ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *ESCHERICHIA COLI* ISOLATES FROM STOOLS OF DIARRHOIEC CHILDREN BELOW 5 YEARS IN ZARIA, NIGERIA

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NIGERIA

NOVEMBER, 2012

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the supervision of Prof. J. O. Ehinmidu, Dr B. O. Olayinka, both of the Department of Pharmaceutics and Pharmaceutical Microbiology, and Dr (Mrs) A. T. Olayinka of the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika.

This work is original and has not been submitted elsewhere for a higher degree. The work of other investigators have been properly acknowledged and referred to accordingly.

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_____
Signature/Date

CERTIFICATION

This thesis entitled "Isolation and antibiotic susceptibility pattern of *Escherichia coli* isolates from stools of diarrhoeic children below 5 years in Zaria, Nigeria" by **Susan Mosebolatan David** meets the regulations governing the award of the degree of Masters of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Prof. A.A. Joshua Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This work is dedicated to the Almighty God the Elohim who has kept me and given me his inspiration.

To my Sweetheart and loving children for their love, patience, encouragement and support

through this pursuit and in loving memory of my parents Late Samuel and Margaret Jaiyeoba.

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Indeed God hath chosen the foolish things of the world to confound the wise and the weak things of this world to confound the things which are mighty. I Cor. 1:27.

ABSTRACT

E. coli has become widespread in distribution and recognised both as harmless commensal and a versatile pathogen. It has emerged as an important cause of diarrhoea illness with diverse phenotypes and pathogenicic mechanisms. *E. coli* and other members of the family enterobacteriaceae are well known to develop or acquire resistance to a variety of antibiotics by different mechanisms. Seventy (70) isolates of *Escherichia coli* from stool of diarrhoeic children below five (5) years of age with suspected gastrointestinal infections attending two hospitals and a primary health care centre in Zaria were screened for their susceptibility to a panel of nine commonly used antibiotics by the disc diffusion method. *Escherichia coli* isolates which were resistant to multiple antibiotics and showed minimum inhibitory concentration between 20 – 100 µg/ml against ciprofloxacin were used for conjugation studies. Curing experiments of the transconjugants and resistant *Escherichia coli* isolates was carried out with acridine orange to determine if the resistant determinants were plamid mediated . High level of resistance was observed to Amoxicillin (100%), Ampicillin (100%), Tetracycline (100%), Sulphamethoxazole/Trimethoprim (97.1%), Ceftazidime (88.6%), Cefuroxime (85.7%) while lower resistance were observed to Gentamicin (35.7%), Ciprofloxacin (40%) and Ceftriaxone (58.6%). Sixteen isolates (22.9%) were found to be resistant to all the 9 antibiotics used. Sixty-eight isolates (97.1%) were identified as being resistant to multiple antibiotics since they were simultaneously resistant to at least three different antibiotics with seventeen (17) different phenotypic patterns. The multiple antibiotic resistance indices (MARI) for the MAR isolates was found to be high, between 0.3 to 1.0 suggesting that the isolates originated from an environment where antibiotics were often used. Plasmid DNA analysis on thirty-five resistant isolates showed 11 plasmid band sizes with molecular weights ranging from 2800 bp to > 5000 bp. Three isolates were found to harbour the IncK epidemiologic plasmid encoding gene for the bla_{CTX-M} of the ESBLs and nineteen resistant isolates were also found to harbour the gyrB gene. The results of this study indicates that resistance to ampicillin, amoxicillin and cotrimoxazole is remarkably high in Zaria, and their continued usefulness in the treatment of *E. coli* infections in children need to be reviewed. In contrast, gentamicin, ceftriaxone and ciprofloxacin were more efficacious but the later (ciprofloxacin) is however contraindicated in children below 12 years of age. Plasmid borne resistant genes among the *Escherichia coli* isolates were more from the stools of children obtained from the General Hospital, Kofan-Gaya, Zaria-city, Nigeria.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Diarrhoeal Diseases

Diarrhoea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than is normal for the individual (WHO, 2009). Diarrhoeal disease has been reported to be a leading cause of morbidity and mortality in children and there are about two billion cases of diarrhoeal disease worldwide (WHO, 2009). Out of the 1.5 million children reported to have been killed by diarrhoeal disease in 2004, 80% were below two years (WHO, 2009). In children below five years, diarrhoeal disease has been described to be the second leading cause of death after pneumonia (Kosek *et al*., 2003). In developing countries, children under three years old have been reported to experience an average of three episodes of diarrhoea every year. Each episode of diarrhoea deprives the child of the nutrition necessary for growth and as a result, diarrhoea has been reported to be a major cause of malnutrition, and malnourished children are predisposed to falling ill due to diarrhoea (WHO, 2012).

The highest age-mortality rate (8.5 children per 1000/year) occurs in children under 1 year of life (Kosek *et al.*, 2003). Improvement in sanitation, nutrition, education and early access to oral rehydration therapy among other measures has been reported to lower the fatality of severe acute infectious diarrhoea from 4.6 million in 1982 to an estimated 1.5–2.5 million in 2010 (Black *et al.*, 2010; O'Ryan *et al.*, 2010). However, diarrhoea remains the second most common cause of death in children under 5 years of age worldwide according to the Global Burden Disease Report of the WHO (Boschi-Pinto *et al.*, 2008; Kosek *et al.*, 2003).

A diversity of recognized microorganisms such as bacteria, viruses and parasites have been reported to be associated with severe acute infectious diarrhoea in children (Albert *et al.*, 1999; O'Ryan *et al.*, 2005; Al-Gallas *et al.*, 2007; DuPont, 2009). However, the etiological information on all the known agents of severe acute infectious diarrhoea in children is scarce. Numerous studies performed in different countries have reported diarrhoeagenic *Escherichia coli* (DEC) pathotypes as being the most frequent and important bacterial pathogens associated with acute infectious diarrhoea in developing countries (Black *et al.,* 2010; O'Ryan *et al.* 2010). The frequency of these pathogens have been reported to vary with geographical location and socioeconomic/sanitary conditions of the region. (Black *et al.*, 2010; O'Ryan *et al.*, 2010). Diarrhoea has been reported to represent 30-40% of the cases of children who require medical attention (Albert *et al.*, 1999; O'Ryan *et al.*, 2005), and about 15-30% have been documented to require hospitalization (Nataro and Karper, 1998; O'Ryan *et al.*, 2010). Rotavirus and *Escherichia coli* are reported to be the two most common causes of diarrhoea in developing countries.(WHO, 2009).

E. coli was first suspected as being a cause of children's diarrhoea in the 1940s, when nursery epidemics of severe diarrhoea were found (Bray, 1945). These specific serotypes, designated enteropathogenic *E. coli*, were epidemiologically incriminated as the cause of the outbreaks. Studies with *E. coli* serotype in volunteer experiments has confirmed that ingestion of large numbers of these organisms resulted in diarrhoea, and the ingested *E. coli* serotype cells were recovered in the faeces of the volunteers (Levine *et al*., 1993).

Three groups of *E. coli* are known to be associated with diarrhoeal diseases: they are: enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC) and Enteropathogenic *E. coli* (EPEC). Enterotoxigenic *E. coli* serotypes have been shown to possess numerous types of

enterotoxin. Such as cytotoxic toxins which damages the mucosal cells, and cytotonic, which induces only the secretion of water and electrolytes. Enteroinvasive *Escherichia coli* (EIEC) has been reported to invade and cause tissue destruction and inflammation of tissue resembling the effects of *Shigella* . A third group of *E.coli* serotypes, called enteropathogenic *E. coli* (EPEC), has been associated with outbreaks of diarrhoea in newborns in nurseries (Doyle *et al*, 1996). More recently identified are verotoxigenic *E. coli* (VTEC) also reffered to as enterohaemorrhagic *E. coli* (EHEC) (Cheesebrough, 2000).

1.2 Statement of research problem

Antibiotic resistance is a global public health issue that is impacted by both human and non-human antimicrobial usage. The continuing emergence, development and spread of pathogenic organisms that are resistant to antibiotics are a cause of increasing concern to health care practice (WHO, 2012). The use of antibiotics has been reported to be one of the factors contributing to the emergence of bacterial resistance (Yagupsky, 2006; WHO, 2011).

E. coli has been reported to be present in the intestinal tracts of both humans and animals. *E. coli* and other members of the family *Enterobacteriaceae* has been reported to develop or acquire resistance to a variety of antibiotics by different mechanisms (Suarez *et al.*, 2005; Torres *et al.*, 2007; Woodford *et al.*, 2007).

In children under five years of age, diarrhoeal disease was reported to be the second leading cause of death that is second only to pneumonia (Kosek *et al*., 2003). The specific mortality of diarrhoea in children younger than five years of age in Africa was estimated to be about 106 per 1000 (Olowe et al., 2003). In Nigeria, more than 315,000 deaths of preschool age children were recorded annually as a result of diarrhoea disease (Babaniyi, 1991; Alabi et al., 1998). Diarrhoea

has been reported to be the second major cause of morbidity among common diseases with more than 300 children dying each day in Nigeria (Ogunsanya *et al*, 1994).

E. coli is one of the main causes of both nosocomial and community acquired infections in humans and is therefore an organism of clinical importance in the lives of children (Karlowsky *et al*., 2004). Diarrhoea disease majorly caused by *E. coli* through water, fresh fruits and faecal-oral contamination, was estimated to kill about 1.8 million people each year (WHO, 2005).

A lot of work has been done on diarrhoea in the world, Africa and in Nigeria, particularly in the Southwestern part of Nigeria. Few documentations are made available in the Northern part of this country concerning the study of *E. coli* in diarrhoeic stools. This therefore brought the need for this study so as to investigate, and to document the prevalence and antibiotic susceptibility of *E. coli* from diarrhoeic stools of children below 5years in Zaria, Nigeria.

1.3 Justification of the Research

The societal knowledge of *E. coli* and its relation to enteric infection is poor. Childhood diarrhoea disease is still a major health problem, especially in developing countries, where it is considered as one of the foremost causes of death in children, accounting for approximately 2 million deaths each year worldwide (Kosek *et al*., 2003; Bryce *et al*., 2005).

Akinyemi *et al*., (1998) reported a high incidence of *E. coli* in children and adult patients with acute gastrioenteritis in Lagos, Southwestern part of Nigeria. Nwanze *et al.,* (2010) reported the antibiotic susceptibility patterns of *E. coli* obtained from diarrhoeal stools from Federal Medical Centre, Owerri, Southeastern part of Nigeria. In his report he stated that the incidence of diarrhoea was more common in males than females, and more prevalent in the age group of 0-

5years. Also that isolates were highly resistant to tetracycline, cotrimoxazole, ampicillin and amoxicillin but sensitive to nitrofurantoin and ofloxacin.

Ifeanyi *et al.,* (2010) also reported *E. coli* as the most frequently isolated bacteria (62.8%) in all the age groups studied in the Federal Capital Territory Abuja, North central part of Nigeria and documented that *E. coli* was significantly associated with diarrhoea at age of 13-24 months.

There is also a report by Chigor *et al.*, (2010) on multi drug resistance and plasmid patterns of *E. coli* 0157 and other *E. coli* isolated from diarrhoeal stool and surface waters from selected sources in Zaria, Nigeria who reported the detection of *E. coli* 0157 in surface waters was 2.2% and its prevalence in children with diarrhoea was 5.4%. Also that the most active antibiotics were gentamicin, chloramphenicol and fluoroquinolones. This report and a few others did not make the prevalence of *E. coli* infection in Zaria clear.

The influence of traditional and cultural practices of some people in this locality, like cattle rearing, use of surface water for drinking, herd watering and fresh produce irrigation, the frequent occurrences of flood in addition to the use of pit latrines and digging of wells close to latrines, septic tanks or sewage systems may increase the risk of diarrhoea in this locality.

The sale of antibiotics in the open market without prescription may be a good factor to compound antibiotic resistance in Zaria leading to selective pressure and the emergence of antibiotic resistant strains. This study is thus designed to highlight the presence of *E. coli* as a common pathogen in the stool of diarrhoeic children, by isolation and determination of its antibiotic susceptibility pattern. There is also the need to document the antibiotic susceptibility profiles and resistant patterns of *E. coli* in this locality so as to be sure that the prescriptions currently given are effective in the treatment of infections.

1.4 Aim of the study

Isolation of *E. coli* from diarrhoeic stools of children below five years and determination of their antibiotic susceptibility profiles with the aim of limiting the incidences of occurrence in this locality.

1.5 Specific objectives

1. Sample collection, isolation and identification of *E. coli* from stools of children under 5 years in selected hospitals in Zaria, Nigeria.
2. Determination of the susceptibility profiles of the *E. coli* isolates to selected antibiotics commonly prescribed in the environment using standard procedures.
3. Determination of the minimum inhibitory concentration of selected antibiotics against the *E. coli* isolates using the agar dilution method.
4. Determination of transfer of resistance factors by conjugation studies and curing with acridine dye.
5. Isolation and classification of plasmid DNA using agarose gel electrophoresis technique and polymerase chain reaction.

1.6 Hypothesis

Isolated *Escherichia coli* is highly susceptible to antibiotics prescribed in this locality and there is no plasmid-mediated resistance among the multi-drug resistant isolates in Zaria.

1.7 LIMITATIONS

The children who were below 5 years of age, who did not present with diarrhoea in the three study areas within the period of July, 2010 to March, 2011 were not considered for the study.

Older children above 5 years and children with histories of use of regular antibiotics for at least five days before presentation were excluded from the study.

Inability to carry out serological studies and lack of facilities for further molecular studies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diarrhoeal diseases

2.1.1 Defination, classification and distribution

Diarrhoea has been defined as the passage of three or more loose or liquid stools per day or more frequent passage than is normal for the individual (WHO, 2009). Diarrhoea that occurs due to infection of the intestinal tract, has been ascribed to a variety of bacterial, viral and parasitic organisms. This infection has been reported to be transmitted through contaminated food or drinking-water, or from person-to-person as a result of poor hygiene (WHO, 2012). There are three reported clinical types of diarrhoea namely:

Acute watery diarrhoea which lasts several hours or days, but less than two weeks.

Persistent diarrhoea which lasts between 2 and 4 weeks and

Chronic diarrhoea which lasts greater than 4 weeks in duration (WHO, 2009).

Diarrhoea infection has been frequently reported to be associated with shortage of clean water for drinking, cooking and cleaning (WHO, 2009). Children who die from diarrhoea are often known to suffer from underlying malnutrition, which makes them more vulnerable to diarrhoea. Each diarrhoeal episode, in turn, makes their malnutrition even worse. Diarrhoea is a leading cause of malnutrition in children below five years (WHO, 2012). Water contaminated with human faeces, for example, from sewage, septic tanks and latrines, is of particular concern in diarrhoeal infection. Animal faeces has also been reported to contain microorganisms that can

cause diarrhoea (WHO, 2009). Diarrhoeal disease can also be spread from person-to-person, aggravated by poor personal hygiene. Food has been reported to be another major cause of diarrhoea when it is prepared or stored in unhygienic conditions. Fish and seafood from polluted water may also contribute to the disease (WHO, 2009).

Globally, 21% of all deaths in children under five years of age are estimated to be due to diarrhoeal infections (Kosek *et al*., 2003). In recent years, studies from several developing countries have shown that diarrhoeal diseases also cause considerable lasting disabilities both in physical growth and fitness, and in cognitive skills and school performance (Guerrant *et al*., 2002). Disabilities linked to diarrhoeal diseases in under developed and developing countries have been estimated to account for 58.7 million years of lost healthy life (termed Disability Adjusted Life Years, DALYs) (Mathers *et al* ., 2007).

2.1.2 Causes of diarrhoea diseases

A number of microorganisms, including a variety of viral, bacterial, and protozoan agents are reported as causative agents of diarrhoeal disease. Bacterial diarrhoeagenic agents include *Salmonella spp., Shigella spp., Yersinia enterocolitica, Campylobacter spp.*, and *Vibrio spp.,* including *Vibrio cholera,* and different strains of *Escherichia coli* (Alam and Asraf., 2003; Guerrant *et al*., 2004). In addition, enterotoxin producing variants of *Bacillus cereus, Clostridium perfringens, and Staphylococcus aureus* cause gastroenteritis. *Clostridium difficile* associated diarrhoea is commonly seen in relation to prolonged antibiotic therapy (Afset , 2007).

Other species like *Aeromonas spp.* and *Plesiomonas spp.* may more rarely be responsible for diarrhoeal symptoms. The two best known protozoan agents that have been reported to be associated with diarrhoea are *Giardia lamblia*, and *Entamoeba histolytica,* but *Cryptosporidium parvum, Cyclospora, cayetanensis, Isospora belli and microsporidia* may also cause such disease (Afset , 2007). Viral agents that have been reported to cause diarrhoea in children are rotavirus, norovirus and enteric adenovirus. Others are caliciviruses, astroviruses and enteroviruses (Cooke, 2010).

2.1.3 Prevention of diarrhoea

Key measures to prevent diarrhoea include:

Access to safe drinking-water

Improved sanitation

Exclusive breastfeeding for the first six months of life

Good personal and food hygiene

Health education about how infections spread

Rotavirus vaccination (WHO, 2009).

2.1.4 Management of diarrhoea

Key measures to treat diarrhoea include the following.

Rehydration: with intravenous fluids in case of severe dehydration or shock and/or oral rehydration salts (ORS) solution for moderate or no dehydration. ORS is a mixture of clean water, salt and sugar, which can be prepared safely at home. It is quite cheap and affordable. ORS is absorbed in the small intestine and replaces the water and electrolytes lost in the faeces.

Zinc supplements: zinc supplements reduce the duration of a diarrhoea episode by 25% and are associated with a 30% reduction in stool volume.

Nutrient-rich foods: the vicious circle of malnutrition and diarrhoea can be broken by continuing to give nutrient-rich foods – including breast milk – during an episode, and by giving a nutritious diet – including exclusive breastfeeding for the first six months of life – to children when they are well.

Consulting a health worker if there are signs of dehydration (WHO, 2009).

Table 2. 1: Indications for Systemic Antibiotics in diarrhoea infections.

Infection	Treatment
Bacterial gastroenteritis complicated by sepsis in neonates and	Usually ampicillin and gentamicin OR ceftriaxone
Ill immunocompromised children with associated infection e.g. urinary tract infection or pneumonia	Nalidixic acid
Dysentery due to *Shigella, Salmonella* spp (non-typhoid)	Fluoroquinolone or ceftriaxone
Campylobacter (First try to exclude ETEC due to the associated with HUS)	Erythromycin or one of above
Amoebiasis	Metronidazole
Giardiasis	Metronidazole
Vibrio cholera if complicated by severe dehydration	Doxycycline(> 6 years) or fluoroquinolone
C. difficile	Metronidazole or oral vancomycin

ETEC = Enterotoxigenic *E. Coli*, HUS = Haemolytic Uraemic Syndrome

Cooke, M.L., 2010.

2.2 *ESCHERICHIA COLI*

2.2.1 General characteristics

The scientific history of *Escherichia coli* (*E. coli*) started with its first description in 1885 by Theodor von Escherich (Gross and Rowe, 1985), a pediatrician and scientist, who in a series of pioneering studies of the intestinal flora of infants, discovered a normal microbial inhabitant of healthy individuals that he named *Bacterium coli commune.* Its actual name became used and officially accepted in 1958, in his honour (Kuhnert *et al.*, 2000).

Escherichia coli is a member of the bacteria family Enterobacteriaceae (Holmes and Gross, 1995). It was first isolated from faeces and named "Bacterium coli commune" in 1885 by Theodor Escherich, but renamed *Escherichia coli* in 1920 by Castellani and Chalmers (Holmes and Gross, 1995).

As described by Ewing (1986) and Holmes and Gross (1995), *E. coli* is an aerobic Gram negative asporogenous rod and usually motile by peritrichous flagella. Some strains, especially those from extra intestinal infections possess a polysaccharide capsule. Optimum temperature for growth of *E. coli* is 36-37°C with most strains growing over the range of 18-44°C in 18 hours.

E. coli produces 1-4 mm diameter colonies after overnight incubation. The colonies may appear mucoid and some strains are haemolytic. Colonies are generally circular, low convex, smooth and colourless (on nutrient agar), rose-pink (on MacConkey's agar), rose pink or red with metallic sheen (on both eosin-methylene blue and endo-agar) colonies. Blood agar is discoloured around the growth, accompanied by haemolysis in some cases. It produces both acid and gas

from fermentable carbohydrates such as lactose, glucose and sucrose. Most strains ferment lactose promptly and some slowly while few do not altogether (Holmes & Gross, 1995).

Indole is usually produced (both at 37°C and 44°C) but it is unable to utilize urea and citrate. It gives positive methyl red (MR) and negative Voges-Proskauer (VP) reactions. Most strains do not produce hydrogen sulphide gas detectable in triple sugar iron (TSI) or Kligler iron (KI) agar but decarboxylate lysine and ornithine; gelatin is not liquefied, phenylalanine is not deaminated and gluconate not oxidised. The guanosine cytosine content is 50-5l mol% (Ewing ,1986).

The species *Escherichia coli* comprises Gram-negative, rod-shaped, non-spore forming, motile bacteria which are about 2 μm long and 0.6 μm in diameter, with a cell volume of 0.6-0.7 μm3 (Kubitschek, 1990; Darnton *et al.*, 2007). They are facultative anaerobes, oxidase-negative, glucose, lactose and sucrose fermenting, with an optimum growth pH of 6.0-7.0 and temperature 37°C. However, some laboratory strains can multiply at temperatures up to 49°C (Fotadar *et al.*, 2005).

Taxonomically, *E. coli* belongs to the family *Enterobacteriaceae*, and it is a commensal bacterium residing as the most common and predominant inhabitant in the intestinal microflora of human and other mammals (Hill and Drasar, 1975; Nataro and Karper, 1998). However, the establishment of the intestinal *E. coli* flora, is rather a complex process influenced by microbial and host interactions, or by internal and external factors that can have a substantial influence on the prevalence and density of *E. coli*, e.g., delivery mode, and feeding habits, life-style, environment and immunological status (Penders *et al.*, 2006; Adlerberth, 2008; Adlerberth and Wold, 2009). *E. coli* is one of the main causes of both nosocomial and community acquired infections in humans. The organism is therefore of clinical importance and can be isolated from

some specimens. It is one of the organisms most frequently isolated from blood (Karlowsky *et al*., 2004).

2.2.2 Serotypes of *E. coli*

Serotyping was for many years the only tool available to identify pathogenetic variants of *E. coli*. This method, established by Kauffmann in the 1940s, is based on heat-stable lipopolysaccharide O antigens and heat-labile flagellar H antigens. Kauffmann also described other heat-labile capsular K antigens [reviewed by Cheasty and Smith in (Cheasty *et al*., 2005)]. Initially about 174 O13 serogroups and 53 H antigens were described (Gross & Rowe., 1985; Scheutz *et al*., 2004).

Now, over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. At one time, serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing haemolytic uremic syndrome (HUS). Nowadays, particularly for diarrhoea gene strains (those that cause diarrhoea) pathogenic *E. coli* are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers (Todar, 2008).

2.2.3 Pathogenesis of *Escherichia coli*

E. coli may cause various types of intestinal and extraintestinal infections. Of extraintestinal infections, urinary tract infections (UTIs), where *E. coli* is the single most prevalent pathogen, are among the most common infections both in the community and in hospitals (Hooton *et al* ., 1997; Stamm & Hooton., 1996). *E. coli* strains which cause UTIs often have specific virulence

factors, while strains without such virulence factors, termed commensal strains, more rarely cause this type of infection. *E. coli* is also one of the most common infectious agents isolated from patients with bacteraemia and sepsis (Fluit *et al*., 2001), and one of the leading causes of neonatal meningitis (de Louvois ., 1994; Klinger *et al*., 2000). In addition, commensal *E. coli* may cause infections in nearly any body site due to an aggravating factor such as a foreign body or host compromise (Russo *et al* ., 2003).

Virulence factors which make certain strains of *E. coli* pathogenic may be classified in two groups according to function (Kaper *et al.*, 2004). One of these consists of colonization and fitness factors making the bacterium able to colonize and survive on the mucosal surface or, more rarely, to invade the host. The second group consists of toxins and effectors which may induce damage to the host. Some of the virulence factors also help the bacterium evade the host immune defences. Depending on their virulence profile *E. coli* strains are classified in a number of extraintestinal and diarrheagenic pathotypes (Kaper *et al*., 2004).

On the whole, they are each encoded by specific genetic elements (plasmids or chromosomal genes) that determine pathogenicity. Based on these, six different strains of *E. coli* have been identified (Todar, 2008) and they are as follows:

- Enteropathogenic *E. coli* (EPEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enteroinvasive *E. coli* (EIEC)
- Enterohemorrhagic *E. coli* (EHEC)
- Enteroaggregative *E. coli* (EaggEC)
- Diffusely adhering *E. coli* (DAEC)

EPEC has been known to cause severe epidemics of diarrhoea in neonatal nurseries since the 1920s with very high mortality. About 14 serotypes were epidemiologically associated with neonatal diarrhoea including 055, 0111 and 0119 (Levine *et al.,* 1984; Robins-Browne, 1987). Published results confirmed that designated *E. coli* by serotype was recovered more frequently from sick children than from children from healthy controls (Levine *et al.,* 1984). The presence of a plasmid in EPEC serves to increase intimin production, a process that is needed for localised adherence to occur (Jerse *et al.,* 1991). A probe constructed from this plasmid known as enteropathogenic *E. coli* adherence factor (EAF), has been used to rapidly identify EPEC strains in stools of patients with diarrhoea including children (Jerse *et al.,* 1991). Resistance to antimicrobial drugs is more common in EAF-positive classic EPEC strains (Tardelli *et al.,* 1991).

Acute diarrhoea owing to sensitive strains of enteropathogenic *E. coli* can be treated or prevented with either trimethoprim or trimethoprim plus sulfamethoxazole (Hill and Pearson, 1988). However, antibiotic treatment (either trimethoprim-sulfamethoxazole or cephalosporins) of diarrhoeal illness owing to enterohemorrhagic *E. coli* O157:H7 may increase the risk of haemolytic-uremic syndrome, perhaps by increasing the release of Shiga-like toxin by the bacteria (Wong *et al.*, 2000).

ETEC, which was first studied in India but now worldwide, possess pili which are capable of hemagglutination in the presence of mannose. Basically there are 2 types of toxins produced by them designated as:

- Heat-labile toxin (LT) 80,000 Daltons
- Heat-stable toxin (ST) 4500 Daltons.

LT has similarity to cholera toxin but ST activates guanylate cyclase and has no biochemical similarity to cholera toxin. ST however is a family toxin and the most common forms that cause disease in humans are ST1a and ST1b. ETEC strains can elaborate either LT or ST only or both forms of toxins (Mckenzie *et al.,* 2006).

EIEC was originally described in Asia and is a rare cause of the dysentery syndrome. It is similar to Shigella by being invasive and able to produce a shiga-like toxin. Its isolation rates are low and its diagnosis in a routine bacteriologic laboratory is difficult (Todar, 2008).

EHEC, first described in Michigan and Oregon (USA) in 1982, (Riley *et al.,* 1983) causes acute hemorrhagic colitis; specifically the serotype 0157:H7. It causes about 0.6 to 2.4% of all cases of diarrhoea and 15 to 36% of cases of hemorrhagic colitis in the UK and US (Riley *et al.,* 1983).

EAggEC appears like "stacked-brick" pattern on the cell surface. It is a cause of acute (<2 weeks) and persistent (2 to 4 weeks) diarrhoea in children in developing countries. (Crarioto *et al.,*1991).

DAEC also has been identified with diarrhoea in children in developing countries and appears to be more pathogenic in older children (Gunzberg *et al.,* 1993).

2.2.4 Public health importance of *E. coli*

2.2.4.1 Faecal contamination

Public and environmental health protection requires the availability of safe drinking water, which means that it must be free of pathogenic bacteria. Determining the source of faecal contamination in aquatic environment is essential for estimating the health risk associated with pollution, and for facilitating measures to remediate polluted waterways (Blanch *et al.*, 2006). *E. coli* constitute a part of the intestinal microflora of human and warm-blooded animals, and

survives long enough in the different aquatic environments, and are easily isolated, enumerated and identified. It has thus been used as an indicator of faecal contamination (Whitlock *et al.*, 2002; McQuaig *et al.*, 2006), and also to determine the quality and safety of water for consumption worldwide. This procedure, recommended by the U.S. Environmental Protection Agency (EPA), is based on epidemiological studies that demonstrate a direct relationship between the density of *E. coli* organisms in water and the occurrence of swimming-associated gastroenteritis (WHO, 2003). Furthermore, this common organism may contribute to the dissemination of antibiotic resistant microorganisms between human and animal populations, and it may also constitute the route by which resistance genes are introduced in environmental bacterial ecosystems (Kim and Aga, 2007; Kummerer, 2009).

2.2.4.2 *E.coli* as an indicator for antimicrobial resistance

Microbial resistance to antibiotics is an increasing public health problem worldwide; since administration of antibiotic agents causes disturbances in the ecological balance between host and microorganisms (Sullivan *et al.*, 2001; Paterson and Bonomo, 2005; Pallecchi *et al.*, 2007), and may promote the emergence of increased number antibiotic-resistant strains which may lead to more severe infections. *E. coli* and other members of the family *Enterobacteriaceae* are well known to develop or acquire resistance to a variety of antibiotics by different mechanisms. However, production of β-lactamases is the most common and clinically significant mechanism of resistance among this bacterial group (Suarez *et al.*, 2005; Torres *et al.*, 2007; Woodford *et al.*, 2007). Accordingly, the intestinal *E. coli* microflora may provide an important reservoir for antibiotic-resistant bacteria, and resistance genes, which may be transmitted further to potentially pathogenic bacteria (Pallecchi *et al.*, 2007). Thus, the ecological impact of different

antimicrobial agents, as well as the development of antimicrobial resistance before it appears in pathogenic strains and in clinical infections, could be studied in the intestinal *E. coli* flora**.**

2.2.4.3 Emerging pathogen with potential to spread virulence

Although *E. coli* strains are termed commensals and part of the normal intestinal microflora of human and warm-blooded animals, they may cause diseases under certain circumstances (Kaper *et al.*, 2004). Diseases reported to be caused by *E. coli* strains are either as a result of specific or non-specific infections. Unspecific infections may occur where the non-pathogenic, commensal *E. coli* strain become harmful, because of the fact that the host immune system is weak, e.g., in preterm-newborn infants, elderly, malnourished and immunocompromised individuals (Kaper *et al.*, 2004). Specific infections are caused by some subsets of *E. coli* strains that represent a versatile and diverse group of microorganisms with several highly adapted clones. These strains have been reported to have acquired specific virulence factors, which confer on them the ability to adapt to new environments and make them capable of causing a wide range of infections in healthy individuals (Kaper *et al.*, 2004).

Interestingly, most of these virulent attributes that distinguish pathogenic *E. coli* from commensals are frequently reported to be encoded on mobile genetic elements such as plasmids, bacteriophages, and transposons that can be mobilized into different strains creating new and successful combinations of virulence factors (Dobrindt *et al.*, 2002; Dobrindt *et al.*, 2003).

Moreover, these novel combinations may be integrated into the genome resulting in a more stable virulence clone to become specific phenotype of *E. coli* capable of causing intestinal and extraintestinal disease (Dobrindt *et al.*, 2002; Dobrindt *et al.*, 2003; Kaper *et al.*, 2004).

2.3 Antibiotic therapy

Bacterial infections are usually treated with antibiotics. However, the antibiotic sensitivities of different strains of *E. coli* vary widely. As Gram-negative organisms, *E. coli* has been reported to be resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which have been reported to be used to treat *E. coli* infection include amoxicillin, as well as other semisynthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides. The high level of water loss with diarrhoea has necessitated the need for fluid replacement with solutions containing appropriate electrolytes.

2.3.1 Penicillins and Cephalosporins

The β-lactam antibiotics are useful and are frequently prescribed antimicrobial agents with similar structure and mechanism of action (Petri, 2006).

2.3.1.1 Mechanism of action of the penicillins and cephalosporins

The β-lactam antibiotics mechanism of action has been incomplete, but numerous researchers have supplied information that allows understanding of the basic antibacterial phenomenon (Ghuysen, 1991; Bayles, 2000). The cell wall of bacteria has been reported to contain heteropolymeric component of peptidoglycan which provides rigid mechanical stability by virtue of its highly cross-linked lattice work structure. The last step of peptidoglycan synthesis of the bacterial cell wall has been reported to be inhibited by the β-lactam antibiotics (Petri, 2006).

Although inhibition of the transpeptidase enzyme has been ascribed as the major active site, there are other reported additional target sites for penicillins and cephalosporins, called penicillin binding proteins (PBPs). All bacteria have been reported to possess several PBPs entities; for example, *S. aureus* and *Escherichia coli* have been documented to have four and seven PBPs respectively. The PBPs affinities for different β-lactam antibiotics varies, though the interactions eventually become covalent. The higher-molecular-weight PBPs of *E. coli* (PBPs 1a and 1b) include the transpeptidases which has been reported to be responsible for the synthesis of the tetrapeptides of the peptidoglycan. Other PBPs in *E. coli* have been reported to be responsible for maintenance of the septum formation during bacterial cell division. Inhibition of the transpeptidase enzyme has been reported to cause spheroplast formation and subsequent rapid lysis of the bacteria cells. However, inhibition of the activities of other PBPs have been reported to delay cell lysis (PBP 2) or to enhance the production of long, filamentous forms of the bacterium (PBP 3). The lethality of penicillin for bacteria appears to involve both lytic and nonlytic mechanisms. Penicillin's disruption of the balance between PBP-mediated peptidoglycan assembly and murein hydrolase activity results in autolysis. Non-lytic killing by penicillin may involve porin-like proteins in the bacterial membrane that collapse the membrane potential (Bayles, 2000).

2.3.2 The cephalosporins

2.3.2.1 Classification of cephalosporins

The classification of cephalosporins by generations is based on general features of antimicrobial activity (Karchmer, 2000). For instance, the first-generation cephalosporins, such as cephalothin and cefazolin, have been reported to have good antibacterial activity against Gram-positive

bacteria and relatively modest activity against Gram-negative microorganisms. Most Gram-positive cocci are susceptible with the exception of enterococci, methicillin-resistant *S. aureus,* and *S. epidermidis*. Most oral cavity anaerobes are shown to be sensitive, except *B. fragilis* group. *Moraxella catarrhalis, E. coli, K. pneumoniae,* and *P. mirabilis* are reported to be susceptible to first generation cephalosporins. The second-generation cephalosporins are documented to have increased activity against Gram-negative microorganisms. Second-generation cephalosporins such as cefoxitin, cefotetan, and cefmetazole has been reported to be active against the *B. fragilis* group. Third-generation cephalosporins are generally reported to be less active than first-generation cephalosporins against Gram-positive cocci, but much more active against the Enterobacteriaceae, including β-lactamase-producing strains. Third-generation cephalosporins such as ceftazidime and cefoperazone are reported to be active against *P. aeruginosa* but less active against Gram-positive cocci. Fourth-generation cephalosporins, such as cefepime, are reported to have an extended spectrum of activity and increased stability from hydrolysis by plasmid and chromosomally mediated β-lactamases. Fourth-generation cephalosporins are reported to be particularly useful for the empirical treatment of serious infections in hospitalized patients when Gram-positive microorganisms, Enterobacteriaceae, and *Pseudomonas* are the etiologies (Petri, 2006).

2.3.3 Aminoglycosides

The aminoglycoside antibiotics includes gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin, and neomycin. These antibiotics are reported to be commonly used to treat infections caused by aerobic Gram-negative bacteria; e.g streptomycin has been widely prescribed for the treatment of tuberculosis. In contrast to most inhibitors of microbial protein

synthesis, which are bacteriostatic, the aminoglycosides are reported to be bactericidal in action. Mutations affecting proteins in the bacterial ribosome, the target for these antibiotics, can confer marked resistance to aminoglycoside action. However, most commonly reported bacterial resistance are acquisition of plasmids or transposon-encoding genes for aminoglycoside-metabolizing enzymes or impaired transport of aminoglycoside into the bacterial cell. (Chambers, 2006).

2.3.3.1 Mechanism of action of aminoglycosides

Aminoglycosides has been known to disrupt the normal cycle of ribosomal function by interfering, at least in part, with the initiation of protein synthesis, leading to the accumulation of abnormal initiation complexes. (Luzzatto *et al.*, 1969). Aminoglycosides are known to also cause misreading of the mRNA template and incorporation of incorrect amino acids into the growing polypeptide chains. Aminoglycosides vary in their capacity to cause misreading presumably owing to differences in their affinities for specific ribosomal proteins. Although there appears to be a strong correlation between bactericidal activity and the ability to induce misreading (Hummel and Bock, 1989), it remains to be established that this is the primary mechanism of aminoglycoside-induced cell death (Chambers, 2006).

2.3.4 Tetracyclines

Tetracycline is a semi-synthetic derivative of chlortetracycline. Tetracyclines are close congeners of polycyclic naphthacenecarboxamide. They have activity against *Ricketttsia*, gram- positive and gram-negative bacteria, aerobes, anaerobes, and *Chlamydia*. Because of this activities, they

are reffered to as “broad spectrum” antibiotics (Chambers, 2006). Tetracyclines are bacteriostatic antibiotics.

2.3.4.1 Mechanism of action of tetracyclines

Tetracyclines are known to inhibit bacterial protein synthesis by binding to the 30S bacterial ribosome and preventing access of aminoacyl tRNA to the acceptor (A) site on the mRNA-ribosome complex. These antibiotics have been reported to enter gram-negative bacteria by passive diffusion through the hydrophilic channels formed by the porin proteins of the outer cell membrane and by active transport *via* an energy-dependent system that pumps all tetracyclines across the cytoplasmic membrane. Entry of these drugs into Gram-positive bacteria has been reported to require metabolic energy, but the details are not well understood. (Chambers, 2006).

2.3.5 Trimethoprim-Sulfamethoxazole

2.3.5.1 Antibacterial Spectrum of sulfamethoxazole/trimethoprim

The antibacterial spectrum of trimethoprim has been reported to be similar to that of sulfamethoxazole, although the former drug usually is 20 to 100 times more potent than the latter. Most Gram-negative and Gram-positive microorganisms are sensitive to trimethoprim, but resistance has been reported to develop when the drug is used alone. *Pseudomonas aeruginosa, Bacteroides fragilis,* and enterococci usually are reported to be resistant to trimethoprim. There is a significant variation reported in the susceptibility of Enterobacteriaceae to trimethoprim in different geographical locations because of the spread of resistance mediated by plasmids and transposons (Petri, 2006).

2.3.5.2 Mechanism of action of sulfamethoxazole/trimethoprim

The antimicrobial activity of the combination of trimethoprim and sulfamethoxazole has been reported to result from its actions on two steps of the enzymatic pathway for the synthesis of tetrahydrofolic acid. Sulfonamide inhibits the incorporation of *para*-aminobenzoic acid (PABA) into folic acid, and trimethoprim prevents the reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is essential for one-carbon transfer reactions, *e.g.,* the synthesis of thymidylate from deoxyuridylate. Selective toxicity for microorganisms is achieved in two ways. Mammalian cells use preformed folates from the diet and do not synthesize the compound. Furthermore, trimethoprim is a highly selective inhibitor of dihydrofolatereductase of lower organisms: About 100,000 times more drug is required to inhibit human reductase than the bacterial enzyme. This relative selectivity is vital because this enzymatic function is essential to all species (Petri, 2006).

2.3.6 Quinolones

The first quinolone, nalidixic acid, was isolated as a by-product of the synthesis of chloroquine. (Petri, 2006). It has been available for the treatment of urinary tract infections for many years. The introduction of fluorinated 4-quinolones, such as ciprofloxacin , moxifloxacin , and gatifloxacin represents a particularly important therapeutic advance because these agents have broad antimicrobial activity and are effective after oral administration for the treatment of a wide variety of infectious diseases. Relatively few side effects are known to accompany the use of these fluoroquinolones, and microbial resistance to their action does not develop rapidly (Andriole, 1993; Hooper, 2000). Rare and potentially fatal side effects, however, have resulted in the withdrawal from the market of temafloxacin (immune hemolytic anemia), trovafloxacin (hepatotoxicity), grepafloxacin (cardiotoxicity), and clinafloxacin (phototoxicity). In all these

cases, the side effects were so infrequent as to be missed by prerelease clinical trials and detected only by postmarketing surveillance (Sheehan and Chew, 2003).

2.3.6.1 Mechanism of Action of Quinolones

The quinolone antibiotics has been reported to target bacterial DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997). For many Gram-positive bacteria (such as *S. aureus*), topoisomerase IV is the primary activity inhibited by the quinolones. In contrast, for many Gram-negative bacteria (such as *E. coli*), DNA gyrase is the primary quinolone target (Alovero e*t al.*, 2000; Hooper, 2000). The individual strands of double-helical DNA must be separated to permit DNA replication or transcription. However, anything that separates the strands results in "overwinding" or excessive positive supercoiling of the DNA in front of the point of separation. To combat this mechanical obstacle, the bacterial enzyme DNA gyrase is responsible for the continuous introduction of negative supercoils into DNA. This is an ATP-dependent reaction requiring that both strands of the DNA be cut to permit passage of a segment of DNA through the break; the break then is resealed (Petri, 2006).

The DNA gyrase of *E. coli* is composed of two 105,000-dalton A subunits and two 95,000-dalton B subunits encoded by the *gyrA* and *gyrB* genes, respectively (Hooper, 2000). The A subunits, which carry out the strand-cutting function of the gyrase, are the site of action of the quinolones. The drugs inhibit gyrase-mediated DNA supercoiling at concentrations that correlate well with those required to inhibit bacterial growth (0.1 to 10 μg/ml). Mutations of the gene that encodes the A subunit polypeptide can confer resistance to these drugs (Hooper, 2000).

Topoisomerase IV also is composed of four subunits encoded by the *parC* and *parE* genes in *E. coli* (Drlica and Zhao, 1997; Hooper, 2000). Topoisomerase IV separates interlinked (catenated) daughter DNA molecules that are the product of DNA replication. Eukaryotic cells

do not contain DNA gyrase (Petri, 2006). However, they do contain a conceptually and mechanistically similar type II DNA topoisomerase that removes positive supercoils from eukaryotic DNA to prevent its tangling during replication. This enzyme has been reported as the target for some antineoplastic agents.(Goodman and Gilman,2006). Quinolones inhibit eukaryotic type II topoisomerase only at much higher concentrations (100 to 1000 µg/ml) (Mitscher and Ma, 2003).

2.3.6.2 Antibacterial spectrum of quinolones

The fluoroquinolones are reported as potent bactericidal agents against *E. coli* and various species of *Salmonella, Shigella, Enterobacter, Campylobacter,* and *Neisseria* (Eliopoulos and Eliopoulos, 1993).

Resistance to quinolones has been reported to develop during therapy *via* mutations in the bacterial chromosomal genes encoding DNA gyrase or topoisomerase IV or by active transport of the drug out of the bacteria (Oethinger *et al.*, 2000). No quinolone-modifying or -inactivating activities have been identified in bacteria (Gold and Moellering, 1996). Resistance has increased after the introduction of fluoroquinolones, especially in *Pseudomonas* and staphylococci (Pegues *et al.*, 1998; Peterson *et al.*, 1998). Increasing fluoroquinolone resistance also is being observed in *C. jejuni, Salmonella, N. gonorrhoeae,* and *S. pneumoniae* (Centers for Disease Control and Prevention, 1994; Thornsberry *et al.*, 1997; Molbak *et al.*, 1999; Smith *et al.*, 1999). Fluoroquinolones are potent broad-spectrum antimicrobial agents that are increasingly used to treat *E. coli* infection. Despite initial optimism, resistance to these antibiotics has increased significantly since their introduction into medicine and agriculture in the late 1980's and early 1990's. Consequently, the mechanism of resistance in fluoroquinolone-resistant bacteria has

been the subject of intense research and, in recent years, dramatic advances have been made in understanding of these mechanisms.

The primary target of fluoroquinolone action documented is DNA gyrase and topoisomerase IV. The 2 enzymes are tetramers, composed of 2 A subunits and 2 B subunits, encoded by the *gyrA* and *gyrB* genes for DNA gyrase (topoisomerase II) and *parC* and *parE* genes for topoisomerase IV. The mechanism of resistance to fluoroquinolones has been mainly linked to mutations in the quinolone-resistance-determining region (QRDR) of *gyrA* and *parC* genes and, less frequently, in the *gyrB* and *parE* genes.

2.4 Antibiotic Resistance

Antibiotic resistance is a global public health issue that is impacted by both human and non-human antimicrobial usage. The continuing emergence, development and spread of pathogenic organisms that are resistant to antimicrobials are a cause of increasing concern (WHO, 2012). Antimicrobial resistance (AMR) is defined as changes in microbial biology that occur in response to antimicrobials (AM) and that reduce or block the effectiveness of drugs, chemicals, or other agents to cure or prevent infections. Antimicrobial resistance is determined by *in vitro* tests of strain-specific cultures in which survival of the bacterial isolates is tested under conditions of increasing antimicrobial concentrations. The *in vitro* concentration at which bacterial survival is significantly affected is then compared to benchmarks that signify impacts on clinical efficacy; thus the MIC (minimum inhibitory concentrations) values are set by the CLSI (Clinical and Laboratory Standards Institute) to reflect a level of resistance that is likely to compromise the efficacy of antimicrobial treatment in an infected patient (Ginocchio 2002; CLSI 2006).

Sir Alexander Fleming, the father of antibiotics, described the phenomena of antibiotic resistance and suggested in the 1940s that extensive use of antibiotics would cause bacteria to develop resistance, and further pointed out that new antibiotics would be necessary to combat this on a regular basis (Todar, 2009).

In some cases, selection for resistance also results in more virulent strains, as in the case of *E. coli* and *S. aureus* (Ohlsen *et al*., 1998; Johnson *et al*., 2005; Mora *et al*., 2005; Stevens *et al*., 2007).

2.4.1 Origin of Antimicrobial Resistance

Bacteria acquired the genetic and physiological tools to resist antimicrobials long before scientists isolated and identified these natural agents in the early 20th century (Wright, 2007). Because many antimicrobials are natural products of fungi and other organisms, bacteria have evolved these mechanisms over millions of years, and thus it is possible to detect antimicrobial resistance in bacteria that have not been exposed to our uses of antimicrobials in medicine (Levy, 1998) . Antimicrobial resistance was evident from the early history of penicillin. The isolation of the first naturally occurring antimicrobial (penicillin) in the 1930s ushered in a major change in clinical and veterinary medicine (Todar, 2009). With the discovery of natural antimicrobial compounds, the balance seemed at last to be tipped against the pathogens, but victory was short-lived. Even in the laboratory, Fleming observed that his bacterial cultures quickly demonstrated resistance to penicillin, and in 1945, he warned that the misuse of penicillin could lead to selection of resistant forms of bacteria (Levy, 1998).

Fleming suggested that resistance to penicillin could be conferred in two ways—either through

changes in the bacterial cell wall, which was the target of penicillin action, or through the selection of bacteria expressing mutant proteins capable of degrading penicillin. Unfortunately, in the early period of its use, penicillin was available orally to the public without prescription until the mid 1950s (Silbergeld, 2007). By 1946, one hospital reported that 14% of the strains of staphylococci isolated from sick patients were penicillin resistant (Barber *et al*. 1949). By the 1950s, this same hospital reported that 59% of the strains of staphylococci were penicillin resistant (Levy, 1998). There are major cost impacts on the health care system to monitor and prevent spread of resistant infections, which have not been fully calculated (Laxminarayan 2007). From time immemorial, animals have been the source of some of the pathogens that can cause diseases in human populations (Orriss, 1997; Fevre, *et al*. 2006).

Microbes have evolved highly effective mechanisms to respond to environmental pressures, such as temperature change, oxygen concentrations, nutrient availability, and toxin exposure, including antimicrobial agents. Thus, exposure of bacteria to sublethal concentrations of antimicrobial inevitably results in the selection of resistant strains, and under conditions of continued antimicrobial pressure, resistant strains will propagate and spread(Silbergeld, 2007). Because most antimicrobials are derived from natural products, bacteria have acquired, through evolution, biochemical responses to resist antimicrobial attack; and antibiotic resistance can be observed in the absence of any deliberate human use of antibiotics. Because of the speed of bacterial reproduction, these changes can be expressed with great efficiency. Thus, through an evolutionary process of microbial response to the pressure of antimicrobial agents, resistance is an inevitable consequence of antimicrobial use, and it is not surprising that observations of resistance came soon after the identification and isolation of the first natural antimicrobial substance (Silbergeld, 2007).

Nongenetic changes typically involve enhanced activity or upregulation of physiological processes such as membrane transport pumps that extrude harmful agents, including antimicrobials. However, the capacity of these response mechanisms is limited, and usually bacteria express only low-level resistance as a consequence (Wright, 2007; Todar, 2009).

Genetically encoded changes are more serious because these can usually confer higher-level resistance to specific agents and they can be transferred among bacteria. Since most antimicrobials are derived from fungal and other natural sources, bacteria have evolved in the presence of these toxins (Wright, 2007), and it is therefore not surprising that, even in the absence of antimicrobial pressure, there are sources of resistance encoded by specific genes within the community repertoire of bacterial genomes (Silbergeld, 2007). In the presence of selection pressure by an antimicrobial, bacterial populations quickly evolve to a resistant phenotype (Smith *et al*., 2002; Tenover, 2006). The speed of this evasion process is hastened by two factors: the rapid rate of bacterial reproduction and the ability of bacteria to transfer genetic information among organisms even across broad phylogenetic categories (Silbergeld, 2007).

2.4.1.1 Resistance spread

Resistance is a trait expressed by specific bacteria and can result from new mutations that occur spontaneously in bacteria due to their rapid rate of cell division or from the selection of resistance genes already present within a bacterial colony (Silbergeld, 2007). In the presence of antimicrobial pressure, strains that express resistance traits through spontaneous mutation are favored in terms of survival, and they will rapidly supplant susceptible strains in microbial populations (Nwosu, 2001).

But in addition, and potentially of greatest significance for public health, bacteria have a third mechanism of rapid evolution towards a resistant phenotype through the sharing of genes that

encode resistance. By this process, resistance can be propagated within and among bacterial strains, species, and genera, including commensals (nonpathogenic) and pathogens, by mobile genetic elements including plasmids, transposons, integrons, gene cassettes, and bacteriophages. In contrast to chromosomal based resistance determinants, these transfers account for more than 95% of antibiotic resistance (Nwosu, 2001).

These events have been detected in resistant *E. coli* isolated from consumer meat products (Sunde *et al*., 2006). This finding is of particular concern because integrons can transfer multiple resistance genes at a time (Zhang *et al*., 2003; Zhang *et al*., 2006).

Bacteria operate at the community level in terms of responding to stress, and therefore they have developed mechanisms to share genetic information, often across broad species divisions. Because it is the community response that is crucial (Summers, 2002; Heuer *et al*. 2006), genetic change in response to antimicrobial pressure is not dependent upon reproduction or cell division, as is the case for most higher organisms. Bacteria can exchange genetic information across broad classes by several mechanisms, these mechanisms are in many cases enhanced by stressors such as pressures that can enhance the rates and efficiency of genetic recombination (Summers, 2002; Heuer *et al*., 2006).

2.4.1.2 Persistence of resistance

The microbial community can serve as a resource, or reservoir, of resistance genes. Earlier theories of microbial genetics assumed that this was unlikely to be a long term phenomenon since the expression of resistance was thought to cost the organism (in terms of increased energy requirements, susceptibility to other stressors, or decreased reproductive rates) such that in the absence of antimicrobial pressure, its occurrence was rare. Current research has cast doubt on the

concept of a "cost of resistance" (Salyers and Amabile-Cuevas 1997). While the prevalence of resistant strains markedly decreases when antimicrobials are no longer present, this is not always the case for several reasons. Resistance may persist due to the clustering of resistance genes on the same transposable elements such that eliminating only one antimicrobial may not reduce the prevalence of the cluster (Aarestrup *et al*., 2000). Also it may in some cases be "cheaper" for a resistant bacterial strain to acquire an additional genetic change that reduces the biological cost of resistance rather than to revert genetically and phenotypically to the "wild" or susceptible state (Levin *et al*., 2000; Wright, 2007).

2.4.2 Mechanisms of bacterial resistance to penicillins and cephalosporins

Although all bacteria has been reported to possess PBPs, some bacteria are reported to display intrinsic resistance to β-lactam antibiotics because of structural differences in their PBPs. (Petri, 2006). The resistant bacteria are known to develop high-molecular-weight PBPs that have decreased affinity for the antibiotic. Bacterial resistance to the β-lactam antibiotics has been reported to be due to the inability of the agent to penetrate bacterial cell walls to its active site of action (Jacoby, 1994). