT,NA,C,CN,AMC
44	TE,S,SXT,C,AMC
45	TE,S,SXT,AMC
46	TE,S,SXT,C,AMC
47	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
48	DO,TE,S,SXT,AMC
49	OFX,ENR,DO,TE,S,SXT,NA,C,CN,AMC
50	DO,TE,S,SXT,NA,C,CN,AMC
51	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
52	DO,TE,S,SXT,C,CN,AMC
53	DO,TE,S,SXT,C,CN,AMC
54	ENR,TE,S,NA,C,CN,AMC
55	TE,S,NA,C,CN,AMC
56	ENR
57	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
58	OFX,CIP,ENR,SXT,NA,C,CN,AMC
59	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
60	OFX,CIP,ENR,TE,S,NA,C,CN,AMC
61	TE,S,CN,AMC
62	CIP,S,SXT,AMC
63	TE,S,SXT,NA,C,CN,AMC
64	C,CN,AMC
65	CRO,F,S,SXT,NA,AMC

66	S,SXT,NA,AMC
67	CIP,ENR,S,SXT,NA,C,CN,AMC
68	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
69	TE,S,SXT,NA,C,CN,AMC
70	OFX,CIP,ENR,TE,S,SXT,NA,C,CN,AMC
71	OFX,CIP,ENR,SXT,NA,C,CN,AMC
72	ENR,S,SXT,NA,C,AMC
73	OFX,CIP,ENR,S,SXT,NA,CN,AMC
74	CIP,ENR,TE,S,SXT,NA,C,CN,AMC

Appendix II: Multiple antibiotic resistance (MAR) indices of resistant E. coli isolates from chickens in Zaria.

S/N	MAR
1	0.27
2	0.27
3	0.33
4	0.73
5	0.60
6	0.33
7	0.47
8	0.53
9	0.13
10	0.27
11	0.40
12	0.53
13	0.13
14	0.33
15	0.40
16	0.27
17	0.33
18	0.60
19	0.40
20	0.40
21	0.40
22	0.80
23	0.33
24	0.47
25	0.73
26	0.33
27	0.40
28	0.40
29	0.33
30	0.40
31	0.47
32	0.47
33	0.40
34	0.53
35	0.53
36	0.40
37	0.53
38	0.40

39	0.27
40	0.33
41	0.60
42	0.33
43	0.27
44	0.33
45	0.67
46	0.33
47	0.67
48	0.53
49	0.67
50	0.47
51	0.47
52	0.47
53	0.40
54	0.67
55	0.53
56	0.67
57	0.60
58	0.27
59	0.27
60	0.47
61	0.20
62	0.40
63	0.27
64	0.53
65	0.67
66	0.47
67	0.67
68	0.53
69	0.40
70	0.53
71	0.60
72	0.07
73	0.07
74	0.07



Appendix III: Percentage resistance of *E. coli* isolates to various antibiotics

Discrete *E. coli* colonies

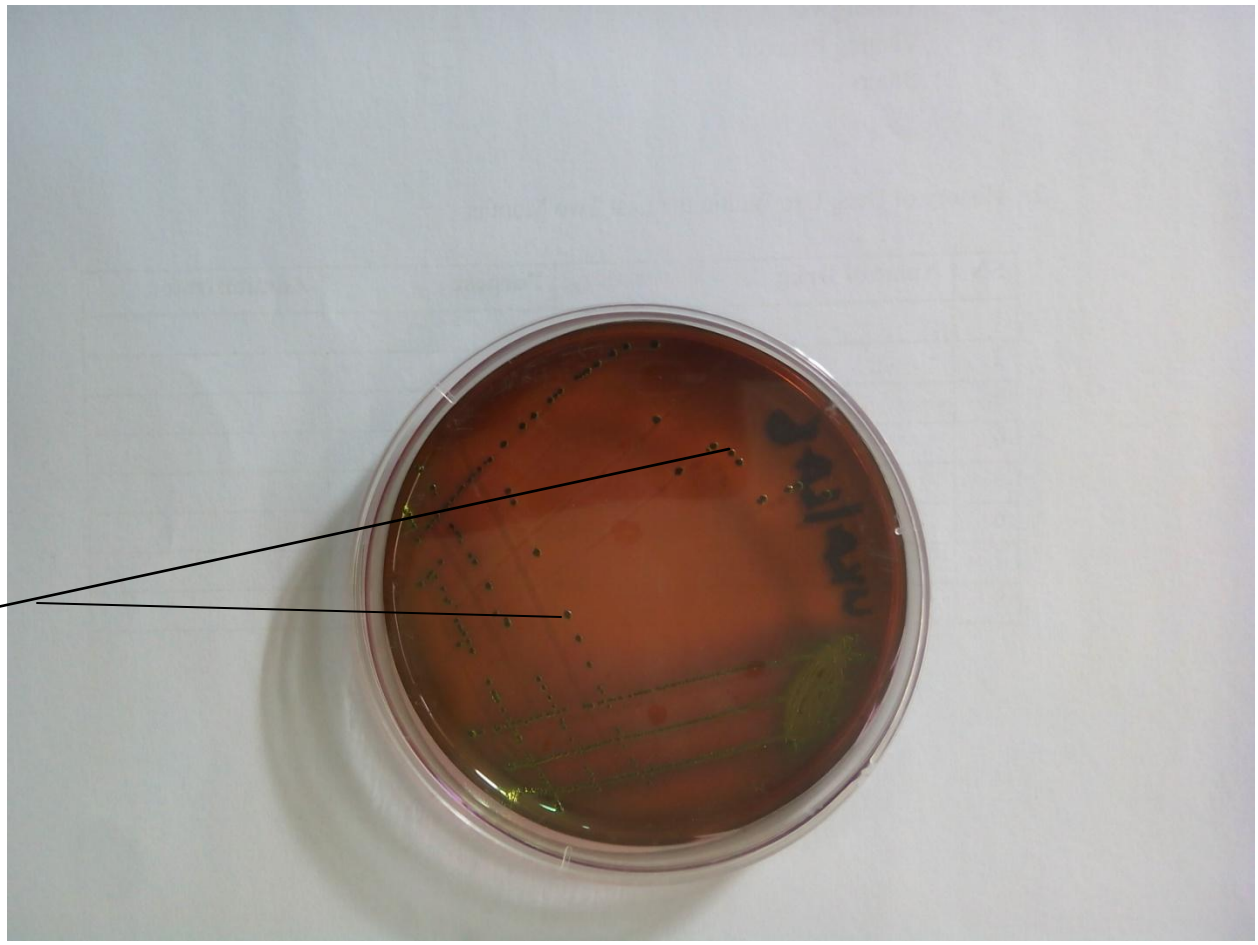


Plate III: Suspected *E. coli* isolate on EMB plate

