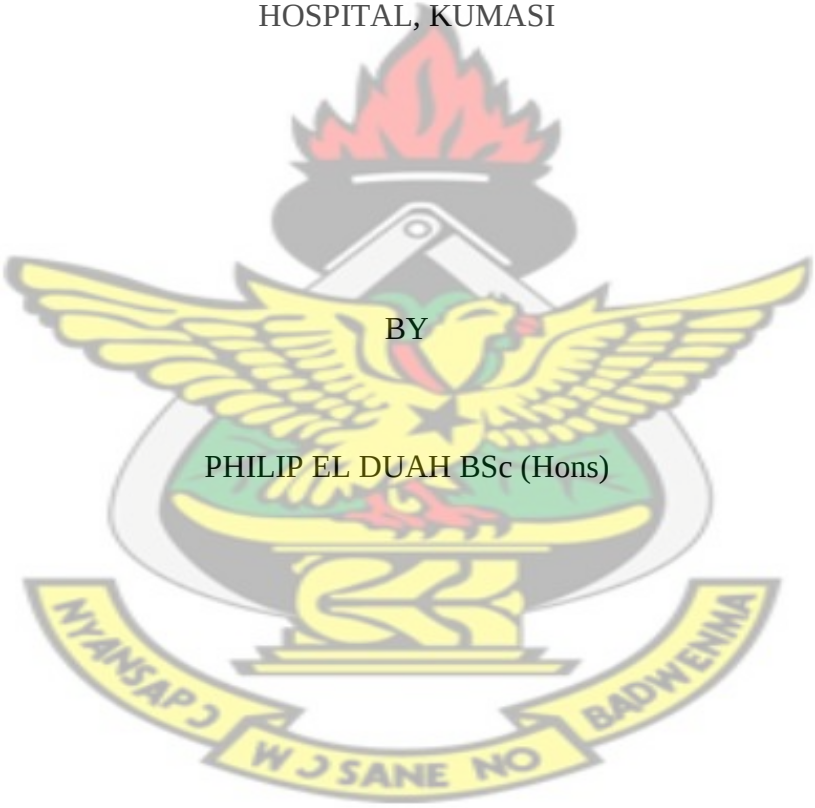
KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

SCHOOL OF GRADUATE STUDIES

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF CLINICAL MICROBIOLOGY

DETECTION OF MUTATIONS IN *gyr*A and *par*C BY MAMA-PCR OF QUINOLONE-RESISTANT ISOLATES OF *Escherichia coli* FROM THE KOMFO ANOKYE TEACHING HOSPITAL, KUMASI



BY

PHILIP EL DUAH BSc (Hons)

DETECTION OF MUTATIONS IN *gyr*A and *par*C BY MAMA-PCR OF QUINOLONE-RESISTANT ISOLATES OF *Escherichia coli* FROM THE KOMFO ANOKYE TEACHING HOSPITAL, KUMASI

By

Philip El Duah BSc (Hons)

A Thesis submitted to the Department of Clinical Microbiology, Kwame Nkrumah University of Science and Technology, in Partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (CLINICAL MICROBIOLOGY)
School of Medical Sciences, College of Health Sciences

July, 2013

DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

PHILIP EL DUAH …… ……..

PG 6067111 Signature Date

Certified by:

PROF. YAW ADU-SARKODIE …… ……

Supervisor Signature Date

Certified by:

PROF. E. H. FRIMPONG …… …….

Head of Department Signature Date

ACKNOWLEDGEMENT

I am most grateful to the almighty God for his sustenance and inspiration.

I am grateful to my supervisor Prof. Yaw Adu-Sarkodie for his guidance and dedication in supervising this research work. To my parents I say I am forever indebted to you. I would also like to extend my profound gratitude to Dr. Augustina Annan of the Kumasi centre for collaborative research in tropical medicine for her support. A big thank you also goes to the Antibiotic Drug use, Monitoring and Evaluation of Resistance (ADMER) organization for funding my work.

My deepest appreciation also goes to the staff of the Microbiology Laboratory at Komfo Anokye Teaching Hospital especially Mr. Robert Awuley Lartey, the Department of Clinical Microbiology and the Kumasi centre for collaborative research in tropical medicine.

ABSTRACT

Antimicrobial resistance is a serious problem on the ascendency which has been documented in some parts of Africa and Ghana. By the World Health Organization's assessment, infections caused by resistant microorganisms often fail to respond to conventional treatment, resulting in prolonged illness and greater risk of death (WHO, 2012). According to Namboodiri *et al*, 2011, data available on the prevalence of resistance to antimicrobial substances in Africa is limited to phenotypic resistance tests with little data on the molecular basis for resistance. Bacterial strains for the study were obtained from isolates of pathological samples of patients visiting the Komfo Anokye Teaching Hospital. Resistance to seven antimicrobials was tested using the disc diffusion method and minimum inhibitory concentration (MIC) of ciprofloxacin was also determined using the agar dilution technique in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Amplification of the quinolone-resistance determining regions was done by mismatch amplification mutation assay (MAMA-PCR) and visualization of PCR products by Agarose gel electrophoresis. The highest number of the isolates were from urine (n=138). A total of 139 of the 200 isolates obtained were from female patients while 55 were from male patients. The ages of patients from whom the isolates were obtained ranged from 2 days old to 88 years old. Out of the antibiotics tested, gentamicin showed the lowest percentage of resistance (47.5%) and ampicillin showed the highest percentage (94%). The number of susceptible isolates to the two quinolones tested was 21%. There were no isolates that were susceptible to nalidixic acid but resistant to ciprofloxacin. The most common phenotype in the study was the pan-resistant (30.5% of isolates) and 2.5% of isolates were susceptible to all antibiotics tested. Testing by MIC yielded 67.5% resistant, 2.5% intermediate and 30% susceptible isolates. MIC50 for ciprofloxacin in this study was >64µg/mL. A very good level of agreement was observed between the two methods used to determine resistance to ciprofloxacin (k=0.91). Five groups were defined by the results according to mutations in *gyr*A and *par*C. The group with the highest number of isolates (67%) was that with Ser-83 substitutions in *gyr*A. The group with the lowest number of isolates (1%) was that with substitutions in Ser-83 and Asp-87 in *gyr*A and Ser-80 in *par*C. Mutations in *gyr*A83 had the highest occurrence (149 isolates) and those in *gyr*A87 had the lowest occurrence (2 isolates). Isolates that had only a single mutation had minimum inhibitory concentration values ranging from 0.5µg/mL to >64µg/mL. With the exception of one isolate, all isolates that had minimum inhibitory concentration values less than 64 µg/ml (22 isolates) had only one mutation and no isolate that had two or more mutations (48 isolates) had a minimum inhibitory concentration value less than 64µg/mL. with the exception of one isolate, all isolates that had mutations in *par*C had MIC values that were greater than 64µg/mL. Mutations in the quinolone resistance development region were found to be associated with all quinolone resistant isolates in this study.

TABLE OF CONTENTS

CHAPTER THREE

CHAPTER FIVE

CHAPTER SIX

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

CHAPTER ONE

1.0 INTRODUCTION

Escherichia coli belong to a group of Gram negative bacteria which are a very important group of organisms in both hospital and community acquired infections the world over. These organisms are more resistant to antibiotics than Gram positive organisms due to the nature of their outer membrane or cell wall (Bastopcu *et al.*, 2008). They are important causes of urinary tract infections, blood stream infections, gastrointestinal infections and various nosocomial infections such as pneumonia (Paterson, 2006).

Quinolones belong to a large family of bactericidal synthetic agents which can be grouped into generations based on their spectrum of activity like the Cephalosporins. Nalidixic acid is the first generation prototype, but the addition of fluorine at position 6 of the main quinolone ring; forming fluoroquinolones, has improved antibacterial activity, leading to the synthesis of many additional compounds. The antibacterial activity of quinolones is due to their ability to inhibit the activity of bacterial DNA gyrase and topoisomerase IV hence disrupting DNA replication (Mims *et al.*, 2006). DNA gyrase and topoisomerase IV are large, complex enzymes composed of 2 pairs of subunits. The subunits of DNA gyrase are GyrA, a 97-kDa protein encoded by the *gyr*A gene, and GyrB, a 90-kDa protein encoded by the *gyr*B gene. The corresponding subunits of topoisomerase IV are ParC (75 kDa) and ParE (70 kDa) (Jacoby, 2005).

A few bacteria are able to function with only DNA gyrase, however a lot of bacteria have both enzymes. In Gram-negative bacteria, gyrase is more susceptible to quinolones than topoisomerase IV, whereas, in gram-positive bacteria, topoisomerase IV tends to be more susceptible than gyrase. Consequently, resistance mutations occur first in *gyr*A in gram-

negative bacteria, but they occur first in *par*C in gram-positive bacteria. Synthetic fluoroquinolones were introduced to help in controlling the threat of Methicillin Resistant *Staphylococcus aureus* (MRSA) but within the duration of a year, a significant number of isolated strains had developed resistance to them. Resistance involves amino acid substitutions in a region of the GyrA or ParC subunit known as the "quinolone-resistance determining region" (QRDR) (Hogg, 2005). Resistance to fluoroquinolones was first reported in 1990s and it has been on the ascendency since then. Although ciprofloxacin proved to be effective against *S. enterica* in a study conducted in Ghana, the resistance rate of enterobacteriaceae other than *S. enterica* against this quinolone increased from zero in 2001–2002 to 50.0% in 2009 (Groß *et al.*, 2011).

Frequent use of ciprofloxacin has been implicated in resistance development among quinolones and other antimicrobial agents (Sabir *et al.*, 2004). Fluoroquinolones like ciprofloxacin possess a broad spectrum antimicrobial activity and good oral bioavailability. This has conferred on them the status of leading antimicrobial agents against a wide variety of infectious diseases (Sabir *et al.*, 2004). Quinolone resistance in infectious diseases particularly due to enterobacteriaceae may lead to treatment failures and give cause for significant concern (Paterson, 2006). Studies conducted in West Africa on quinolone resistance in commensal or diarrhoea causing *Escherichia coli* before 2004 reported no or very low incidences of resistance to nalidixic acid and the fluoroquinolones (Namboodiri *et al.*, 2011) but has been on ascendency since then. Quinolone resistance in enterobacteriaceae normally comes about as a result of alterations in DNA gyrase and/or topoisomerase IV. Resistance can also come about due to impaired access to the target enzymes, which occurs either because of changes in porin expression or because of efflux mechanisms (Paterson, 2006).

1.1 Justification

There is evidence that using antimicrobials judiciously may reduce the rate of emergence of resistance. In this regard, information from regular surveillance of antimicrobial resistance in conjunction with data on the use of antimicrobials serves as a powerful tool for the containment of resistance (WHO, 2002). A lot of work has been done on Quinolone resistance globally. However, the amount of data originating from African and West African studies in particular pertaining to the molecular basis for Quinolone resistance is limited (Namboodiri *et al.*, 2011).

1.2 AIM OF THE RESEARCH PROJECT

1.3 Main objective

To determine the presence of quinolone resistance causing mutations in isolates of *Escherechia coli* from the Komfo Anokye Teaching Hospital, Kumasi.

1.4 Specific objectives

- To isolate *E. coli* from various clinical specimens.
- To determine prevalence of resistance of *E. coli* to quinolones and other antibiotics.
- To determine in-vitro minimum inhibitory concentration of ciprofloxacin on *E. coli* isolates.

- To determine whether mutations exist in the quinolone resistance determining region of *gyr*A and *par*C of *E. coli* isolates from the Komfo Anokye Teaching Hospital.
- To examine the relationship between resistance to quinolones and the presence of single nucleotide polymorphisms.

CHAPTER TWO

2.0 LITEREATURE REVIEW

2.1 Background of *Escherichia coli*

Escherichia coli is a bacterium that is commonly found in the gut of warm blooded organisms (Kapoor, 2010). *E. coli* was first discovered in 1885 by Theodor Escherich, a German bacteriologist whilst he was searching for the cause of fatal intestinal diseases in children. He studied infant faeces for the causative organism and amongst the microbes he encountered was a quick-growing rod-shaped bacterium he named *Bacterium coli commune*. The bacterium is one of the most versatile of all bacterial pathogens. Some strains are important members of the normal gut flora in man and animals whereas others possess certain characteristics that grant them the capability to cause infections in the intestinal tract or at other sites such as the urinary tract (Mims *et al.*, 2006). The strains that form part of the normal gut flora are usually harmless and can be beneficial to their hosts in some ways such as producing vitamin K_2, or by preventing pathogenic bacteria from getting established within the intestine. However, certain strains can also cause conditions such as food poisoning in the gut (Kapoor, 2010). For many years it was thought that the bacterium was simply a commensal organism of the large intestine. This view however changed when a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Todar, 2012). *E. coli* are not strictly confined to the intestine but can be excreted into the environment in faeces and have the ability to survive for short periods outside the body. This makes it an ideal organism to serve as an indicator when testing environmental samples such as water for contamination with faeces (Kapoor, 2010). The term coliform is given to *E. coli* and other bacteria that can ferment

lactose to produce acid and gas within 48 hours such as *Enterobacter* and *Klebsiella* spp which all belong to a broad Family called enterobacteriaceae (Burton and Engelkirk, 2003).

2.2 CHARACTERISTICS AND CLASSIFICATION OF *E. coli*

2.2.1 Structural characteristics

According to Bergey's Manual of Determinative Bacteriology, *E. coli* are rod shaped bacteria which mostly fall within the size range of 0.5 by 1.0 to 3.0 microns. Their shape may vary from almost coccoid forms to long rods and may also occur singly, in pairs and in short chains. *E. coli* come in both motile and non-motile forms with motile strains exhibiting peritrichous flagellation. They are Gram negative, usually not encapsulated and do not form spores (Breed *et al.*, 1957).

2.2.2 Biochemical characteristics

E. coli belong to a large group of enteric bacteria which are rod shaped, mostly motile by peritrichous flagella and possess the ability to ferment glucose and other sugars to give a variety of products. Due to the similarity of the members of this group in terms of appearance, they are distinguished from one another mainly by means of their biochemical characteristics. A series of tests can be performed on any unknown isolate such as its ability to utilize certain substrates like lactose and citrate, convert tryptophan to indole, and hydrolyse urea. On the basis of the isolates response to each test, a characteristic profile can be developed and matched against those of known species for identification (Hogg, 2005).

2.2.2.1 Indole Production

Bacteria use several means to communicate with one another and with their eukaryotic hosts. In certain cases, such social interactions among bacteria permit them to synchronize their behavior as a group and in effect behave like multicellular organisms (Bassler and Losick, 2006).

Bacteria communicate with one another using small chemical signal molecules in a process called quorum sensing (Yang *et al.*, 2006). Much like in higher organisms, the information provided by such chemical signaling is essential for synchronizing the activities of large groups of cells and the process entails the production, release, detection, and response to small molecules. The molecules involved are like hormones and are called autoinducers. The process permits bacteria to monitor their environment and to alter their behavior as a group in response to changes (Waters and Bassler, 2005). Indole is among the numerous signal molecules that are employed by both Gram-positive and Gram-negative bacteria and has a wide range of biological roles in different bacterial strains (Lee and Lee, 2010). Indole is an aromatic heterocyclic organic compound. It consists of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring to form a bicyclic structure (Biswal *et al.*, 2012). Indole is produced by the action of an enzyme called tryptophanase which can reversibly convert the amino acid tryptophan into indole, pyruvate and ammonia during growth of enteric bacteria and accumulates in the medium within which it is being cultured (Newton and Snell, 1965; Hirakawa *et al.*, 2009). Indole is a compound that has diverse functions in the bacterial signaling process. It is involved in the transition of growth from the exponential phase to the stationary phase (Piñero-Fernandez *et al.*, 2011). Indole is

involved in drug resistance in *E. coli* by inducing the expression of several xenobiotic exporter genes which are not expressed under normal conditions (Hirakawa *et al.*, 2005)

2.2.2.2 Lactose fermentation

Escherichia coli are capable of utilizing several compounds as sources of carbon. Glucose however is the preferred carbon source of *E. coli* (Martínez-Gómez *et al*., 2012). Glucose is central to the reactions that take place in glycolysis and is preferentially metabolised since it is more energy efficient to do so and the enzymes involved are permanently switched on or constitutive (Hogg, 2005). Lactose is a disaccharide found in milk which consists of galactose joined to glucose by a β-1,4-glycosidic linkage. Lactose is hydrolyzed to these constituent monosaccharides by lactase in human beings and by b-galactosidase in bacteria (Berg *et al.*, 2002). When *E. coli* grows in a medium that contains both glucose and lactose, the cell has a regulatory mechanism that suppresses the synthesis of lactose-metabolising enzymes until all the glucose has been used up (Hogg, 2005). The lactose or *lac* operon contains three structural genes and is controlled by the *lac* repressor. The enzymes produced by these genes are β- galactosidase which the bacterium uses to convert the disaccharide lactose into its constituent sugars, β-galactoside permease; the protein responsible for lactose uptake and β-galactoside transacetylase, whose function is still uncertain. These enzymes are required to metabolise lactose and so *E.coli* only induces the transcription of these enzymes when lactose is available (Prescott, 2002; Hogg, 2005).

2.3 PATHOGENIC *Escherichia coli*

Over 700 antigenic types or serotypes of *E. coli* have been recognized based on O, H, and K antigens. Serotyping is important in distinguishing the small number of strains that

actually cause disease. *E. coli* is responsible for infections in humans such as urinary tract infections, neonatal meningitis, sepsis and intestinal diseases. These three diseases depend on a specific array of pathogenic determinants (NIH, 2011; Motayo *et al.*, 2012).

2.3.1 Urinary Tract Infections

Most urinary tract pathogens originate in the faecal flora, but only the aerobic and facultative species such as *E. coli* possess the attributes required to colonize and infect the urinary tract. Only certain serogroups of *E. coli* are able to cause urinary tract infections such as O serotypes O1, O2, O4, O6, O7 and O75 and K serotypes K1, K2, K3, K5, K12 and K13. These serotypes are called uropathogenic *E. coli* (UPEC). The ability of these strains to cause urinary tract infections is attributable to a variety of genes in chromosomal pathogenicity islands, such as the genes associated with colonization of the periurethral areas (Mims *et al.*, 2006)

2.3.2 Sepsis

E coli has the tendency to reach the bloodstream and cause sepsis when normal host defenses are reduced. Newborns may be highly susceptible to *E coli* sepsis due to the fact that they lack IgM antibodies. Sepsis may occur secondary to urinary tract infection (Jawetz *et al.*, 2010).

2.3.3 Meningitis

E coli are one of the most common causes of neonatal meningitis; many features of which are similar to group B streptococcal disease. The pathogenesis involves vaginal *E. coli* colonization of the infant through ruptured amniotic membranes or during childbirth. Failure of protective maternal IgM antibodies to cross the placenta and the special susceptibility of newborns play a role. Neonatal meningitis cases are 75% of the time

caused by strains possessing the K1 capsular polysaccharide that contains sialic acid and is structurally identical to the group B polysaccharide of *Neisseria meningitidis*, another cause of meningitis (Ahmad *et al.*, 2010).

2.3.4 *E coli*-Associated Diarrhoeal Diseases

Five classes of diarrhoeal causing *E. coli* are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEC). Each class belongs to a serological subgroup and shows distinct features in pathogenesis. ETEC possesses fimbrial adhesins like CFA I, CFAII, K88 and K99. They are noninvasive and produce LT and or ST toxin. They cause watery diarrhoea in infants and travelers with no inflammation or fever. EIEC has non-fimbrial adhesins and is possibly outer membrane protein invasive. They do not produce shiga toxin but cause dysentery-like diarrhoea, severe inflammation and fever. EPEC is characterized by non fimbrial adhesins (intimin), moderate invasiveness, non-production of LT or ST and usually causes infantile diarrhoea similar to ETEC, some inflammation but no fever. EAggEC is noninvasive and produce ST-like toxin (EAST) and a hemolysin. They cause persistent diarrhoea in young children without inflammation or fever. EHEC is moderately invasive, does not produce LT or ST but does produce shiga toxin and causes pediatric diarrhoea, copious bloody discharge, intense inflammatory response and may be complicated by hemolytic uremia (NIH, 2011).

2.4 DIAGNOSIS OF *E. coli* INFECTIONS

Specimens for diagnosis include urine, blood, pus, spinal fluid, sputum, or other material, as indicated by the localization of the disease process. *E. coli* usually has the highest

occurring rate in urine and the lowest in blood with other body sites having intermediate rates (Shah *et al.*, 2002; Jawetz *et al.*, 2010; Motayo *et al.*, 2012). *E. coli* has also been found to have a higher occurrence in females than in males (Motayo *et al.*, 2012) due to a variety of factors such as differences in the anatomy of male and female genitalia (Puri and Malhotra, 2009). *E. coli* are readily isolated in culture and grow on both blood agar and differential media. With differential media, rapid preliminary identification of gram-negative enteric bacteria is often possible (Cheesbrough, 2006; Jawetz *et al.*, 2010). In UTIs, the bacteria can reach numbers higher than 10^5/mL, which makes them readily detectable by Gram stain even in a urine specimen that has not been centrifuged. For the diagnosis of intestinal disease, it is important to distinguish the virulent types from the numerous other *E coli* strains normally found in stool. Several immunoassay and nucleic acid amplification methods have been described that are able to detect the toxins LT, ST and Stx or genes associated with virulence. These methods are effective but are very expensive. A screening test for EHEC makes use of the fact that the O157:H7 serotype does not use sorbitol, unlike most other *E coli*, and is negative on sorbitol MacConkey agar which contains sorbitol instead of lactose. This provides an indicator medium from which colorless colonies can be selected and then confirmed with O157 antisera (Ahmad *et al.*, 2010).

2.5 ANTIBIOTIC RESISTANCE IN *E. coli*

2.5.1 Background to Antibiotic Resistance

The continuing incidence of pathogenic microorganisms that exhibit resistance to first-line antimicrobials is progressively becoming a concern in health delivery systems worldwide. This emergence is accompanied by higher levels of mortality and morbidity

with its attendant problems for both patients and health care services. This is largely due to increased healthcare costs which comes about as a result of additional diagnostic testing, prolonged stay in health care facilities, prolonged convalescence associated with antibiotic treatment failures leading to increased intensity and duration of treatment, the need to develop new antimicrobial agents, and the implementation of broader infection control and public health interventions aimed at getting the spread of antibiotic-resistant pathogens under control. (Kollef and Fraser, 2001; WHO, 2002). Emerging antibiotic resistance in members of the family enterobacteriaceae is of great significance and requires immediate attention. Resistance related to production of extended-spectrum β-lactamases (ESBLs) is a particular problem in the treatment of infections due to enterobacteriaceae. However, other mechanisms of resistance are also coming up and the combined effect of these is leading to multidrug resistance and the looming threat of pan resistant species development (Paterson, 2006). Subpopulations of major bacterial species may exist among populations of resistant isolates according to their level of susceptibility (ONERBA, 2008).

2.5.2 Causes of Antibiotic Resistance

Reasons for increased antibiotic resistance include suboptimal use of antimicrobials for prophylaxis and treatment of infection, noncompliance with infection-control practices, prolonged hospitalization, increased number and duration of intensive-care-unit stays, multiple comorbidities in hospitalized patients, increased use of invasive devices and catheters, ineffective infection-control practices, transfer of colonized patients from hospital to hospital, grouping of colonized patients in long-term-care facilities, antibiotic use in agriculture particularly for growth enhancements, use of disinfectants in farm and

household chores, and increasing national and international travel. The level of resistance depends on the population of organisms that spontaneously acquire resistance mechanisms as a result of selective pressure either from antibiotic use or otherwise, the rate of introduction from the community of those resistant organisms into health care settings, and the proportion that is spread from person to person (Walsh, 2000; Mathew *et al.*, 2007; Byarugaba, 2010).

2.5.3 Mechanisms of Antibiotic Resistance

Antibiotic resistance can be characterized either as intrinsic or acquired. In intrinsic resistance, microorganisms naturally do not have target sites for the drugs and are therefore unaffected by them or they naturally have low permeability to those agents due to the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to deliver their action. In acquired resistance, a naturally susceptible microorganism attains ways of being unaffected by the drug (Byarugaba, 2010).

2.5.3.1 Resistance To β-Lactam Antibiotics

All β-lactam antimicrobial agents have central, four-member β-lactam ring in common and the principal mode of action of inhibiting the synthesis of the bacterial cell wall. Additional ring structures or substituent groups added to the β-lactam ring serve to further group the agents into penicillins, cephems, carbapenems, or monobactams (CLSI, 2012). The β-lactam ring is important in the inactivation of a set of transpeptidases that catalyze the final cross-linking reactions during the synthesis of peptidoglycan in bacteria. The effectiveness of these antibiotics depends on their ability to reach the penicillin-binding protein (PBP) unaltered and their ability to bind to the PBPs as well.

Resistance to β-lactams in many bacteria is usually due to the hydrolysis of the antibiotic by a β-lactamase or the modification of PBPs or cellular permeability (Byarugaba, 2010).

2.5.3.2 Resistance to Tetracyclines

The tetracyclines are a group of drugs that differ in physical and pharmacologic characteristics but have virtually identical antimicrobial properties and give complete cross-resistance (Jawetz *et al.*, 2010). They inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. The tetracyclines are broad-spectrum antibiotics that exhibit activity against a wide range of Gram-positive bacteria, Gram-negative bacteria and atypical organisms like chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites (Byarugaba, 2010). Tetracycline resistance is normally due to the acquisition of new genes by means of either a mobile plasmid or a transposon. The development of resistance is primarily due to either energy-dependent efflux of tetracycline or protection of the ribosomes from the action of tetracycline (Roberts, 2006). A third mechanism of resistance, tetracycline modification, has been identified, but its clinical relevance is still unclear (Speer *et al.*, 1992). The Efflux mechanism occurs by means of an export protein from the major facilitator superfamily (MFS). These export proteins are membrane-associated proteins which are coded for by *tet* efflux genes and export tetracycline from the cell. By exporting the drug out of the cell, the intracellular drug concentration reduces and thus the ribosomes within the cell are protected (Byarugaba, 2010). Ribosome protection occurs through the action of ribosome protection proteins that guard the ribosomes from the action of tetracyclines (Taylor and Chau, 1996). Ribosome protection proteins are proteins found in the cytoplasm that bind to the ribosome and causes a change in the conformation of the

ribosome. This change prevents tetracycline from binding to the ribosome but does not affect protein synthesis. They confer resistance mainly to doxycycline and minocycline (Byarugaba, 2010).

2.5.3.3 Resistance to chloramphenicol

Chloramphenicol binds to the 50S subunit of the ribosome. It interferes with the binding of new amino acids to the nascent peptide chain, largely because chloramphenicol inhibits peptidyl transferase. Chloramphenicol is mainly bacteriostatic, and growth of microorganisms resumes when the drug is withdrawn. Microorganisms resistant to chloramphenicol produce the enzyme chloramphenicol acetyltransferase which successively converts the drug to 3-acetyl and 1,3-diacetyl derivatives and destroys drug activity. The production of this enzyme is usually under control of a plasmid (Shaw, 1983; Jawetz *et al.*, 2010). Resistance to chloramphenicol may sometimes be due to decreased outer membrane permeability or active efflux activity in the gram-negative bacteria (Butaye *et al.*, 2003).

2.5.3.4 Resistance to Aminoglycosides

Aminoglycosides include a group of drugs which are characterized by the presence of an aminocyclitol ring linked to amino sugars in their structure and have a broad spectrum of activity against bacteria. Examples of these drugs include streptomycin, kanamycin, gentamycin, tobramycin, and amikacin, which are broad spectrum in action (Byarugaba, 2010). All aminoglycosides have free amino and hydroxyl groups that are essential when binding to ribosomal proteins. There are a number of enzymes that have the ability to acetylate the amino groups and phosphorylate or adenylate the hydroxyl groups.

Aminoglycosides that are modified in this manner are no longer able to bind to ribosomes and can no longer inhibit protein synthesis. In the most important form of aminoglycoside resistance, the compound is modified and resistance is due partly to the poor uptake of the altered compound (Schmitz *et al.*, 1999).

2.5.3.5 Resistance to Sulfonamides and Trimethoprim

The sulfonamides are a group of compounds which have the basic mechanism of action of competitive inhibition of para-aminobenzoic acid (PABA) utilization. Using the sulfonamides in conjunction with trimethoprim results in the inhibition of sequential metabolic steps and possible antibacterial synergism (Jawetz *et al.*, 2010). Resistance in sulfonamides usually comes about by the development of alternative, drug-resistant forms of dihydropteroate synthase (DHPS). Sulfonamide resistance in gram-negative bacilli is mostly due to the acquisition of either of the two genes *sulI* and *sulII* which encode sulfonamide resistant forms of dihydropteroate synthase (Enne *et al.*, 2001). The *sulI* gene is normally found linked to other resistance genes in class 1 integrons, while *sulII* is usually located on small nonconjugative plasmids or large transmissible multi-resistance plasmids (Byarugaba, 2010). Trimethoprim is an analog of dihydrofolic acid which is an essential component in the synthesis of amino acid and nucleotides that competitively inhibits the enzyme dihydrofolate reductase (DHFR). There are a number of mechanisms by which bacteria can develop resistance to these agents which include thymineless mutation, impermeability, alteration in chromosomal dihydrofolate reductase and the plasmid-encoded production of an additional dihydrofolate reductase which is insensitive to inhibition by antifolate agents (Thomson, 1993; Byarugaba, 2010).

2.5.3.6 Resistance to Quinolones

Quinolones are synthetic analogs of nalidixic acid. The earlier quinolones did not achieve systemic antibacterial levels when taken orally and thus were only effective as urinary antiseptics (Jawetz *et al.*, 2010). The fluorinated derivatives called fluoroquinolones contain a substitution of a fluorine atom at position 6 of the quinolone molecule which greatly improved their activity against gram-positive and gram-negative bacteria as well as anaerobes (Byarugaba, 2010). Fluoroquinolones are broad-spectrum antimicrobials which work by inhibiting bacterial DNA replication. Resistance usually comes about spontaneously due to point mutations that result in amino acid substitutions within the topoisomerase subunits GyrA, GyrB, ParC or ParE, decreased expression of outer membrane porins, overexpression of multidrug efflux pumps and possession of plasmids that protect cells from the lethal effects of quinolones (Hopkins *et al.*, 2005; Jacoby, 2005). Gyrase is more susceptible to inhibition by quinolones than topoisomerase IV in gram-negative bacteria, whereas, in gram-positive bacteria, topoisomerase IV is usually the main target. Consequently, resistance mutations occur first in gyrA in gram-negative bacteria, but they occur first in parC in gram-positive bacteria (Jacoby, 2005). Different levels of resistance have been reported for ciprofloxacin from different parts of the world. The differences in level of resistance can be attributed to several factors such as the rate of consumption of the drug (Al Johani *et al.*, 2010; Al-Agamy *et al.*, 2012). Resistance to the quinolones has also been found to always occur in nalidixic acid first before the fluoroquinolones (Namboodiri *et al.*, 2011)

2.5.3.6.1 Mechanisms of Resistance in Target Enzymes

Resistance involves amino acid substitutions in a region of the GyrA or ParC subunit of the gyrase or topoisomerase IV enzymes respectively termed the "quinolone-resistance determining region" (QRDR). This region occurs on the DNA-binding surface of the enzyme (Cabral *et al.*, 1997), and, for *E. coli*, DNA gyrase includes amino acids between positions 51 and 106 (Friedman *et al.*, 2001) , with alteration at positions 83 and 87 often associated with clinical resistance. The QRDR in DNA gyrase is near tyrosine 122, which is covalently bound to phosphate groups on DNA in the initial strand-breaking reaction. After a first level mutation has reduced the susceptibility of DNA gyrase in a gram-negative organism, an additional mutation in gyrA or mutations in gyrB or parC can further increase the level of resistance. Mutations in gyrB or parC alone would be ineffective in causing resistance in a bacterial cell with wild-type GyrA due to the fact that the most-susceptible target sets the level of susceptibility (Jacoby, 2005).

2.5.3.6.2 Efflux Mechanism of Resistance

In Gram-negative and gram-positive bacteria, nonspecific, energy-dependent efflux systems, some of which are expressed constitutively and others of which are controlled by global regulatory systems or are inducible by mutation are present (Jacoby, 2005). Up regulation of efflux pumps, which export quinolones and other antimicrobials out of the bacterial cell also results in development of resistance. A typical illustration involves association of mutations in the gene encoding a repressor of the *acr*AB pump genes, *acr*R, with quinolone resistance (Wang *et al.*, 2001). On the other hand, mutations that inactivate marR, a repressor of *mar*A allow MarA to activate *acrA*B and *tol*C which is postulated to act as an outer membrane efflux channel, and a gene that reduces translation

of the porin ompF, an intrinsic efflux system, thus collectively decreasing influx and increasing efflux of quinolones (Alekshun and Levy, 1997). Target alterations and efflux activation are often found together in resistant clinical isolates. In *E. coli*, when the AcrAB efflux pump is deleted, mutations in *gyr*A rarely increase the MICs of quinolones at all (Oethinger *et al.*, 2000). When the efflux system is fully functional, single mutations in *gyr*A are still unable to produce a significant increase in resistance and are therefore clinically classified as susceptible or exhibit a ciprofloxacin MIC of ≤1 μg/mL. When a second mutation in *gyr*A or a mutation in *par*C occurs however, a clinical level of resistance or MIC of ≥4 μg/mL is attained (Heisig and Tschorny, 1994; Deguchi *et al.*, 1997; Jacoby, 2005). In general, the more resistant a clinical isolate, the more quinolone resistance–associated mutations it contains (Lindgren *et al.*, 2003).

2.5.3.6.3 Mechanism of Resistance Due to Plasmids

Resistance to quinolones can also be acquired horizontally through transferable quinolone resistance genes or other DNA (Namboodiri *et al.*, 2011). The plasmid-mediated quinolone resistance gene is known as *qnr*. The gene product Qnr was found to be a 218-aa protein belonging to the pentapeptide repeat family and shared sequence homology with the immunity protein McbG, which is thought to be involved in the protection of DNA gyrase from the action of microcin B17, a bacterial toxin (Tran and Jacoby, 2002). Purified Qnr-His6 has been shown to protect *Escherichia coli* DNA gyrase directly from inhibition by ciprofloxacin (Tran *et al.*, 2005). Two additional plasmid mediated quinolone resistance mechanisms have been described and include aac(6′)-Ib-cr which encodes a variant aminoglycoside acetyltransferase with two amino acid alterations which permits it to inactivate ciprofloxacin through the acetylation of its piperazinyl

substituent. Two genes, oqxAB and qepA also encode efflux pumps that pump out quinolones. All of these genes determine relatively small increases in the MICs of quinolones, but these changes are sufficient to cause the selection of mutants with higher levels of resistance (Strahilevitz *et al.*, 2009).

2.5.3.7 Multidrug Resistance

Multidrug resistance among many organisms has become a big problem in the management of infectious diseases. It is increasingly being reported in bacteria and is often mediated by transferable genetic elements such as plasmids, transposons, and integrons (Dessen *et al.*, 2001; Byarugaba, 2010). In a multicentre study to determine the prevalence of antibiotic-resistant faecal *E. coli* from adult volunteers from urban areas in Kenya, Mexico, Peru and the Philippines, and non-urban locations in Curacao, Mexico, Venezuela, Ghana, Zimbabwe and the Philippines, resistance to ciprofloxacin ranged from 1–63% with the highest percentage being associated with urban populations of Asia and South America. In Peru and the Philippines the prevalence of gentamicin resistance was more than 20%. Higher prevalence of resistance to ampicillin, oxytetracycline and trimethoprim was found in urban areas compared with non-urban areas of Asia, Africa and South America, respectively. Antibiotic resistance in faecal *E. coli* from the adult volunteers was emerging for cefazolin, gentamicin and ciprofloxacin and was high for the older drugs ampicillin, oxytetracycline, trimethoprim and chloramphenicol (Nys *et al.*, 2004). In a study that characterized antibiotic resistance among diarrhoeagenic *E. coli* from children less than 5 years of age from Kenya, isolates exhibited high-level multidrug resistance to WHO recommended antibiotics. Resistance rates to tetracycline, ampicillin and co-trimoxazole were 70.7, 65.9 and 68.3%, respectively. These figures

were very similar to resistance prevalence among *E. coli* from healthy children (Bii *et al.*, 2005). The presence of multiple virulence genes was associated with multidrug resistance. Conjugation experiments have been used to show that resistance towards ampicillin, tetracycline, trimethoprim, sulphamethoxazole and chloramphenicol could be transferred en bloc among commensal microflora suggesting that mobile genetic elements may be involved in the dissemination of MDR phenotypes (Bartoloni *et al.*, 2006). In this respect, it is possible that *E. coli* is capable of surviving in extra-intestinal environments and may acquire other MDR traits from soil and water bacteria (Kariuki, 2010)..

2.6 MEASUREMENT OF ANTIMICROBIAL ACTIVITY

Determination of the susceptibility of pathogenic bacteria to antimicrobial drugs can be performed by one of two main methods namely dilution or diffusion. It is important that the methods used be standardized such that all the factors that affect antimicrobial activity are controlled; in the United States, the tests are performed according to the methods of the Clinical and Laboratory Standards Institute (CLSI) Using an appropriate standard test organism and a known sample of drug for comparison, these methods can be employed to estimate either the potency of antibiotic in the sample or the susceptibility of the microorganism (Jawetz *et al.*, 2010). Other standards institutes are the British Society of Antimicrobial Chemotherapy (BSAC) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

2.6.1 Diffusion Method

Paper disks, impregnated with a specified amount of an antimicrobial, are placed on agar medium which has been uniformly seeded with the organism being tested. A

concentration gradient of the antimicrobial forms by diffusion from the disk and the growth of the test organism is inhibited at a distance from the disc that is related, among other factors, to the susceptibility of the organism (Vandepitte *et al.*, 2003). Disk diffusion tests based solely on the presence or absence of a zone of inhibition without consideration of the size of the zone are not acceptable for determination of susceptibility to antimicrobials. Reliable results can only be obtained with disk diffusion tests that use the principle of standardized methodology and zone diameter measurements correlated with minimal inhibitory concentrations (MICs) with strains known to be susceptible or resistant to various antimicrobial agents (CLSI, 2012). Use of a single disk for each antibiotic with careful standardization of the test conditions permits the report of susceptible or resistant for a microorganism by comparing the size of the inhibition zone against a standard of the same drug. Inhibition around a disk containing a certain amount of antimicrobial drug does not imply susceptibility to that same concentration of drug per milliliter of medium, blood, or urine (Jawetz *et al.*, 2010). Drs. Bauer, Kirby, Sherris, and Turck are the ones who established the test currently in use by painstakingly testing all of the variables used in the procedure, such as the media, temperature, and depth of agar and published their paper describing the procedure (Cavalieri *et al.*, 2005).

2.6.2 Dilution Method

The minimal inhibitory concentration (MIC) of an antimicrobial agent is described as the lowest concentration of the antimicrobial agent that prevents a given bacterial isolate from multiplying and producing visible growth in the test system. The concentration is determined in the laboratory by incubating a known quantity of bacteria with specified dilutions of the antimicrobial agent. Using CLSI interpretive criteria, the results are

interpreted as susceptible, intermediate, or resistant. MIC tests can be performed using broth or agar media (Cavalieri *et al.*, 2005). The test is a quantitative method that estimates antimicrobial activity. The MIC value obtained in the test is compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response (Vandepitte *et al.*, 2003). The basics of the broth and agar dilution methods used by the CLSI are derived mostly from information generated by International Collaborative Study and the results obtained using these tests are greatly influenced by methodology, which must be carefully controlled in order to obtain reproducible results (CLSI, 2006). MIC50 is the lowest concentration of the antibiotic at which 50% of the isolates being tested are inhibited (Bayram *et al.*, 2011).

2.7 MOLECULAR METHODS OF DETERMING RESISTANCE

Recent advances in molecular biology have led to the development of tests for antibiotic susceptibility that employ genotypic assays (Rolain *et al.*, 2004). Nucleic acid-based assays for the detection of resistance may have some advantages over phenotypic assays such as knowledge of the spread of resistance determinants across the globe. However, they do also have a number of limitations. New mechanisms of resistance may be missed and the number of different genes involved makes it more costly to generate an assay. Again, it is difficult to implement proper quality control measures for molecular assays and this sometimes leads to attainment of questionable results. Phenotypic assays are still the method of choice for most resistance determinations. In some cases the presence of a resistance gene is highly predictive for clinical outcome of antimicrobial therapy such as the presence of a β-lactamase gene in *Neisseria gonorrhoeae* which correlates well with the outcome of penicillin treatment (Fluit *et al.*, 2001). However, the presence of a

resistance gene does not necessarily lead to treatment failure (Milatovic and Braveny, 1987).

2.7.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is one of the most commonly used molecular techniques for detecting certain DNA sequences of interest. It depends on the fact that DNA synthesis requires primers base-paired to the template DNA. Using synthetic oligonucleotides that are complementary to the DNA on either side of the gene being studied, specific synthesis of the target gene can be obtained. Samples are subjected to repeated cycles of denaturation, annealing of primers and extension by a thermostable DNA polymerase leading to replication of a duplicate DNA sequence in an exponential manner, to a point which will be visibly detectable by gel electrophoresis with the aid of a DNA-intercalating chemical which fluoresces under UV light (Hogg, 2005; Dale and Park, 2010).

2.7.2 DNA Hybridization

This is based on the fact that the DNA pyrimidines specifically pair up with purines. Therefore, a labeled probe with a known specific sequence can pair up with opened or denatured DNA from the test sample. When a mixture of single-stranded DNA formed by heating double-stranded DNA is cooled and held at a temperature of about 25°C below the melting temperature, strands with complementary base sequences will reassociate to form stable dsDNA, whereas non-complementary strands will remain single. If this hybridization occurs, the probe labels this with a detectable radioactive isotope, antigenic substrate, enzyme or chemiluminescent compound. On the other hand if no target sequence is present or the isolate does not have the specific gene of interest, no

attachment of probes will occur, and therefore no signals will be detected (Prescott, 2002).

2.7.3 Modifications of PCR and DNA Hybridization

Several modifications have been introduced which improves the sensitivity and specificity of these standard procedures (Parsons and Heflich, 1997).

2.7.3.1 Allele Selection for the Detection of Point Mutations

Allele-selection techniques can be classified into three categories which comprise those that preferentially destroy the wild-type allele, those that preferentially amplify the mutant or rare allele, and those that spatially separate the mutant from the wild-type allele (Parsons and Heflich, 1997).

2.7.3.1.0 Selective Amplification of Rare or Mutant Allele

The various techniques employ a PCR primer that has fewer mismatches when hybridized to the rare allele than to the abundant allele as the basis for the genotypic selection and the oligonucleotide primer that forms a 3′ mismatch with the DNA template resists primer extension by *Thermus aquaticus* DNA polymerase (Ugozzoli and Wallace, 1991; Parsons and Heflich, 1997). Allele specific amplification requires the use of a polymerase that lacks a 3'-5' exonuclease activity generally because that property would remove the 3'-terminal mismatch. In this regard, *Taq* polymerase is mostly used (Parsons and Heflich, 1997). Several allele specific amplification methods have been applied to the detection of relatively large mutant fractions. These methods include using the tetra-priming approach (Ye *et al.*, 1992), Polymerase Amplification of Specific

Alleles (PASA) (Bottemaa and Sommer, 1993), Amplification Refractory Mutation System (ARMS) (Little, 2001) and the mismatched polymerase chain reaction (Qiang *et al.*, 2002). Although these techniques belong to relatively different groups of methods, they do have certain advantages in common for genotypic selection. They are relatively simple to perform, rapid, cost-effective, and do not require a restriction endonuclease cleavage site in the mutational target. One disadvantage is the small target size. Again, some of the methods can only detect a single base substitution, while others are able to detect two different nucleotides at the same position. Some modifications of allele specific amplification effectively increase the mutational target size (Parsons and Heflich, 1997).

2.6.3.1.1 Mismatch Amplification Mutation Assay (MAMA) PCR

Among the allele-specific selection methods, the Mismatch Amplification Mutation Assay (MAMA) is the one with the highest reported sensitivity (Cha *et al.*, 1992). MAMA is based on the use of a mutant-specific primer that contains one deliberate mismatch to the mutant allele but two mismatches to the wild-type allele. The rationale behind MAMA PCR is that the single nucleotide mismatch at the 3' extremity of the annealed reverse primer prevents *Taq* polymerase from extending the primer. The absence of the specific PCR product in conjunction with a positive internal PCR control shows a deviation from the wild-type DNA sequence (Cha *et al.*, 1992; Qiang *et al.*, 2002; Karami *et al.*, 2008). MAMA PCR provides a means for routine detection of mutations such as those in *gyr*A which is associated with resistance to ciprofloxacin without the need for sequencing the *gyr*A gene (Zirnstein *et al.*, 1999). Although methods such as restriction fragment length polymorphism (RFLP), single-strand

conformational polymorphism (SSCP) analysis and sequencing of the relevant gene regions have been used to detect such mutations, the procedures are labour intensive and time consuming (Qiang *et al.*, 2002; Karami *et al.*, 2008). Sometimes, a single-base mismatch at the 3' terminus is not enough to obtain the desired level of discrimination, especially when the ratio of mutant to wild-type sequence is low. A 3'-terminal mismatch together with an additional mismatch 1, 2, or 3 bases from the 3' terminus can increase the level of discrimination. The intentional introduction of a second mutation 1-3 bases from the 3' terminus will sometimes be necessary to destabilize the 3' end and provide even greater differentiation (Kwok *et al.*, 1994).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

Samples for the study were obtained from the Microbiology Laboratory of the Komfo Anokye Teaching Hospital, Kumasi. Antimicrobial susceptibility testing was performed at the diagnostic laboratory of the Department of Clinical Microbiology at the School of Medical Sciences, KNUST and Molecular testing was done at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR).

3.2 Ethical Clearance

Ethical approval for the study was obtained from the Committee on Human Research, Publications and Ethics, School of Medical Sciences, KNUST and Komfo Anokye Teaching Hospital, Kumasi.

3.3 Sample size

The appropriate sample size for the study was estimated at 199 by statistical power analysis with GPower version 3.1 using an effect size of 23% (0.23), the lowest effect size deemed to be clinically significant as obtained from a review of literature and the additional variables shown in figure 1. This number was approximated to 200 in this study.

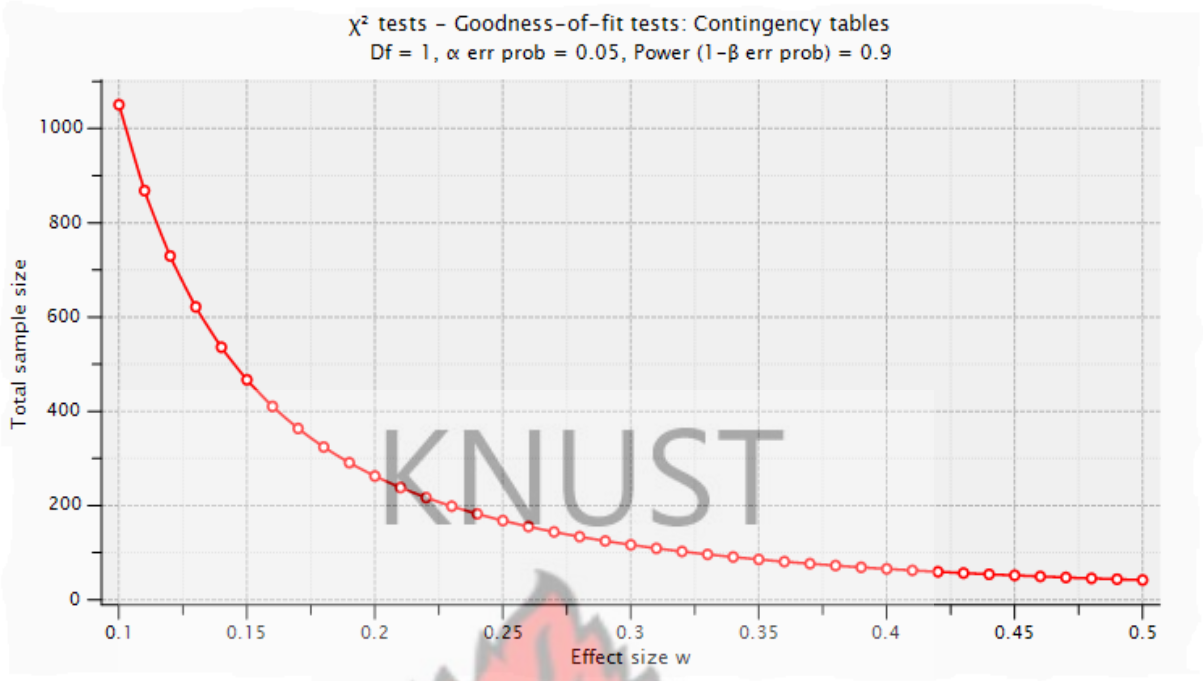


Figure 1: Appropriate number of samples for the study

3.4 Isolates

All *E. coli* that were isolated by the Microbiology Laboratory of the Komfo Anokye Teaching Hospital as part of their routine diagnostics were included in the study until the desired sample size was obtained. A total of 200 non-duplicate isolates of *E. coli* were obtained within a period of six months. The isolates were obtained from urine, blood and other sources such as wounds, abscesses, pus, sputum, ear and all sorts of aspirates from various sites.

3.5 Identification of *E. coli*

3.5.1 Colonial Morphology

Isolates that appeared as pink or yellow colonies on MacConkey or CLED agar respectively were considered to be potential *E. coli* (Cheesbrough, 2006).

Plate 1: Lactose fermenter on McConkey **Plate 2: Lactose fermenter on CLED**

3.5.2 Biochemical Tests

3.5.2.1 Indole test

The indole test was performed by inoculating the test organism into 5 ml of sterile peptone water in a bijou bottle. After overnight incubation at 37°C, a few drops of Kovac's reagent were added. The appearance of a red surface layer confirmed the presence of *E. coli*. *E. coli* ATCC 25922 was used as the positive control organism.

Plate 3: Positive and negative indole tests

3.5.2.2 Citrate utilization test

Slopes of Simmon's citrate agar in test tubes prepared as recommended by the manufacturer were first streaked with the test organism and then the butt stabbed. The tubes were incubated at 37°C for 48 hours. A bright blue colour in the medium indicates a positive test and no change in colour indicates the presence of *E. coli*.

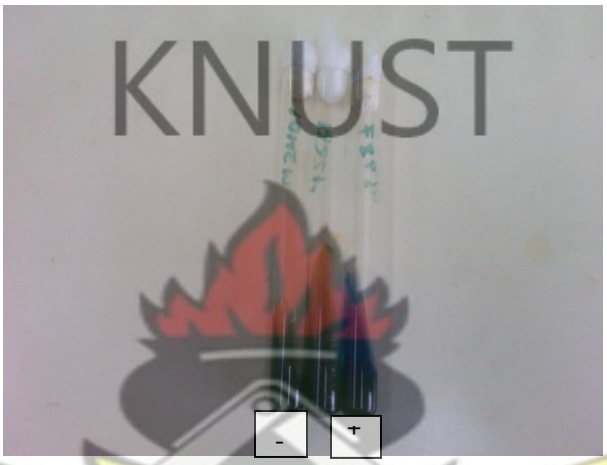


Plate 4: Positive and negative citrate utilization test

3.6 Storage of Isolates

Confirmed *E. coli* isolates were sub-cultured from the MacConkey and CLED plates onto nutrient agar and then inoculated into 20% v/v glycerol broth consisting of Brain-Heart infusion broth and glycerol then stored at -20°C.

3.7 Sub-culturing

Stored frozen isolates were thawed at room temperature and sub-cultured on nutrient agar to obtain pure growth.

3.8 Antimicrobial Susceptibility Testing (AST)

AST of each isolate was performed by the disk diffusion and minimum inhibitory concentration methods, using the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2006; CLSI, 2012).

3.8.1 Disk Diffusion Test

3.8.1.0 Preparation of Mueller-Hinton Agar

Mueller-Hinton agar (Oxoid, UK) was prepared according to the manufacturer's instructions. Immediately after autoclaving, the agar was allowed to cool in a 50 °C water bath. The medium was poured into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of about 4 mm. The agar plates were allowed to cool further to room temperature. The plates that were not used the same day were stored in a refrigerator at 4 °C.

3.8.1.1 Turbidity Standard Preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard was used. This was prepared by adding a 0.5-mL aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl2 \cdot 2H2O$) to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The density of the turbidity standard was verified by measuring absorbance using a nephelometer (Becton Dickinson). The BaSO4 suspension was transferred into Bijou bottles in 4 mL aliquots and stored in the dark until use.

3.8.1.2 Inoculum Preparation

Inoculum preparation was done by the direct colony suspension method. This was done by picking at least four morphologically similar colonies of *E. coli* from an 18 to 24 hour nutrient agar plate and emulsifying them in autoclaved distilled water. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard.

3.8.1.3 Inoculation of Test Plates

A sterile cotton swab was dipped into the adjusted suspension within 15 minutes after adjusting the turbidity. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess fluid from the swab. The dried surface of a Mueller Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. The streaking was repeated two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid of the petri dish was left ajar for three minutes to allow for any excess surface moisture to be absorbed.

3.8.1.4 Application of Disks to Inoculated Agar Plates

Disks containing ampicillin (10µg), nalidixic acid (30µg), gentamicin (10µg) tetracycline (30µg), ciprofloxacin (5µg), chloramphenicol (30µg) and sulfamethoxazole-trimethoprim (25µg) were obtained from Oxoid, Uk and used in this study. Each disk was placed individually with sterile forceps and pressed down to ensure complete contact with the agar surface. The disks were distributed evenly with at least 24 mm distance from center to center. The plates were inverted and placed in an incubator set to 37°C.

3.8.1.5 Reading Plates and Interpreting Results

After 18 hours of incubation, each plate was examined and the diameters of uniformly circular zones of complete inhibition on confluent lawn of growth were measured to the nearest whole millimeter including the diameter of the disk using a ruler which was held on the back of the inverted petri dish. The petri dish was held above a black, nonreflecting background and was illuminated with reflected light.

3.8.2 MIC Testing

The minimum inhibitory concentration of ciprofloxacin against *E. coli* was tested.

3.8.2.1 Weighing Antimicrobial Powders

The volume of diluent required to obtain the final concentration of stock solution of ciprofloxacin was calculated using the formula below:

$$\text{Volume} = \frac{\text{Weight (mg)} \bullet \text{Potency (µg/mg)}}{\text{Concentration (µg/ml)}}$$

Potency of the anhydrous ciprofloxacin (Fluka analytical) was calculated using the formula Potency= (Assay purity) x (1-Water Content). Assay purity and water content values were provided by the manufacturer on the certificate of analysis of the product.

3.8.2.2 Preparing Stock Solutions

Ciprofloxacin stock solutions were prepared at concentrations of 5120 µg/mL. A minimum amount of 0.1N HCL was used to solubilize the antimicrobial powder and

topped up to the desired volume with the same diluent. 10 mL volumes of the stock solution were dispensed into sterile bijou bottles and stored at -80ºC until use.

3.8.2.3 Dilution of Antimicrobial Agents

Ciprofloxacin stock solutions were diluted to the various working concentration using the scheme shown in Table 1.

Table 1: Scheme for Preparing Dilutions of Antimicrobial Agents

Antimicrobial solution					Intermediate conc. (µg/ml)	Final conc. At 1:10 dilution in Agar (µg/ml)
Step	Conc. (µg/mL)	Source	Volume (mL)	Diluent (mL)		
	5120	Stock	-	-	5120	512
1	5120	Stock	2	2	2560	256
2	5120	Stock	1	3	1280	128
3	5120	Stock	1	7	640	64
4	640	Step 3	2	2	320	32
5	640	Step 3	1	3	160	16
6	640	Step 3	1	7	80	8
7	80	Step 6	2	2	40	4
8	80	Step 6	1	3	20	2
9	80	Step 6	1	7	10	1
10	10	Step 9	2	2	5	0.5
11	10	Step 9	1	3	2.5	0.25
12	10	Step 9	1	7	1.25	0.125

3.8.2.4 Preparing Agar Dilution Plates

Appropriate dilutions of ciprofloxacin solution were added to molten test agars that had been allowed to equilibrate in a water bath to 45ºC. The agar and antimicrobial solution were mixed thoroughly by palming and poured quickly into sterile petri dishes on a level surface to give an agar depth of 3 to 4 mm. The agar was allowed to solidify at room

temperature and used immediately. Plates that could not be used immediately were stored at 4ºC and plates stored at 4ºC were allowed to equilibrate to room temperature before use.

3.8.2.5 Agar Dilution Scheme

A 1:10 dilution of ciprofloxacin to molten agar was used by adding 2 mL of the desired concentration of antimicrobial to 18 mL of molten agar. Plates without any antimicrobial solution incorporated were used as growth controls.

3.8.2.6 Dilution of Inoculum Suspension

Inoculum suspensions adjusted to 0.5 McFarland's standard were prepared as for the disk diffusion method and diluted 1:10 in sterile distilled water. 2 µL of the dilution was delivered per spot on the test plate. Inoculum dilutions were used within 15 minutes of preparation.

3.8.2.7 Inoculating Agar Dilution Plates

An aliquot of each inoculum was applied to the agar surface with a micropipette. A growth-control plate was inoculated first then the lowest test concentration through to the highest concentration then a second growth control plate. The inoculated plates were allowed to stand at room temperature until the spots were dry. The plates were inverted and incubated at 37ºC for 18 hours.

3.8.2.8 Determining Agar Dilution End points

The lowest concentration of ciprofloxacin that completely inhibited the growth of the test organism was recorded as the MIC.

3.8.3 Quality Control

Quality control testing was done with each batch of tests performed for both disk diffusion and MIC testing using *E. coli* ATCC 25922.

3.9 Analysis of the Quinolone-Resistance Determining Regions

3.9.1 DNA extraction

3.9.1.1 Preparation of Luria Bertani Broth (LB)

Luria Bertani broth was prepared according to the manufacturer's instructions and 1mL aliquots transferred into microcentrifuge tubes and autoclaved at 121°C for 15 minutes.

3.9.1.2 Preparation of the *E. coli* suspension

A sterile inoculating loop was used to pick a colony or section of *E. coli* without removing the agar. The loop was inserted into the LB broth and the cells were removed from the loop by stirring for a few seconds inside a lamina flow hood. The broth was capped loosely and incubated at 37° C for 20 hours with shaking.

3.9.1.3 Extraction by heat lysis

Cells from 1 mL of overnight culture were harvested by centrifugation at 13,000 rpm in an Eppendorf centrifuge for 5 min. After the supernatant was decanted, the pellet was resuspended in 500 µl of DNase-RNase-free distilled water (Thermo Scientific) by vortexing. The cells were lysed by heating to 95°C for 10 min in an Eppendorf heating block and cellular debris were removed by centrifugation for 5 min at 13,000 rpm. The supernatant was carefully transferred to a fresh microcentrifuge tube and stored at −20°C (Pitout *et al.*, 1998; Qiang *et al.*, 2002).

3.9.2 DNA amplification

Four PCR reactions were carried out on each template and 1 µL of template DNA was added to a final volume of 50 µL containing 0.35 µM forward primer, 0.25 µM MAMA primer, 0.10 µM control primer, 25µl of DreamTaq Green PCR Master Mix (2X) and nuclease-free water to 50µl. The reaction was performed on a thermal cycler (Applied Biosystems, USA) by an initial denaturation at 95°C for 3 min and 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 40 s, with a final step of 72°C for 5 min. PCR products were visualized on horizontal 1.0% agarose gels in 1X TAE buffer, loaded with 9 µL of reaction mix and stained with ethidium bromide nucleic acid stain. O'GeneRuler 100bp DNA ladder (Thermo Scientific) was used during electrophoresis to estimate the sizes of the bands obtained. DNA primers used in this study were those used by Qiang *et al* in 2002 and are shown below:

Forward *gyrA*, 5′-GACCTTGCGAGAGAAATTACAC-3′ (forward, position: 7–28)

Control *gyrA*, 5′-GATGTTGGTTGCCATACCTACG-3′ (reverse, position: 546–525)

MAMAgyrA83, 5'-T CGT GTC ATA GAC CGG GC-3'

MAMAgyrA87, 5'-G CGC CAT GCG GAC GAT CGT TTC-3'

Forward *parC*, 5′-CGGAAAACGCCTACTTAAACTA-3′ (forward, position: 41–62)

Control *parC*, 5′-GTGCCGTTAAGCAAAATGT-3′ (reverse, position: 506–488)

MAMAparC80, 5'-AT CGC TTC ATA ACA GGC TCT-3'

MAMAparC84, 5'-C CAT CAG GAC CAT CGC CTC-3'

3.10 Data Analysis

WHONET 5.6 was used to calculate the percentage of isolates that showed resistance, susceptibility and intermediate results to the antibiotics that were tested. The software was also used to:

1. Determine the proportion of isolates obtained from different specimens that were resistant, susceptible and intermediate to the various antibiotics tested.

2. Compare the results obtained for the two quinolones that were tested in the study.

3. Determine multi-resistance patterns among the isolates that were tested in the study.

4. Determine the MIC50 value for ciprofloxacin.

Microsoft Excel was used to generate graphs to illustrate some results and also to calculate the generalized kappa statistic which was used to compare the results for ciprofloxacin obtained by the disk diffusion method and agar dilution method.

CHAPTER FOUR

4.0 RESULTS

4.1 Bacterial Isolates

A total of 200 non-duplicate isolates were obtained in this study. The isolates were obtained from both males and females of varying ages and from various clinical specimens including blood, urine, wounds, abscesses, ear, pus, sputum, cyst and aspirates. The highest number of the isolates were from urine (n=138) and the lowest was from a cyst (n=1) (Table 2).

Table 2. Number and Percentage of *E. coli* isolates from different specimen types

Specimen type	Number of isolates	Percentage (%)
Abscess	3	2
Aspirate	2	1
Blood	17	9
Cyst	1	1
Ear	3	2
Pus	4	2
Sputum	3	2
Urine	138	69
Wound	29	15

A total of 139 of the 200 isolates obtained were from female patients while 55 were from male patients and 6 isolates were obtained from patients whose gender were unknown (Figure 2) as they were not indicated in the patients records obtained from the Microbiology laboratory of the Komfo Anokye Teaching Hospital.

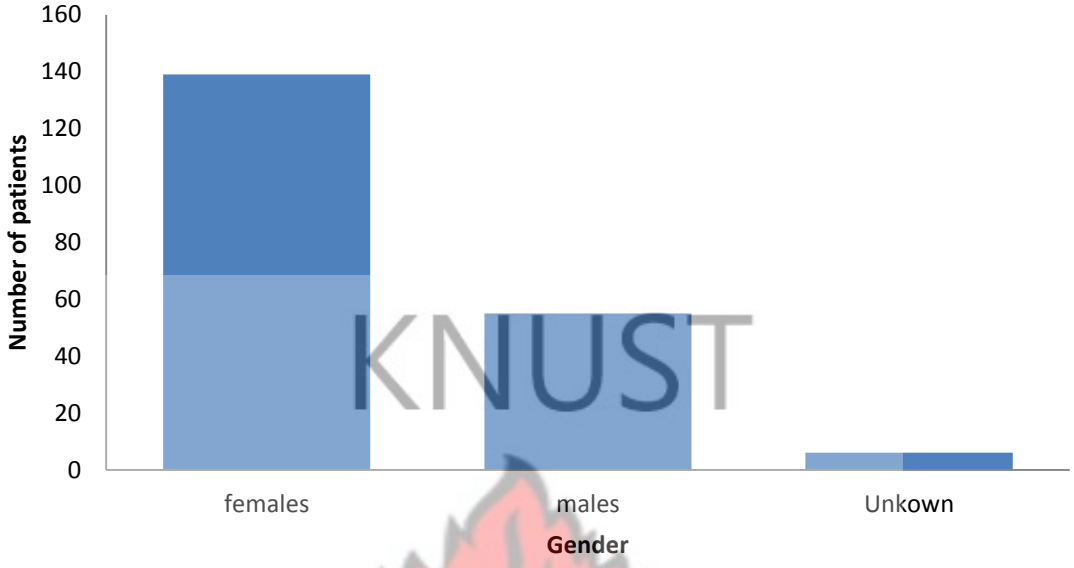


Figure 2. Gender distribution of isolates obtained in the study

The ages of patients from whom the isolates were obtained ranged from 2 days old to 88 years old. However, the ages of 13 individuals from whom isolates were obtained could not be determined from patients records (Table 3).

Table 3. Age distribution of patients from whom isolates were obtained

Age category	Number of isolates	Percentage
Adult (Above 18 years)	139	70
Newborn (Below 1 month)	2	1
Pediatric (1 month-18 years)	46	23
Unknown	13	17

4.2 Antimicrobial susceptibility testing

Out of the antibiotics tested, gentamicin showed the lowest percentage of resistance (47.5%) and ampicillin showed the highest percentage (94%) closely followed by tetracycline (90%). Chloramphenicol and ciprofloxacin showed similar percentages of resistance at 63% and 66% respectively and of the two quinolones tested, nalidixic acid showed the highest percentage (75%). Neither chloramphenicol nor tetracycline showed intermediate susceptibility against any of the isolates tested (0%) (Table 4).

Table 4. %RIS and test measurement analysis of *E. coli* isolates with various antibiotics

Antibiotic name	Code	Breakpoints (mm)	%R	%I	%S	%R 95%C.I.
Gentamicin	GEN	13 - 14	47.5	0.5	52	40.4-54.6
Nalidixic acid	NAL	14 - 18	75	3.5	21.5	68.3-80.7
Trimethoprim/ Sulfamethoxazole	SXT	11 - 15	89.5	1	9.5	84.2-93.2
Ampicillin	AMP	14 - 16	94	1.5	4.5	89.5-96.7
Chloramphenicol	CHL	13 - 17	63	0	37	55.9-69.6
Ciprofloxacin	CIP	16 - 20	66	2	32	58.9-72.4
Tetracycline	TCY	12 - 14	90	0	10	84.8-93.6

%R= Percentage resistance, %I= Percentage intermediate, %S= Percentage susceptibility, C.I= Confidence interval, mm= millimeters

Various bacterial subpopulations were observed based on the zone diameters obtained for the various antibiotics tested in the study. Two principal subpopulations were observed by the ampicillin testing. One group is the wild-type susceptible population with large

zone diameters (17, 18 and 20mm) and the second is a group with high level resistance to ampicillin (zone diameter = 6mm) (Figure 3).

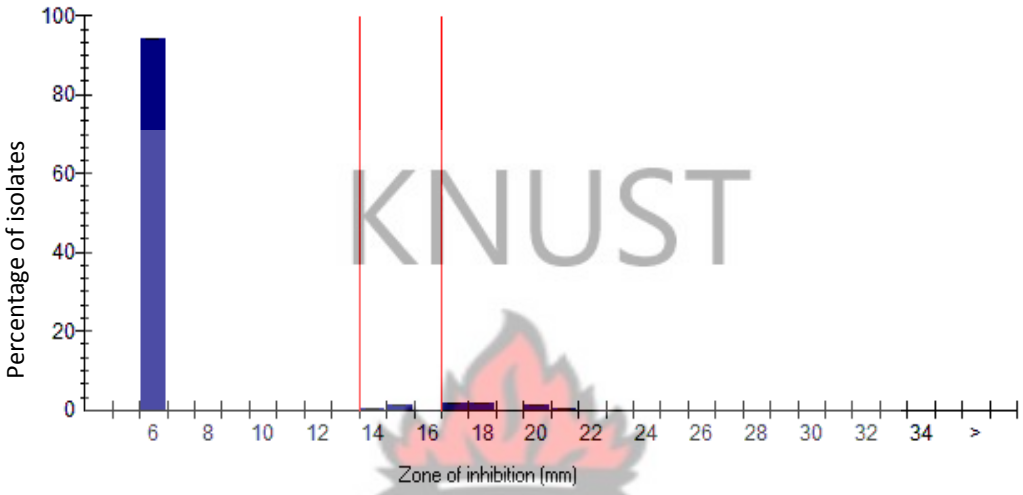


Figure 3. Zone diameters of ampicillin against *E. coli* isolates

The zone diameters for gentamicin also depict two distinct populations; the first being the wild-type susceptible with large zone diameters (17, 18 and 19mm) and the second being the resistant group with zone diameters 6, 8, 9 and 10 millimeters (Figure 4).

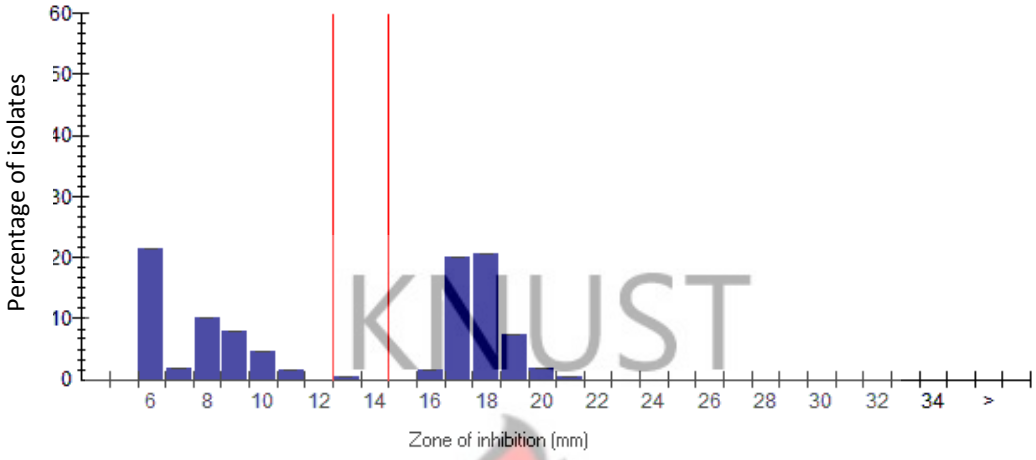


Figure 4. Zone diameters of gentamicin against *E. coli* isolates

Zone diameters of nalidixic acid illustrate two groups within the resistant category; one group with high level resistance (zone diameter=6mm) and the other with relatively low resistance (zone diameter= 13mm). One distinct population is evident in the susceptible category which is the wild-type group with large zone diameters (22, 23 and 24mm) (Figure 5).

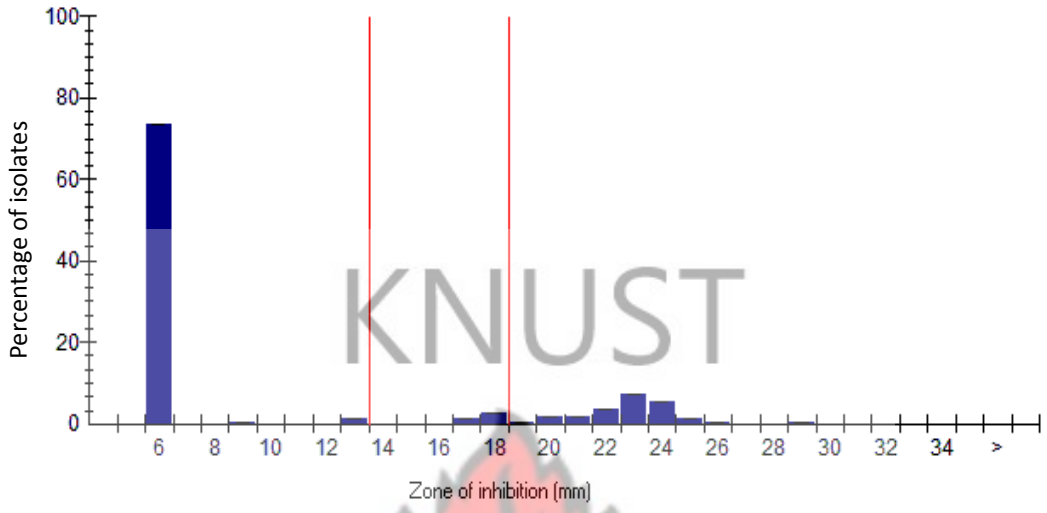


Figure 5. Zone diameters of nalidixic acid against *E. coli* isolates

With respect to zone diameters of ciprofloxacin, three groups were observed in the susceptible category which comprise a group with decreased susceptibility and possess relatively smaller zone diameters (21, 22 and 23mm), a second group with moderately good susceptibility with zone diameters ranging from 27 to 32mm and a third group which is the wild-type susceptible isolates with relatively larger zone diameters (35 and 36mm). Two groups can be seen in the resistant category which comprise one with high reistance (zone diameter=6mm) and the other is low-level resistance (zone diameters=10, 11 and 12mm) (Figure 6).

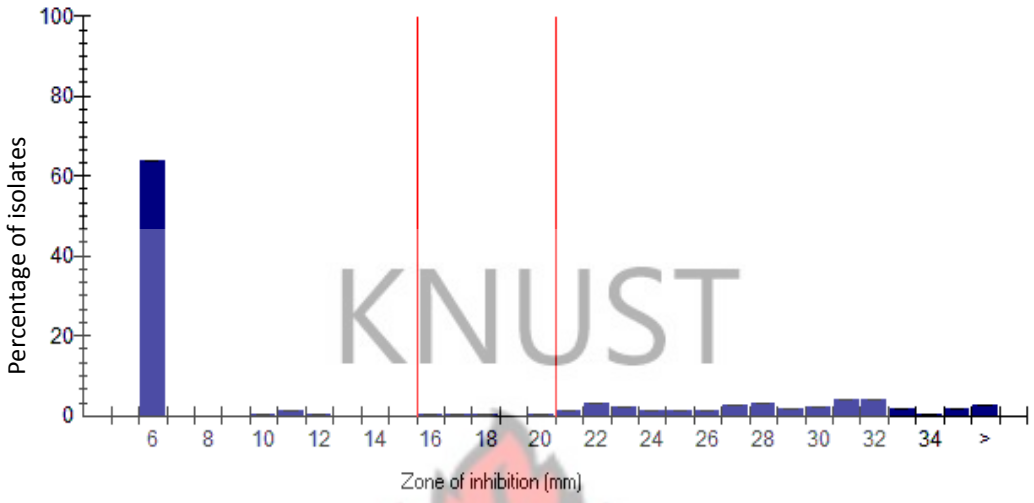


Figure 6. Zone diameters of ciprofloxacin against *E. coli* isolates

Trimethoprim/Sulfamethoxazole and tetracycline both show similar distributions of subpopulations which consist of a group with high susceptibility and zone diameters ranging from 25 to 28mm for Trimethoprim/Sulfamethoxazole and 22 and 23mm for tetracycline and a group with high level resistance and zone diameters of 6mm for Trimethoprim/Sulfamethoxazole and 6, 7 and 8mm for tetracycline (figures 7 and 8). Chloramphenicol however showed two distinct groups for the resistant category with zone diameters of 6 and 10mm and one group in the susceptible category which is the wild type isolates with large zone diameters ranging from 23 to 27mm (Figure 9).

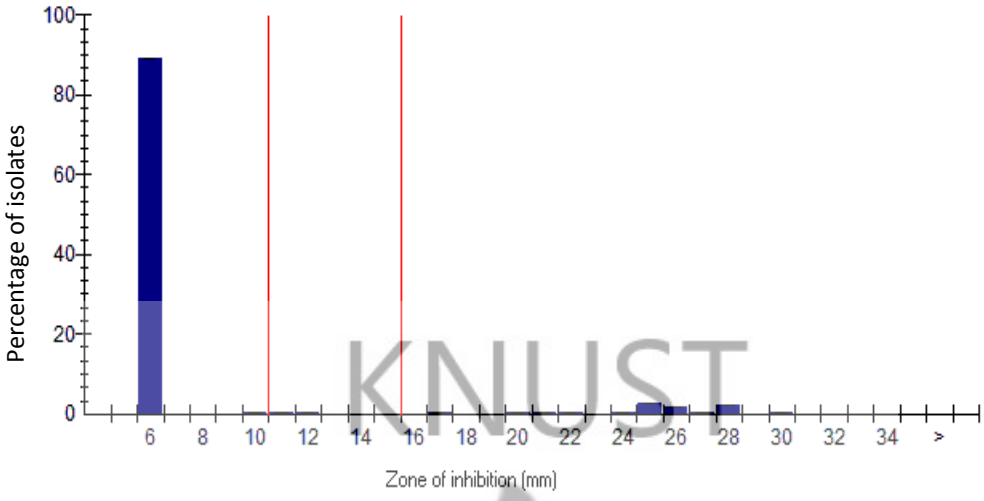


Figure 7. Zone diameters of Trimethoprim/Sulfamethoxazole against *E. coli* isolates

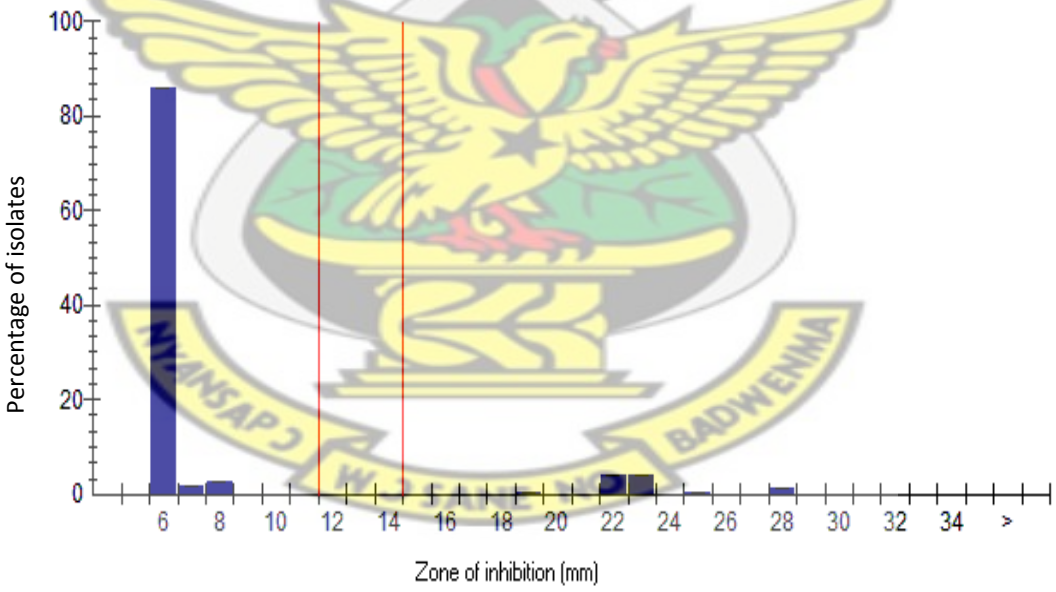


Figure 8. Zone diameters of tetracycline against *E. coli* isolates

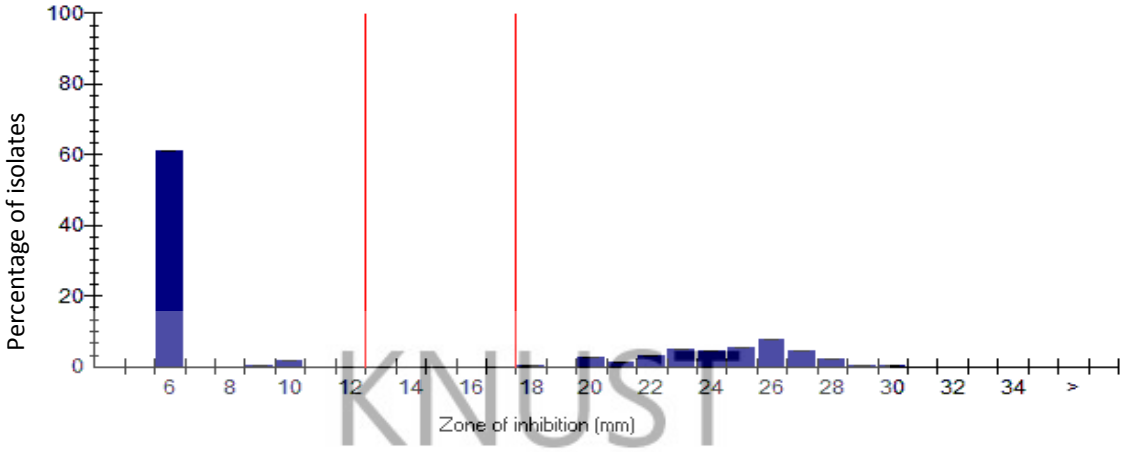


Figure 9. Zone diameters of chloramphenicol against *E. coli* isolates

4.2.1 %RIS and test measurement analysis of *E. coli* isolated from different sources

Ciprofloxacin and chloramphenicol had the same level of resistance against *E. coli* that were isolated from blood (58.8%) which was the lowest recorded. The highest level of resistance was recorded for ampicillin, trimethoprim/sulfamethoxazole and tetracycline, all of which showed a resistance level of 82.4% (Table 5).

Table 5. %RIS and test measurement analysis of *E. coli* isolated from blood

Antibiotic name	Code	Breakpoints (mm)	%R	%I	%S	%R 95%C.I.
Gentamicin	GEN	13 - 14	64.7	0	35.3	38.6-84.7
Nalidixic acid	NAL	14 - 18	76.5	0	23.5	49.8-92.2
Trimethoprim/ Sulfamethoxazole	SXT	11 - 15	82.4	5.9	11.8	55.9-95.4
Ampicillin	AMP	14 - 16	82.4	0	17.6	55.9-95.4
Chloramphenicol	CHL	13 - 17	58.8	0	41.2	33.4-80.6
Ciprofloxacin	CIP	16 - 20	58.8	0	41.2	33.4-80.6
Tetracycline	TCY	12 - 14	82.4	0	17.6	55.9-95.4

%R= Percentage resistance, %I= Percentage intermediate, %S= Percentage susceptibility, C.I.= Confidence interval, mm= millimeters

Gentamicin recorded the lowest level of resistance against isolates that were isolated from urine (45.7%). The highest level of resistance was observed against ampicillin (94.9%) and this was followed by trimethoprim/sulfamethoxazole and tetracycline both of which showed 89.1% resistance (Table 6).

Table 6. %RIS and test measurement analysis of *E. coli* isolated from urine

Antibiotic name	Code	Breakpoints (mm)	%R	%I	%S	%R 95%C.I.
Gentamicin	GEN	13 - 14	45.7	0.7	53.6	37.3-54.4
Nalidixic acid	NAL	14 - 18	76.1	4.3	19.6	68.0-82.8
Trimethoprim/ Sulfamethoxazole	SXT	11 - 15	89.1	0.7	10.1	82.4-93.6
Ampicillin	AMP	14 - 16	94.9	1.4	3.6	89.4-97.7
Chloramphenicol	CHL	13 - 17	62.3	0	37.7	53.6-70.3
Ciprofloxacin	CIP	16 - 20	67.4	2.2	30.4	58.8-75.0
Tetracycline	TCY	12 - 14	89.1	0	10.9	82.4-93.6

%R= Percentage resistance, %I= Percentage intermediate, %S= Percentage susceptibility, C.I.= Confidence interval, mm= millimeters

Gentamicin again showed the lowest level of resistance against isolates from all other sources apart from blood and urine (46.7%) with ampicillin and tetracycline also showing the highest level of resistance (95.6%) followed by trimethoprim/sulfamethoxazole (93.3%) (Table 7).

Table 7. %RIS and test measurement analysis of *E. coli* isolated from other sources apart from blood and urine

Antibiotic name	Code	Breakpoints (mm)	%R	%I	%S	%R 95%C.I.
Gentamicin	GEN	13 - 14	46.7	0	53.3	32.0-62.0
Nalidixic acid	NAL	14 - 18	71.1	2.2	26.7	55.5-83.1
Trimethoprim/ Sulfamethoxazole	SXT	11 - 15	93.3	0	6.7	80.6-98.2
Ampicillin	AMP	14 - 16	95.6	2.2	2.2	83.7-99.2
Chloramphenicol	CHL	13 - 17	66.7	0	33.3	51.0-79.6
Ciprofloxacin	CIP	16 - 20	64.4	2.2	33.3	48.7-77.7
Tetracycline	TCY	12 - 14	95.6	0	4.4	83.7-99.2

%R= Percentage resistance, %I= Percentage intermediate, %S= Percentage susceptibility, C.I.= Confidence interval, mm= millimeters

4.2.2 Comparison of quinolones tested

Isolates with large zone diameters for nalidixic acid (>24mm) and large zone diameters for ciprofloxacin (>30mm) were recorded in the study and represent the wild-type phenotype for *E. coli* against the quinolones tested (Figure 10). All isolates in the upper right quadrants of Figures 10 and 11 (21%) represent those isolates susceptible to the two quinolones tested. All isolates in the upper left quadrants of Figures 10 and 11 represent isolates that were resistant to nalidixic acid but susceptible to ciprofloxacin (7.5%). Isolates in the lower left quadrants of Figures 10 and 11 represent those that were susceptible to nalidixic acid but resistant to ciprofloxacin and these had the highest percentage (66%) (Figure 11) with most of them showing high resistance levels (zone diameter=6mm) (Figure 10). There were no isolates that were susceptible to nalidixic acid but resistant to ciprofloxacin (Figures 10 and 11)

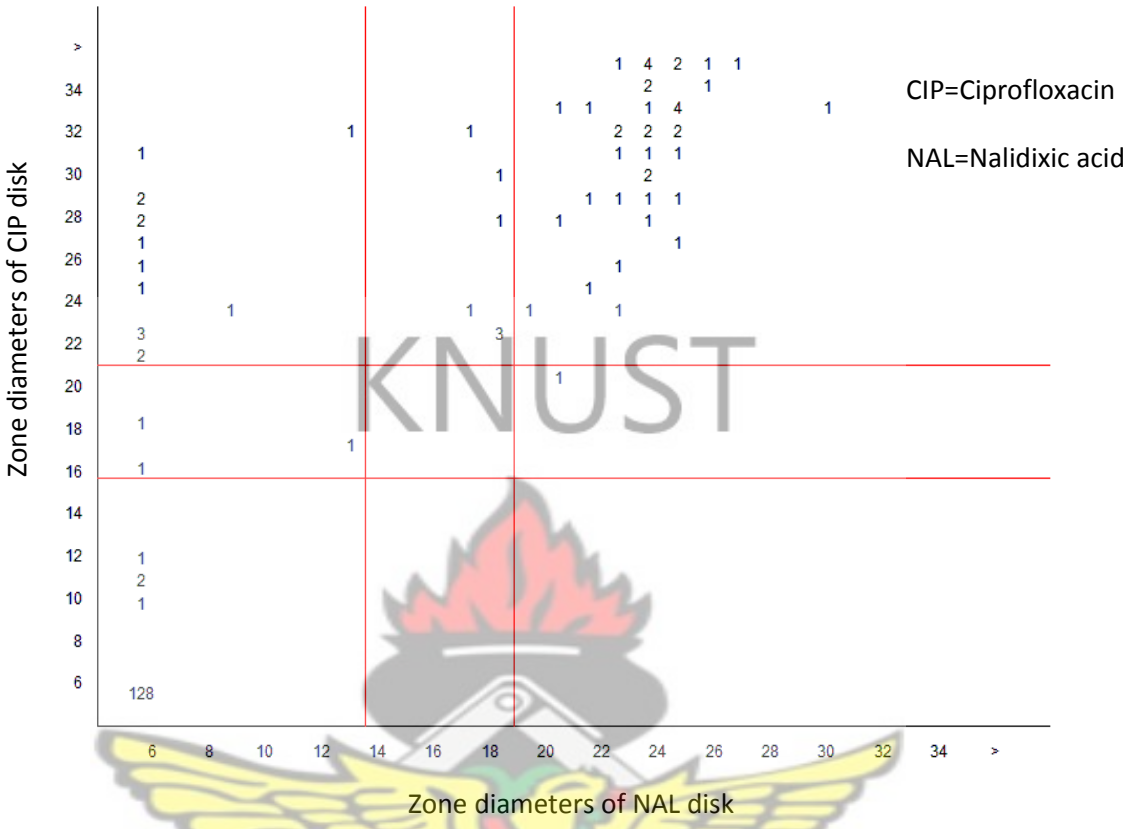


Figure 10. Zone diameter distribution of isolates tested against both nalidixic acid and ciprofloxacin

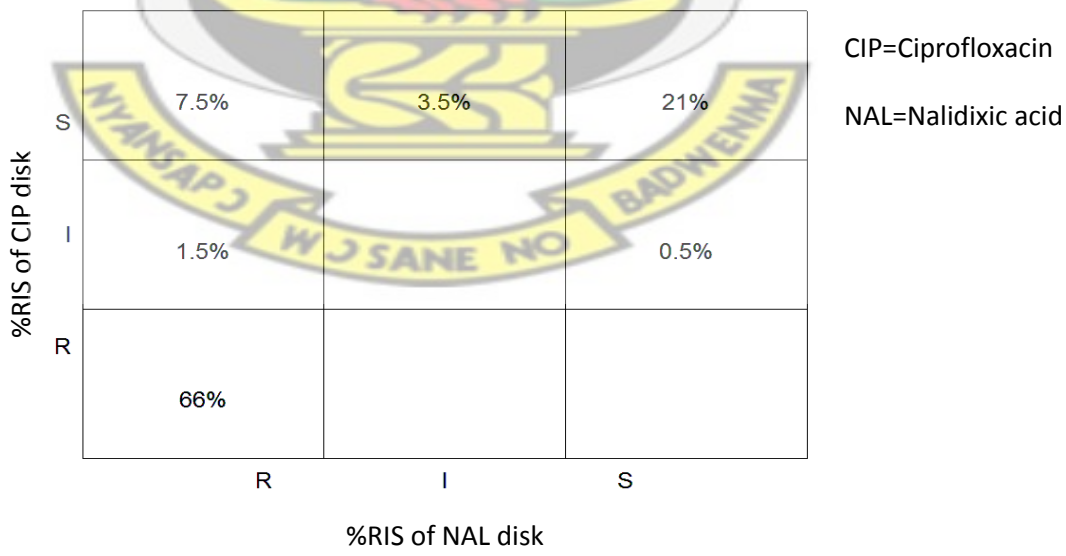


Figure 11. %RIS distribution of isolates tested against both nalidixic acid and ciprofloxacin

4.2.3 Multidrug resistant *E. coli*

The most common phenotype in the study was the pan-resistant (30.5% of isolates) which comprise those resistant to all drugs tested followed by GEN NAL SXT AMP CIP TCY (Non-susceptible to gentamicin, nalidixic acid, trimethoprim/sufamethoxazole, ampicillin, ciprofloxacin and tetracycline) which was exhibited by 13% of the isolates tested. 2.5% of isolates were susceptible to all antibiotics tested. All quinolone resistant isolates were also resistant to at least one other non-quinolone antibiotic (Table 8)

Table 8. Distribution of resistance profiles of *E. coli*

Resistance profile	MDR	XDR	PDR	Isolates No	Isolates %
				5	2.5
AMP				2	1
AMP TCY				1	0.5
SXT AMP				1	0.5
NAL AMP				1	0.5
AMP CHL TCY				1	0.5
SXT CHL TCY	MDR	Possible XDR		1	0.5
SXT AMP TCY				8	4
SXT AMP CHL				2	1
NAL AMP CIP				2	1
NAL SXT TCY				1	0.5
GEN NAL SXT				1	0.5
SXT AMP CIP TCY	MDR	Possible XDR		1	0.5
SXT AMP CHL TCY	MDR	Possible XDR		21	10.5
NAL AMP CIP TCY				3	1.5
NAL SXT AMP TCY				4	2
NAL SXT AMP CIP				2	1
NAL AMP CHL CIP TCY	MDR	Possible XDR		2	1
NAL SXT CHL CIP TCY	MDR	Possible XDR		1	0.5
NAL SXT AMP CIP TCY	MDR	Possible XDR		13	6.5
NAL SXT AMP CHL TCY	MDR	Possible XDR		12	6
GEN NAL AMP CIP TCY	MDR	Possible XDR		1	0.5
GEN NAL AMP CHL CIP	MDR	Possible XDR		1	0.5
GEN NAL SXT AMP CIP	MDR	Possible XDR		2	1
NAL SXT AMP CHL CIP TCY	MDR	Possible XDR		20	10
GEN NAL SXT AMP CIP TCY	MDR	Possible XDR		26	13
GEN NAL SXT AMP CHL TCY	MDR	Possible XDR		3	1.5
GEN NAL SXT AMP CHL CIP	MDR	Possible XDR		1	0.5
GEN NAL SXT AMP CHL CIP TCY	MDR	Possible XDR	Possible PDR	61	30.5

GEN=Gentamicin
NAL=Nalidixic acid
SXT=Trimethoprim/Sulfamethoxazole
AMP=Ampicillin
CHL=Chloramphenicol
CIP=Ciprofloxacin
TCY=Tetracycline

Code=Resistant or Intermediate
Space=Susceptible
No=Number
%=Percentage
MDR=Multidrug Resistance
XDR=Extensive Drug Resistance
PDR=Pandrug Resistance

4.2.4 Minimum inhibitory concentration

Three groups were observed in the MIC testing of ciprofloxacin. The first group comprised those that were susceptible to ciprofloxacin and had MIC values of 0.5 and 1µg/mL. The second group consisted of those that ranged from intermediate to moderately high resistant isolates and had MIC values ranging from 2 to 32µg/mL. The third group was made up of the high-level resistant isolates that had MIC values >64µg/mL. Testing by MIC yielded 67.5% resistant, 2.5% intermediate and 30% susceptible isolates (Figure 12). MIC50 for ciprofloxacin in this study was >64µg/mL.

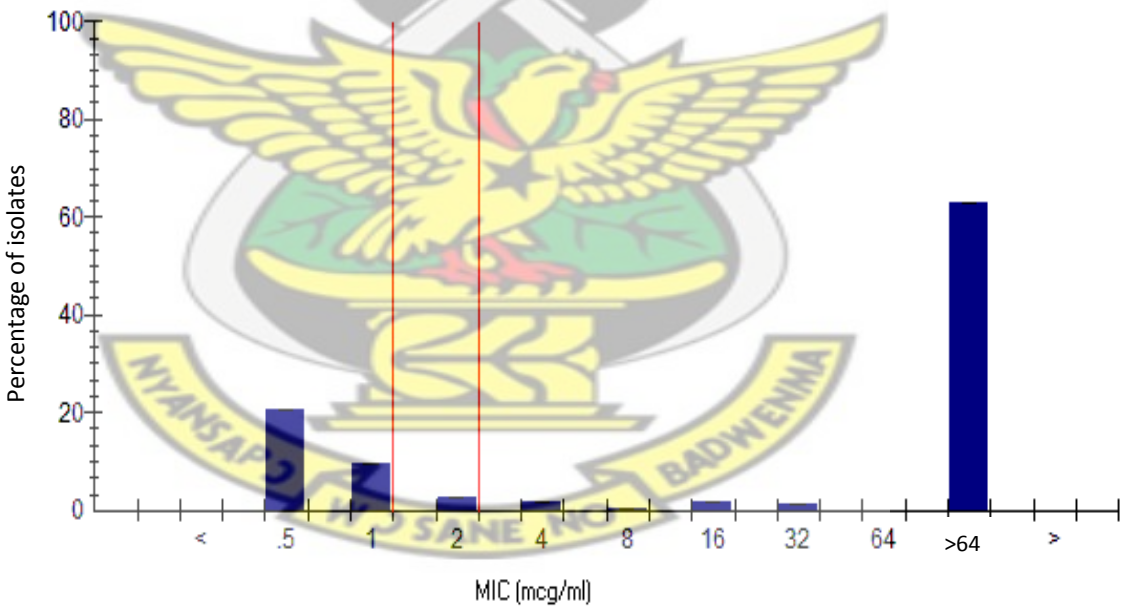


Figure 12. MIC of ciprofloxacin against *E. coli* isolates

4.2.5 Comparison of disk diffusion and minimum inhibitory concentration methods

There was very good levels of agreement between the results obtained by the disk diffusion and agar dilution methods for ciprofloxacin by the generalized kappa statistic (k=0.91).

4.2.6 Analysis of quinolone resistance development region

One hundred and forty nine isolates that were resistant to nalidixic acid underwent analysis by mismatch amplification mutation assay polymerase chain reaction. Five groups were defined by the results according to mutations in *gyr*A and *par*C. The first group consisted of 100 isolates (67%) with Ser-83 substitutions in *gyr*A (Figure 13).

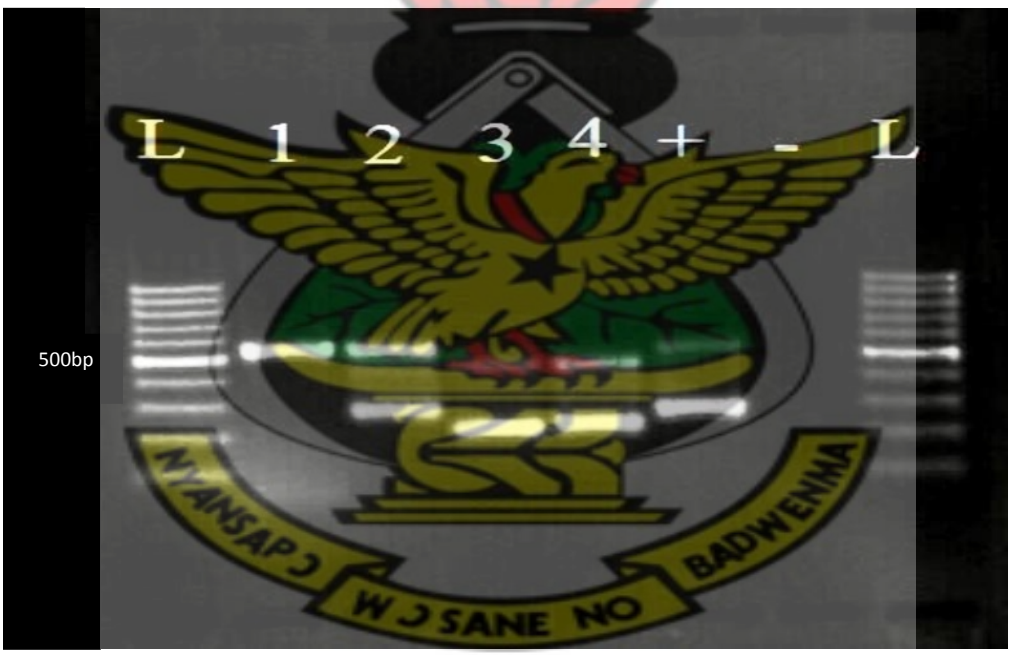


Figure 13. Agarose gel of MAMA PCR of *E. coli* showing a mutation in *gyr*A83. Lane 1 depicts a single control band of 540bp obtained from strains with mutations in *gyr*A83. Lane 2 depicts a double band of 540/259 or 540/274 obtained from strains with no mutations in *gyr*A87. Lanes 3 and 4 depict double bands of 446/217 or 446/238 obtained from strains with no mutations in *par*C80 and *par*C84 respectively. + depicts PCR product of *E. coli* ATCC25922 and – depicts PCR product of a negative control. L is the molecular weight standard (Thermo Scientific).

The second group consisted of 19 isolates (13%) with substitutions in Ser-83 in *gyr*A and Ser-80 in parC (Figure 14)

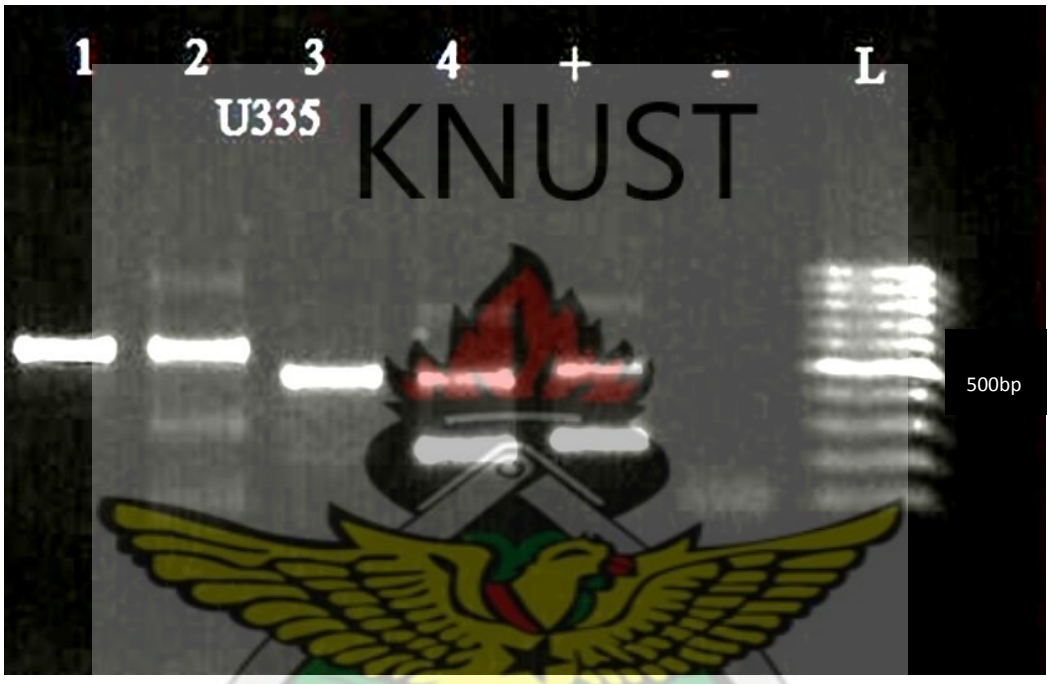


Figure 14. Agarose gel of MAMA PCR of *E. coli* showing mutations in *gyr*A83 and *par*C80. Lane 1 depicts a single control band of 540bp obtained from strains with mutations in *gyr*A83. Lane 2 depicts a double band of 540/259 or 540/274 obtained from strains with no mutations in *gyr*A87. Lane 3 depicts a single band of 446bp obtained from strains with mutations in *par*C80. Lane 4 depicts a double band of 446/217 or 446/238 obtained from strains with mutations in *par*C84. + depicts PCR product of *E. coli* ATCC25922 and – depicts PCR product of a negative control. L is the molecular weight standard (Thermo Scientific).

The third group consisted of 15 isolates (10%) which had Ser-83 substitutions in *gyr*A and Glu-84 substitutions in *par*C (Figure 15). The fourth group consisted of 13 isolates (9%) with substitutions in Ser-83 in *gyr*A together with substitutions in Ser-80 and Glu-

84 in *par*C (Figure 16). The fifth group consisted of 2 isolates (1%) with substitutions in Ser-83 and Asp-87 in *gyr*A and Ser-80 in *par*C (Figure 17) (Figure 18).

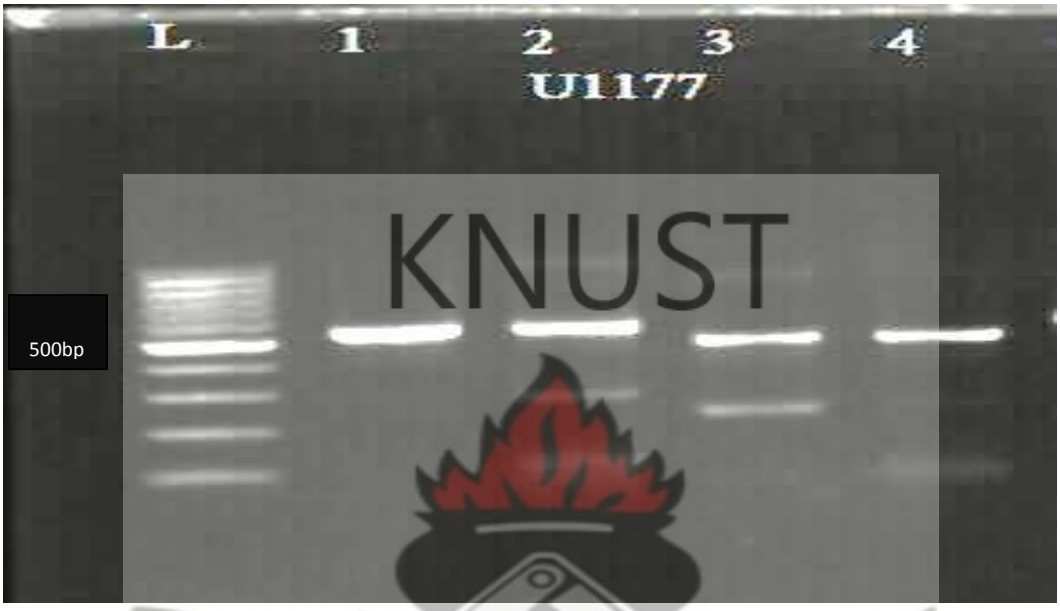


Figure 15. Agarose gel of MAMA PCR of *E. coli* showing mutations in *gyr*A83 and *par*C84. Lane 1 depicts a single control band of 540bp obtained from strains with mutations in *gyr*A83. Lane 2 depicts a double band of 540/259 or 540/274 obtained from strains with mutations in *gyr*A87. Lane 3 depicts a double band of 446/217 or 446/238 obtained from strains with no mutations in *par*C80. Lanes 4 depicts a single band of 446bp obtained from strains with mutations in *par*C84. + depicts PCR product of *E. coli* ATCC25922 and – depicts PCR product of a negative control. L is the molecular weight standard (Thermo Scientific).

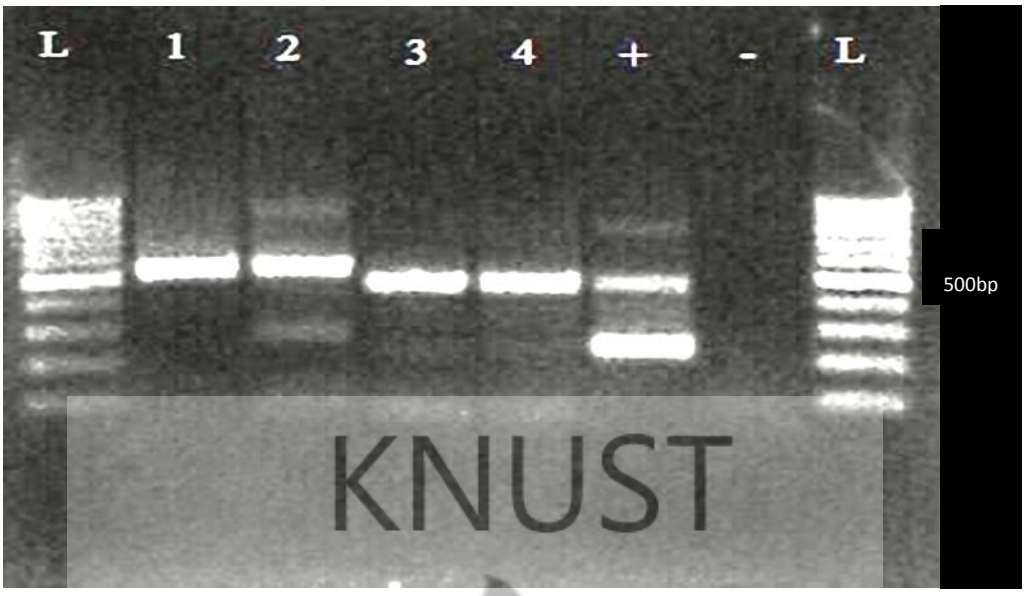


Figure 16. Agarose gel of MAMA PCR of *E. coli* showing mutations in *gyr*A83, *par*C80 and *par*C84. Lane 1 depicts a single control band of 540bp obtained from strains with mutations in *gyr*A83. Lane 2 depicts a double band of 540/259 or 540/274 obtained from strains with mutations in *gyr*A87. Lanes 3 and 4 depict sing bands of 446bp obtained from strains with mutations in *par*C80 and *par*C84 respectively. + depicts PCR product of *E. coli* ATCC25922 and – depicts PCR product of a negative control. L is the molecular weight standard (Thermo Scientific).

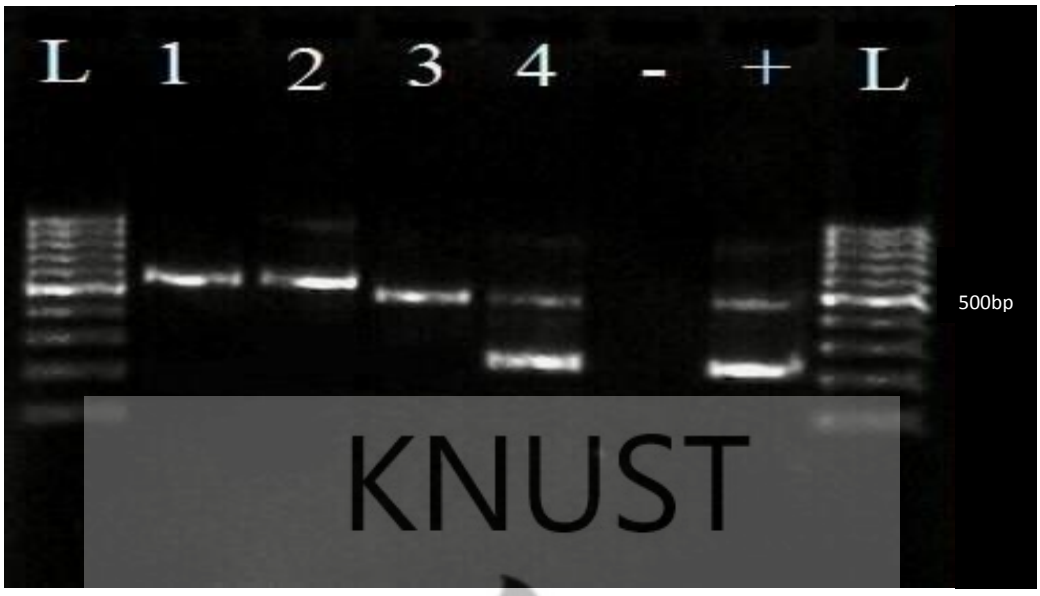


Figure 17. Agarose gel of MAMA PCR of *E. coli* showing mutations in *gyr*A83, *gyr*A87 and *par*C80. Lanes 1 and 2 depict a single control band of 540bp obtained from strains with mutations in *gyr*A83 and *gyr*A87 respectively. Lanes 3 depicts a single band of 446bp obtained from strains with mutations in *par*C80 and lane 4 depicts a double band of 446/217 or 446/238 obtained from strains with no mutations in *par*C84. + depicts PCR product of *E. coli* ATCC25922 and – depicts PCR product of a negative control. L is the molecular weight standard (Thermo Scientific).

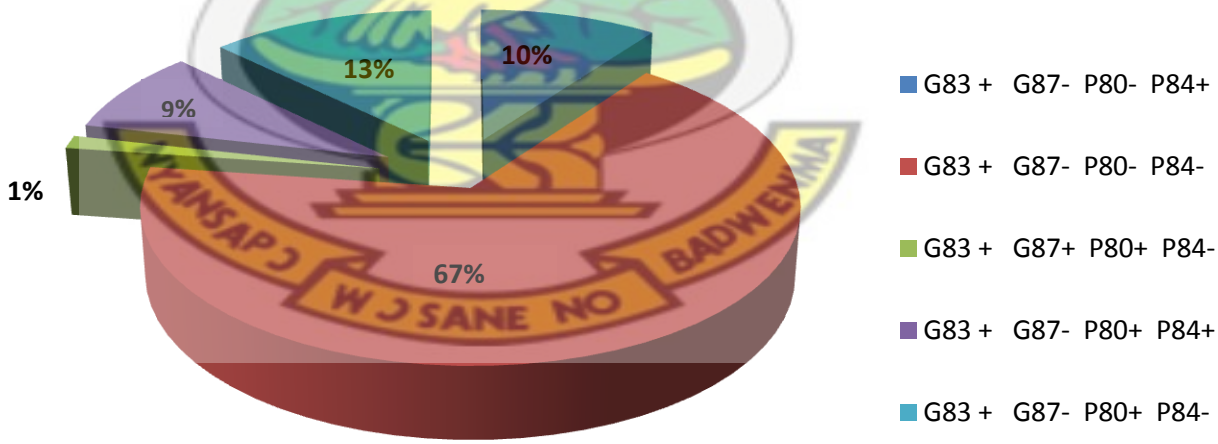


Figure 18. Percentage distribution of mutation combinations in *E. coli* isolates

Mutations in *gyr*A83 had the highest occurrence (149 isolates) and those in *gyr*A87 had the lowest occurrence (2 isolates). Mutations in *par*C80 and *par*c84 had similar occurrence with mutations in *par*C80 occurring in 34 isolates and mutations in *par*C84 occurring in 28 isolates (Figure 19)

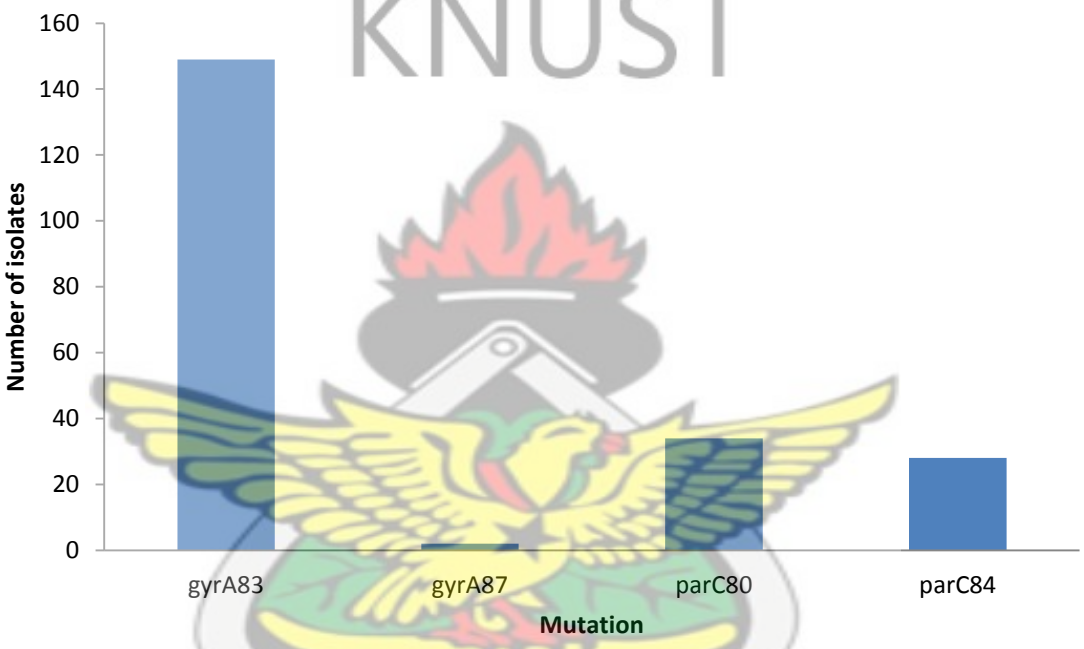


Figure 19. Distribution of mutations in *gyr*A and *par*C of *E. coli*

Isolates that had only a single mutation had minimum inhibitory concentration values ranging from 0.5µg/mL to >64µg/mL. With the exception of one isolate, all isolates that had minimum inhibitory concentration values less than 64 µg/ml (22 isolates) had only one mutation and no isolate that had two or more mutations (48 isolates) had a minimum inhibitory concentration value less than 64µg/mL. Again, with the exception of one

isolate, all isolates that had mutations in *par*C had MIC values that were greater than 64µg/mL (Table 9).

Table 9. Comparison of ciprofloxacin MIC and *E. coli* genotype

CIP MIC (µg/mL)	Result of PCR				NO of isolates
	*gyr*A83	*gyr*A87	*par*C80	*par*C84	
0.5	+	-	-	-	3
1	+	-	-	-	6
2	+	-	-	-	5
4	+	-	-	-	2
8	+	-	-	-	1
16	+	-	-	-	2
16	+	-	-	+	1
32	+	-	-	-	3
>64	+	-	-	-	78
>64	+	-	-	+	14
>64	+	-	+	-	19
>64	+	+	+	-	2
>64	+	-	+	+	13
					Total=149

CIP MIC: Minimum inhibitory concentration of ciprofloxacin

CHAPTER FIVE

5.0 DISCUSSION

5.1 Distribution of *E. coli* isolates

E. coli has been isolated from various body sites both in healthy and diseased individuals (Mims *et al.*, 2006). The frequency of isolation of *E. coli* in disease condition has been previously found to be highest in urine followed by isolation from other sources like wounds and pus, then blood and lastly cerebrospinal fluid (Shah *et al.*, 2002; Motayo *et al.*, 2012). A similar pattern of distribution was observed in this study with isolates from urine having the highest occurrence (61%) followed by isolates from wounds and other body sources (30%) and least occurrence in blood (9%). *E. coli* has also been found to be more frequently isolated from females than from males (Motayo *et al.*, 2012), a pattern that was also observed in this study with 139 isolates coming from females and 55 coming from males. This pattern can be attributed to the fact that females in general are more predisposed to urinary tract infections than males due to several factors like anatomical differences in genitalia (Puri and Malhotra, 2009). Generally, a higher number of isolates were obtained from adults than from children in the study which also conforms with other studies (Motayo *et al.*, 2012).

5.2 Antimicrobial susceptibility testing

The rapidly increasing rate of resistance development to previously effective antibiotics is becoming a major concern worldwide. The fluoroquinolones, which were previously a reliable class of antibiotics against the enterobacteriaceae have in recent studies showed

rising levels of resistance (Groß *et al.*, 2011). The percentage of isolates resistant to ciprofloxacin in this study was 66% by the disk diffusion method and 67.5% by the agar dilution method. These levels of resistance are comparable to levels obtained in other recent studies that also had high levels of resistance (60-80%) (Al Johani *et al.*, 2010) but higher than those obtained in other recent studies (38.5%) (Al-Agamy *et al.*, 2012). The differences in rates of resistance can be due to a variety of factors that might be peculiar to the particular location such as the rate of consumption of ciprofloxacin in the various areas (Al-Agamy *et al.*, 2012). Development of resistance to quinolones has been reported to always occur in the prototype nalidixic acid first before the fluoroquinolones (Namboodiri *et al.*, 2011) and this was demonstrated in this study. Among the quinolone resistant isolates obtained in this study, 66% were resistant to ciprofloxacin which is similar to that reported by Namboodiri *et al* in 2011 that 67% of quinolone resistant *E. coli* were ciprofloxacin resistant. Generally, the level of susceptibility to the various antibiotics tested was very poor with levels of resistance as high as 94% for ampicillin and 90% for tetracycline and Trimethoprim/Sulfamethoxazole. These levels are significantly higher than previously reported in other studies that had 70.7%, 65.9% and 68.3% for tetracycline, ampicillin and co-trimoxazole respectively (Bii *et al.*, 2005). Chloramphenicol and ciprofloxacin also showed high levels of resistance though relatively better than those of the other broad spectrum antibiotics tested. Gentamicin was found to be the least compromised by resistance among the antibiotics tested with a level of resistance of 47.5%. This level is also similar to that observed by Motayo *et al* in 2012 which was 41.3%. Despite the fact that gentamicin showed moderately lower levels of resistance, it was still significantly higher than some previously reported levels (6%) (Aslani *et al.*, 2007).

Different subpopulations of isolates were observed in this study according to the output of resistance testing against the various antibiotics. Ampicillin and nalidixic acid showed two distinct subpopulatons against *E. coli* in the study conducted. Gentamicin exhibited two distinct subpopulations in the resistant category and one group in the susceptible category. Four groups were evident in the susceptible category and two in the resistant category for ciprofloxacin. Trimethoprim/Sulfamethoxazole and tetracycline both showed similar distributions of subpopulations which consisted of a group with high susceptibility and a group with high level resistance. Chloramphenicol however showed two distinct groups for the resistant category and one group in the susceptible category. Other reports have also shown various distributions of subpopulations. In one report, *E. coli* susceptibility to nalidixic acid was made up of a susceptible subpopulation and a highly resistant subpopulation. Three populations were observed for *E. coli* isolates toward ciprofloxacin and a trimodal susceptibility pattern to co-trimoxazole consisting of a susceptible population, a highly resistant population and an intermediate population located between. *E. coli* behaviour towards gentamicin also showed a trimodal distribution of zone diameters with a susceptible population, a resistant to intermediate population located between and a highly resistant population (ONERBA, 2008). The minimum inhibitory concentration values for ciprofloxacin were generally high with an MIC50 value greater than 64µg/mL recorded. Other studies conducted on *E. coli* with regard to its minimum inhibitory concentration have also reported relatively high MIC50 values (64 µg/mL) (Diwan *et al.*, 2012). More reliable results for antimicrobial susceptibility testing can only be obtained when there is a good agreement between disk diffusion testing and minimum inhibitory concentration (CLSI, 2012). This was the case in this study in which there was a very good agreement between results obtained for

ciprofloxacin by disk diffusion testing and agar dilution methods (K=0.91). All the quinolone resistant isolates in the study were also resistant to at least one other unrelated antibiotic and resistance to quinolones was implicated in a vast majority of the multidrug resistant strains isolated in the study. In a similar study conducted by Namboodiri *et al* in 2011, quinolone resistance was also found to be almost always associated with multiply-resistant *E. coli*. In their study, quinolone- resistant *E. coli* were also found to be resistant to at least one other antimicrobial and all but three of the quinolone-resistant isolates were resistant to four or more non-quinolone antibiotics.

5.3 Analysis of quinolone resistance determining region

Mutations in *gyr*A and *par*C have been documented to be associated with resistance to quinolones in Gram-negative and Gram-positive organisms and mutations particularly in *gyr*A have been found associated with development of clinical resistance (Jacoby, 2005). In this study, the highest number of mutations was detected in Ser-83 (n=149) and at least one amino acid substitution in the GyrA protein at position 83 and/or 87 was detected in the nalidixic acid resistant isolates that underwent genotyping. This is similar to results obtained in other studies that also found *gyr*A83 to have the highest frequency of mutation (Sáenz *et al.,* 2003) and at least one mutation in the *gyr*A gene either at position 83 or 87 or both (Karami *et al.,* 2008). With the exception of one isolate, all isolates that had minimum inhibitory concentration values less than 64 µg/ml in this study had only one mutation and no isolate that had two or more mutations had a minimum inhibitory concentration value less than 64µg/mL. Again, with the exception of one isolate, all isolates that had mutations in *par*C had MIC values that were greater than 64µg/mL. This outcome somewhat suggests the cumulative effect of mutations on development of

resistance (Lindgren *et al.*, 2003) however 78 isolates that had only a single mutation also had minimum inhibitory concentration values greater than 64µg/mL. These isolates that have fewer mutations than expected may perhaps also be affected by active efflux and impermeability to the drug in addition to the mutation in *gyr*A (Alekshun and Levy, 1997). Although the approach described in this study will detect a number of different mutations in the wild-type sequence of *E. coli* at the position of interest, it has some limitations. First, it does not identify the nature of the mutation detected. Secondly, changes at the third base position of a codon would be detected by this method but these substitutions will not necessarily result in an amino acid substitution in the gene product due to the degenerate nature of the genetic code. In this regard, detection of such silent mutations by this method could, in principle, lead to wrong conclusions being drawn about amino acid substitution in the gene product.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study has demonstrated that *E. coli* from the Komfo Anokye Teaching Hospital has high level of resistance to quinolones and other broad spectrum antibiotics. The highest level of resistance was against ampicillin and the lowest was against gentamicin. Several subpopulations of *E. coli* exist among the isolates from KATH based on their resistance patterns to the various antibiotics. The quinolones were implicated in a majority of the multidrug resistant strains observed in the study. Minimum inhibitory concentration of ciprofloxacin against *E. coli* was generally high and there was a very good agreement between the disk diffusion and agar dilution method of susceptibility testing. Mutations in the quinolone resistance development region were found to be associated with all quinolone resistant isolates in this study.

6.2 Recommendation

1. Health care institutions should perform antimicrobial susceptibility testing as often as possible before prescribing antibiotics.
2. Ciprofloxacin should not be routinely prescribed for UTIs due to high level of resistance.
3. A similar study should be conducted for other microorganisms for which ciprofloxacin is routinely prescribed

4. Further molecular testing with broader range of MIC testing and detection of other resistance genes such as those for Efflux systems should be done.

REFERENCES

1. Ahmad, N., Drew, W. L. and Plorde, J. J. (2010). Sherris Medical Microbiology, The McGraw-Hill Companies, Inc.

2. Al-Agamy, M. H. M., Atef Mohamed Shibl and Radwan, H. H. (2012). "Detection of mutations in quinolone-resistant determining regions in clinical isolates of Escherichia coli from Saudi Arabia." African Journal of Biotechnology **11**(5).

3. Al Johani, S. M., Akhter, J., Balkhy, H., El-Saed, A., Younan, M. and Memish, Z. (2010). "Prevalence of antimicrobial resistance among gram-negative isolates in an adult intensive care unit at a tertiary care center in Saudi Arabia." Ann Saudi Med **30**(5): 364–369.

4. Alekshun, M. N. and Levy, S. B. (1997). "Regulation of Chromosomally Mediated Multiple Antibiotic Resistance: the mar Regulon." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **41**(10): 2067–2075.

5. Aslani, M. M., Salmanzadeh-Ahrabi, S., Alikhani, Y. M., Jafatri, F., Zali, R. M. and Mani, M. (2007). "Molecular detection and antimicrobial resistance of diarrheagenic Escherichia coli strains isolated from diarrheal cases." Saudi Med J 2008 **29**(3): 388-392.

6. Bartoloni, A., Pallecchi, L., Benedetti, M., Fernandez, C., Vallejos, Y., Guzman, E., Villagran, A. L., Mantella, A., Lucchetti, C., Bartalesi, F., Strohmeyer, M., Bechini, A., Gamboa, H., Rodríguez, H., Falkenberg, T., Kronvall, G., Gotuzzo, E., Paradisi, F. and Rossolini, G. M. (2006). "Multidrug-resistant Commensal Escherichia coli in Children, Peru and Bolivia." Emerg Infect Dis **12**(6): 907–913.

7. Bassler, B. L. and Losick, R. (2006). "Bacterially Speaking." Cell **125**(2): 237–246.

8. Bastopcu, A., Yazgi, H., Uyanik, M. H. and Ayyildiz, A. (2008). "Evaluation of Quinolone Resistance in Gram Negative Bacilli Isolated from Community- and Hospital-Acquired Infections." The Eurasian Journal of Medicine **40**: 58-61.

9. Bayram, Y., Korkoca, H., Aypak, C., Parlak, M., Cikman, A., Kilic, S. and Berktas, M. (2011). "Antimicrobial Susceptibilities of Brucella Isolates from Various Clinical Speciemens." Int J Med Sci **8**(3): 198-202.

10. Berg, J., Tymoczko, J. and Stryer, L. (2002). Biochemistry

11. Bii, C. C., Taguchi, H., Ouko, T. T., Muita, L. W., Wamae, N. and Kamiya, S. (2005). "Detection of virulence-related genes by multiplex PCR in multidrug-resistant diarrhoeagenic Escherichia coli isolates from Kenya and Japan." Epidemiol. Infect **133**: 627–633.

12. Biswal, S., Sahoo, U., Sethy, S., Kumah, H. K. S. and Banerjee, M. (2012). "INDOLE: THE MOLECULE OF DIVERSE BIOLOGICAL ACTIVITIES." Asian Journal of Pharmaceutical and Clinical Research **5**(1): 1-6.

13. Bottemaa, C. D. K. and Sommer, S. S. (1993). "PCR amplification of specific alleles: Rapid detection of known mutations and polymorphisms." Mutation Research **288**(1): 93–102.

14. Breed, R. S., Murray, E. G. D. and Smith, N. R. (1957). BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY. BALTIMORE, THE WILLIAMS & WILKINS COMPANY.

15. Burton, G. R. W. and Engelkirk, P. G. (2003). Microbilogy for health sciences, Lippincott Williams & Wilkins.

16. Butaye, P., Cloeckaert, A. and Schwarz, S. (2003). "Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria." Int J Antimicrob Agents **22**(3): 205-210.

17. Byarugaba, D. K. (2010). Mechanisms of Antimicrobial Resistance. Antimicrobial Resistance in Developing Countries

18. A. d. J. Sosa, D. K. Byarugaba, C. F. Amábile-Cuevaset al, Springer New York Dordrecht Heidelberg London**:** 15-26.

19. Cabral, J. H. M., Andrew P. Jackson, Clare V. Smith, Nita Shikotra, Maxwell, A. and Liddington, R. C. (1997). "Crystal structure of the breakage–reunion domain of DNA gyrase." Nature **388**: 903-906.

20. Cavalieri, S. J., Rankin, I. D., Harbeck, R. J., Sautter, R. L., McCarter, Y. S., Sharp, S. E., Ortez, J. H. and Spiegel, C. A. (2005). Manual of Antimicrobial Susceptibility Testing, Library of Congress Cataloging-in-Publication Data.

21. Cha, R. S., Zarbl, H., Keohavong, P. and Thilly, W. G. (1992). "Mismatch Amplification Mutation Assay (MAMA): Application to the c-H-ras Gene." Genome Research **2**: 14-20.

22. Cheesbrough, M. (2006). District Laboratory Practice in Tropical Countries. New York, Cambridge University Press.

23. CLSI (2006). "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically." **Vol 26** (7th edition): Approved Satandard M7-A7.

24. CLSI (2012). "Performance Standards for Antimicrobial Disk Susceptibility Tests; ." **Vol. 32**(11th edition): Approved Standard M02-A11

25. Dale, J. W. and Park, S. F. (2010). Molecular Genetics of Bacteria, A John Wiley & Sons, Ltd., Publication.

26. Deguchi, T., Fukuoka, A., Yasuda, M., Nakano, M., Ozeki, S., Kanematsu, E., Nishino, Y., Satoshi Ishihara, Ban, Y. and Kawada, Y. (1997). "Alterations in the GyrA Subunit of DNA Gyrase and the ParC Subunit of Topoisomerase IV in Quinolone-Resistant Clinical Isolates of *Klebsiella pneumoniae*." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **31**(3): 699–701.

27. Dessen, A., Guilmi, A. M. D., Vernet, T. and Dideberg, O. (2001). "Molecular mechanisms of antibiotic resistance in gram-positive pathogens." Curr Drug Targets Infect Disord **1**(1): 63-77.

28. Diwan, V., Chandran, S. P., Tamhankar, A. J., Lundborg, C. S. and Macaden, R. (2012). "Identification of extended-spectrum β-lactamase and quinolone resistance genes in Escherichia coli isolated from hospital wastewater from central India." J Antimicrob Chemother.

29. Enne, V. I., Livermore, D. M., Stephens, P. and Hall, L. M. C. (2001). "Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction." Lancet **357**(9271): 1325–1328.

30. Fluit, A. C., Visser, M. R. and Schmitz, F.-J. (2001). "Molecular Detection of Antimicrobial Resistance." Clinical Microbiology Reviews **14**(4): 836-871.

31. Friedman, S. M., Lu, T. and Drlica2, K. (2001). "Mutation in the DNA Gyrase A Gene of Escherichia coli That Expands the Quinolone Resistance-Determining Region." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **45**(8): 2378–2380.

32. Groß, U., Sylvarius K. Amuzu, Ring de Ciman, Iparkhan Kassimova, Lisa Groß, Wolfgang Rabsch, Ulrike Rosenberg, Marco Schulze, August Stich and Zimmermann, O.

(2011) "Bacteremia and Antimicrobial Drug Resistance over Time, Ghana." Emerging Infectious Diseases **Vol. 17,** DOI: 10.3201/edi1710.110327.

33. Heisig, P. and Tschorny, R. (1994). "Characterization of Fluoroquinolone-Resistant Mutants of *Escherichia coli* Selected In Vitro." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **38**(6): 1284-1291.

34. Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T. and Yamaguchi, A. (2005). "Indole induces the expression of multidrug exporter genes in Escherichia coli." Mol Microbiol **55**(4): 1113-1126.

35. Hirakawa, H., Kodama, T., Takumi-Kobayashi, A., Honda, T. and Yamaguchi, A. (2009). "Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic Escherichia coli O157:H7." Microbiology **155**(2): 541-550.

36. Hogg, S. (2005). Essential Microbiology. John Wiley & Sons Ltd. Great Britain, Antony Rowe, Ltd, Chippenham, Wiltshire.

37. Hopkins, K. L., Davies, R. H. and Threlfall, E. J. (2005). "Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments." Int J Antimicrob Agents **25**(5): 358-373.

38. Jacoby, G. A. (2005). "Mechanisms of Resistance to Quinolones." Clinical Infectious Diseases **41**(Supplement 2): S120-S126.

39. Jawetz, Melnick and Adelberg (2010). Medical Microbiology. M. Geo. F. Brooks, M. Karen C. Carroll, P. Janet S. Butel and P. Stephen A. Morse, McGraw-Hill.

40. Kapoor, K. (2010). Illustrated Dictionary of Microbiology, Oxford Book Company, Regd. Off 267-B, Scheme, Opp. Narayan Niwas, Gopalpura By Pass Road, Jaipur-302018 Rajasthan (India) Ph: 0141-2594705,Telefax: 2597527.

41. Karami, A., Naghavi, K., Sorouri, R., Ranjbar, R. and Khalilpour, A. (2008). "Use of a MAMA-PCR Method to detect gyrA Mutations in Nalidixic Acid Resistant Clinical Isolates of Escherichia coli." Iranian J Publ Health **37**(1): 42-47.

42. Kariuki, S. (2010). Antimicrobial Resistance in Enteric Pathogens in Developing Countries. Antimicrobial Resistance in Developing Countries. A. b. d. J. Sosa, D. K. Byarugaba, C. F. A. bile-Cuevaset al, Springer New York Dordrecht Heidelberg London**:** 177-199.

43. Kollef, M. H. and Fraser, V. J. (2001). "Antibiotic Resistance in the Intensive Care Unit." Annals of Internal Medicine **134**(4): 298-314.

44. Kwok, S., Chang, S.-Y., Sninsky, J. J. and Wang, A. (1994). "A guide to the design and use of mismatched and degenerate primers." Genome Research **3**: S39-S47.

45. Lee, J.-H. and Lee, J. (2010). "Indole as an intercellular signal in microbial communities." FEMS Microbiology Reviews **34**(4): 426–444.

46. Lindgren, P. K., Karlsson, A. and Hughes, D. (2003). "Mutation Rate and Evolution of Fluoroquinolone Resistance in *Escherichia coli* Isolates from Patients with Urinary Tract Infections." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **47**(10): 3222–3232.

47. Little, S. (2001). "Amplification-refractory mutation system (ARMS) analysis of point mutations." Current Protocols in Human Genetics **9.8.**: 1–9.8.12.

48. Martínez-Gómez, K., Flores, N., Castañeda, H. M., Martínez-Batallar, G., Hernández-Chávez, G., Ramírez, O. T., Encarnación, G. G. and Bolivar, F. (2012). "New insights into Escherichia coli metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol." Microbial Cell Factories **11**(46).

49. Mathew, A. G., Cissell, R. and Liamthong, S. (2007). "Antibiotic Resistance in Bacteria Associated with Food Animals: A United States Perspective of Livestock Production." Foodborne Pathogens and Disease **4**(2): 115-133.

50. Milatovic, D. and Braveny, I. (1987). "Development of resistance during antibiotic therapy." Eur J Clin Microbiol **6**(3): 234-244.

51. Mims, C., Dockrell, H. M., Goering, R. V., Roitt, I., Wakelin, D. and Zuckerman, M. (2006). Medical Microbiology. Great Britain, Mosby.

52. Motayo, O. B., Ogiogwa, I. J., Okerentugba, P. O., Innocent-Adiele, H. C., Nwanze, J. C., Onoh, C. C. and Okonko, I. O. (2012). "Antimicrobial Resistance Profile of Extra-Intestinal Escherichia coli Infections in a South Western Nigerian City." Journal of Microbiology Research **2**(5): 141-144.

53. Namboodiri, S. S., Opintan, J. A., Lijek, R. S., Newman, M. J. and Okeke, I. N. (2011). "Quinolone resistance in Escherichia coli from Accra, Ghana." BioMed Central **11**(44): 1-9.

54. Newton, W. A. and Snell, E. E. (1965). "Formation and Interrelationships of Tryptophanase and Tryptophan Synthetases in Escherichia coli." Journal of Bacteriology **89**(2): 355–364.

55. NIH (2011) "How pathogenic E. coli bacterium causes illness." ScienceDaily.

56. Nys, S., Okeke, I. N., Kariuki, S., Dinant, G. J., Driessen, C. and Stobberingh, E. E. (2004). "Antibiotic resistance of faecal Escherichia coli from healthy volunteers from eight developing countries." Journal of Antimicrobial Chemotherapy **54**: 952-955.

57. Oethinger, M., Kern, W. V., Jellen-Ritter, A. S., McMurry, L. M. and Levy, S. B. (2000). "Ineffectiveness of Topoisomerase Mutations in Mediating Clinically Significant Fluoroquinolone Resistance inEscherichia coli in the Absence of the AcrAB Efflux Pump." Antimicrob. Agents Chemother **44**(1): 10-13.

58. ONERBA (2008). Resistance to antimicrobials in France. Subpopulation analysis of major bacterial species, according to their susceptibility level. **Chapter VI-5**.

59. Parsons, B. L. and Heflich, R. H. (1997). "Genotypic selection methods for the direct analysis of point mutations." Mutation Research **387** 97–121.

60. Paterson, D. L. (2006). "Resistance in gram-negative bacteria: Enterobacteriaceae." Association for Professionals in Infection Control and Epidemiology, Inc. and Elsevier, Inc. **34**(1): S20-28.

61. Piñero-Fernandez, S., Chimerel, C., Keyser, U. F. and Summers, D. K. (2011). "Indole Transport across Escherichia coli Membranes." Journal of Bacteriology **198**(8): 1793-1798.

62. Pitout, J. D. D., Thomson, K. S., Hanson, N. D., Ehrhardt, A. F., Coudron, P. and Sanders, C. C. (1998). "Plasmid-Mediated Resistance to Expanded-Spectrum Cephalosporins among Enterobacter aerogenes Strains." Antimicrob Agents Chemother **42**(3): 596–600.

63. Prescott, L. M. (2002). Microbiology, The McGraw−Hill Companies.

64. Puri, R. and Malhotra, J. (2009). "Recurrent Urinary Tract Infection (UTI) in Women." South Asian Federation of Obstetrics and Gynecology **1**(1): 10-13.

65. Qiang, Y. Z., Qina, T., Fub, W., Chengb, W. P., Lia, Y. S. and Yia, G. (2002). "Use of a rapid mismatch PCR method to detect gyrA and parC mutations in ciprofloxacin-resistant

clinical isolates of Escherichia coli." Journal of Antimicrobial Chemotherapy **49**(3): 549-552.

66. Roberts, M. C. (2006). "Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution." FEMS Microbiology Reviews **19**(1): 1-24.

67. Rolain, J. M., Mallet, M. N., Fournier, P. E. and Raoult, D. (2004). "Real-time PCR for universal antibiotic susceptibility testing." Journal of Antimicrobial Chemotherapy **54**: 538–541.

68. Sabir, N., Khan, E., Sheikh, L. and Hasan, R. (2004). "Impact of Antibiotic usage on resistance in Microorganisms; Urinary Tract Infections with E-coli as a case in point." Journal of Pakistan medical association **54**(472).

69. Sáenz, Y., Zarazaga, M., Briñas, L., Ruiz-Larrea, F. and Torres, C. (2003). "Mutations in gyrA and parC genes in nalidixic acid-resistant Escherichia coli strains from food products, humans and animals." Journal of Antimicrobial Chemotherapy **51**: 1001–1005.

70. Schmitz, F. J., Verhoef, J., Fluit, A. C. and The SENTRY participants group (1999). "Prevalence of Aminoglycoside Resistance in 20 European University Hospitals Participating in the European SENTRY Antimicrobial Surverillance Programme." Eur J Clin Microbiol Infect Dis **18**: 414-421.

71. Shah, A. A., Hasan, F. and Hameed, A. (2002). "Study on the Prevalence of Enterobacteriacae in hospital acquired and community acquired infections." Pakistan J. Med. Res. **41**(1).

72. Shaw, W. V. (1983). "Chloramphenicol acetyltransferase: enzymology and molecular biology." Critical Review Biochemistry **14**(1): 1-46.

73. Speer, B. S., Shoemaker, N. B. and Salyers, A. A. (1992). "Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance." Clinical Microbiology Reviews **5**(4): 387-399.

74. Strahilevitz, J., Jacoby, G. A., Hooper, D. C. and Robicsek, A. (2009). "Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat." Clin Microbiol Rev **22**(4): 664–689.

75. Taylor, D. E. and Chau, A. (1996). "Tetracycline Resistance Mediated by Ribosomal Protection." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY **40**(1): 1-5.

76. Thomson, C. J. (1993). "Trimethoprim and brodimoprim resistance of gram-positive and gramnegative bacteria." Journal of chemotherapy **5**(6): 458-464.

77. Todar, K. (2012). Todar's Online Textbook of Bacteriology. 21/10/2012.

78. Tran, J. H. and Jacoby, G. A. (2002). "Mechanism of plasmid-mediated quinolone resistance." Proceedings of the National Academy of Sciences of the United States of America **99**(8): 5638–5642.

79. Tran, J. H., Jacoby, G. A. and Hooper, D. C. (2005). "Interaction of the Plasmid-Encoded Quinolone Resistance Protein Qnr with Escherichia coli DNA Gyrase." Antimicrob. Agents Chemother **49**(1): 118-125.

80. Ugozzoli, L. and Wallace, R. B. (1991). "Allele-specific polymerase chain reaction." Methods **2**(1): 42–48.

81. Vandepitte, J., Verhaegen, J., Engbaek, K., Rohner, P., Piot, P. and Heuck, C. C. (2003). Basic laboratory procedures in clinical bacteriology. Geneva, WHO.

82. Walsh, C. (2000). "Molecular mechanisms that confer antibacterial drug resistance." Nature **406**: 775–781.

83. Wang, H., Dzink-Fox, J. L., Chen, M. and Levy, S. B. (2001). "Genetic characterization of highly fluoroquinolone-resistant clinical Escherichia coli strains from China: role of acrR mutations." Antimicrob Agents Chemother **45**(5): 1515–1521.

84. Waters, C. M. and Bassler, B. L. (2005). "QUORUM SENSING: Cell-to-Cell Communication in Bacteria." Annual Review of Cell and Developmental Biology **21**: 319-346.

85. WHO (2002). Surveillance standards for antimicrobial resistance. Communicable Disease Surveillance Response**:** 2-3.

86. WHO (2012). "Antimicrobial Resistance." **Fact Sheet Number 194**.

87. Yang, S., Lopez, C. R. and Zechiedrich, E. L. (2006). "Quorum sensing and multidrug transporters in Escherichia coli." PNAS **103**(7): 2386–2391.

88. Ye, S., Humphries, S. and Green, F. (1992). "Allele specific amplification by tetra-primer PCR." Nucleic Acids Research **20**(5): 1152.

89. Zirnstein, G., Swaminathan, B., Li, Y. and Angulo, F. (1999). "Ciprofloxacin Resistance in Campylobacter jejuni Isolates: Detection of gyrA Resistance Mutations by Mismatch Amplification Mutation Assay PCR and DNA Sequence Analysis." Journal of Clinical Microbiology **37**(10): 3276-3280.

APPENDICES

Plate 5: Inoculum spots on Mueller Hinton agar plates

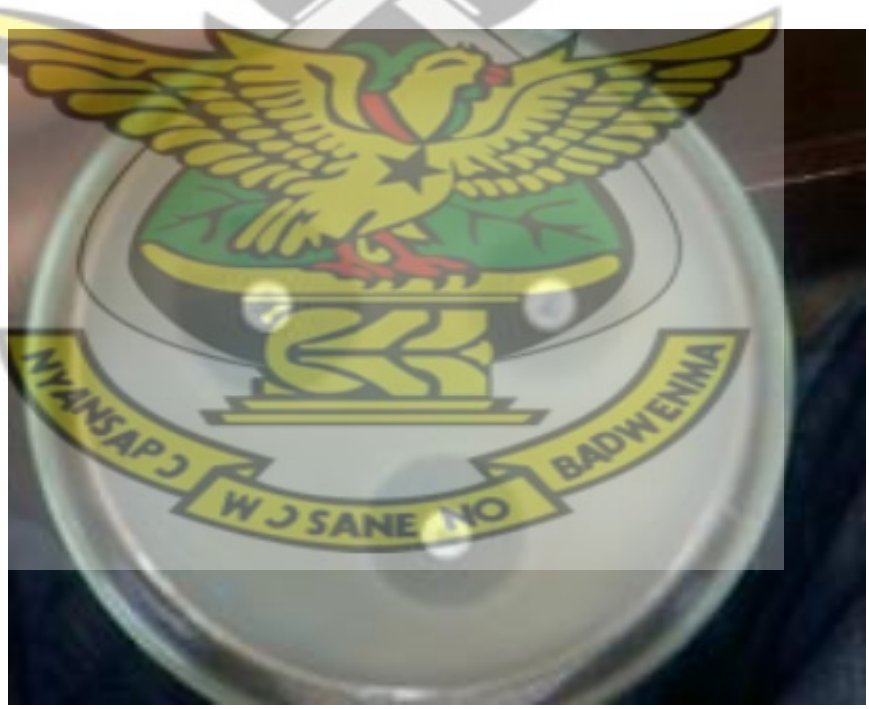


Plate 6: Zone diameters of disk diffusion test

Plate 7: McFarland's turbidity standard

Appendix 1: Calculation of generalized Kappa statistic

BY CATEGORY	**CAT1**	**CAT2**	**CAT3**
num = sum_xi*(m-xi)	**5**	**8**	**3**
den = nm(m-1)pq	**85.9**	**9.8**	**89.4**
gen kappa_cat1 =	**0.942**	**0.179**	**0.966**

****

OVERALL

num = nm^2 - sum_x^2	**16**
den = nm(m-1)sum_pq	**185.1**
gen kappa =	**0.914**
(p(e))	**0.537**
(sum p^3)	**0.321**

$SE_{Fleiss1}$ [a]	**0.076**	**$SE_{Fleiss2}$ [b]**	**0.065**
z =	**11.992**	**z =**	**13.972**
p calc =	**0.000000**	**p calc =**	**0.000000**
CI_{Lower} =	**0.764**	**CI_{Lower} =**	**0.785**
CI_{Upper} =	**1.063**	**CI_{Upper} =**	**1.042**