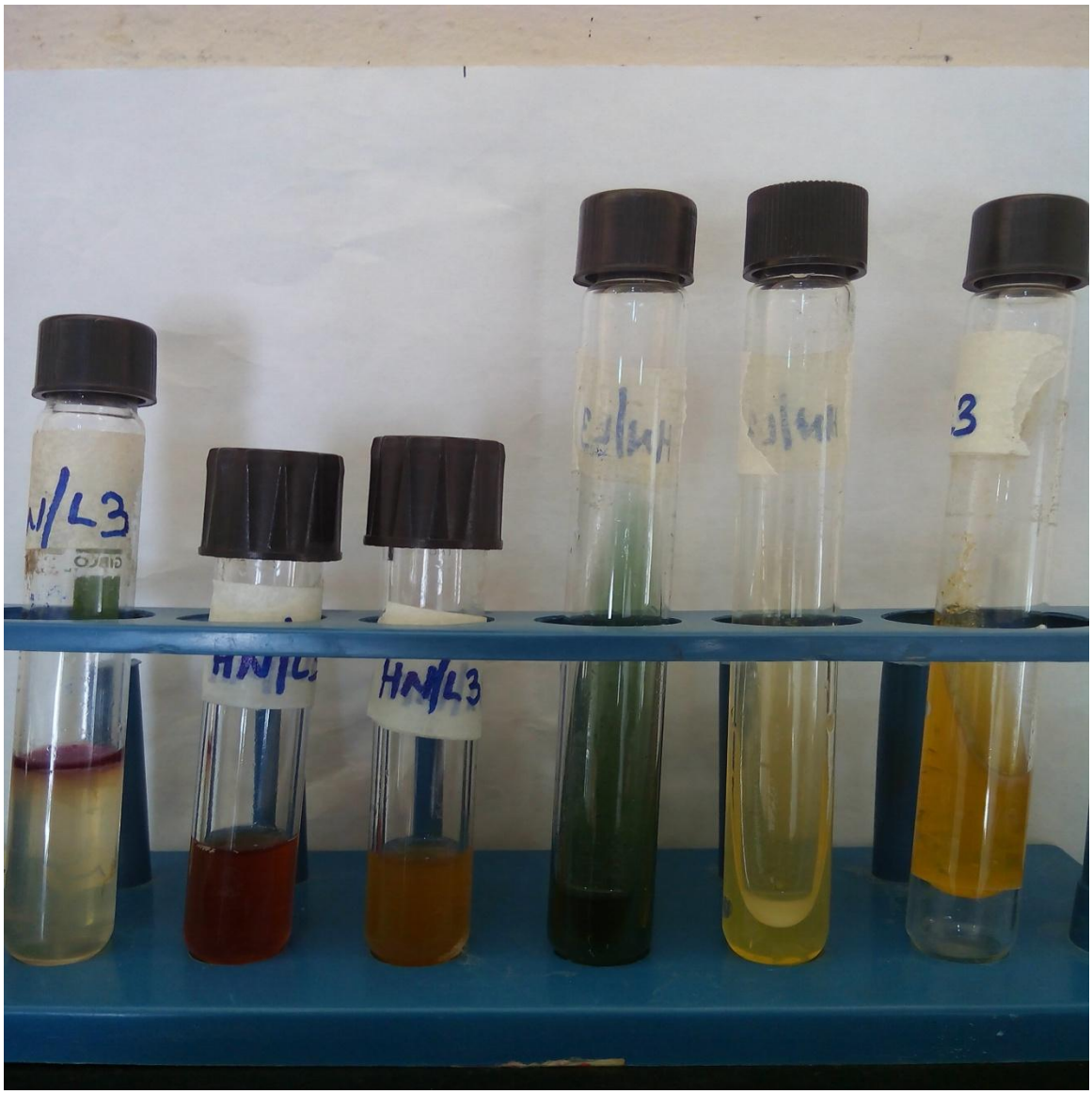


Plate IV: Conventional biochemical tests of +ve *E. coli* isolate

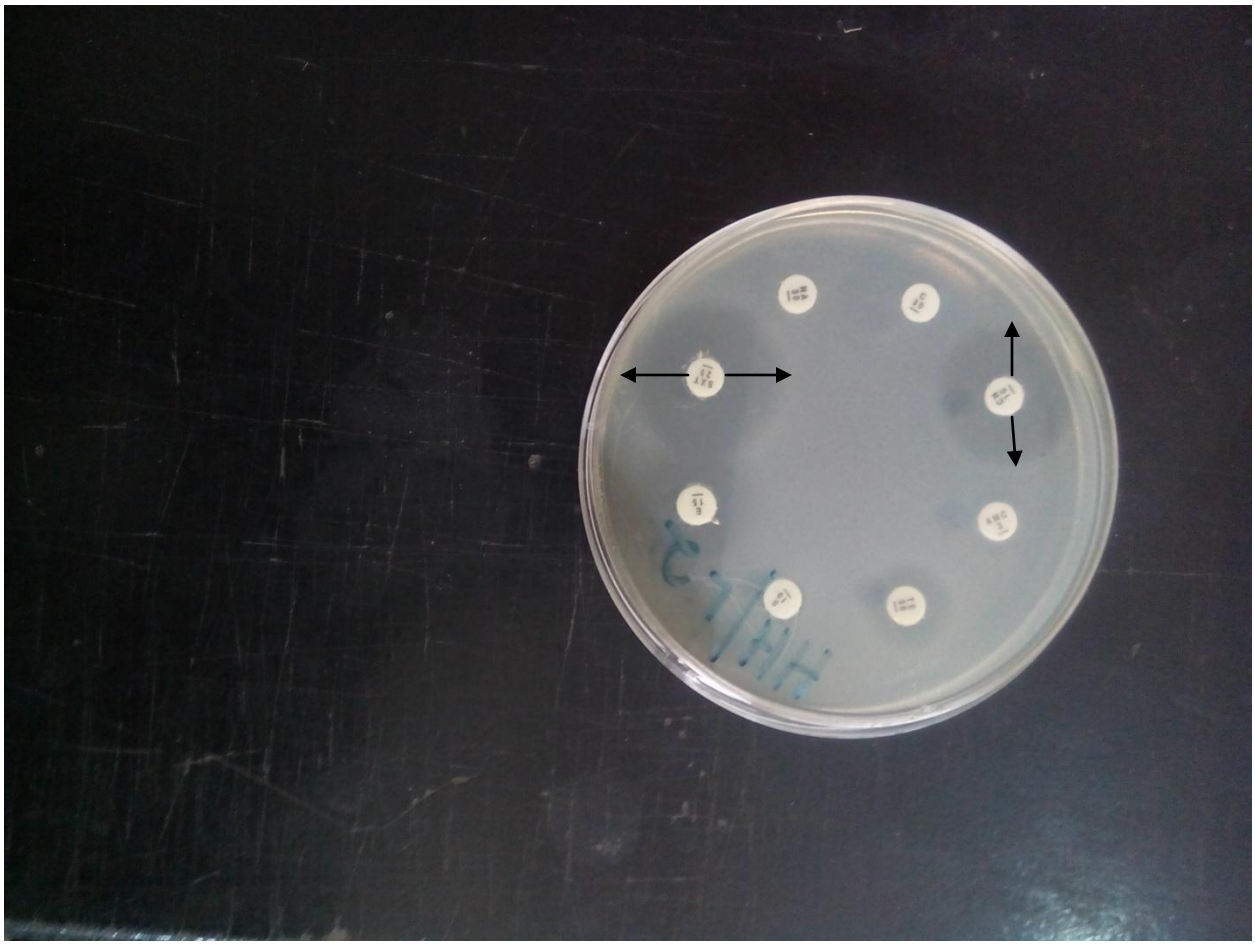


Plate V: Disk diffusion test of a resistant isolate with arrows showing zones of inhibition.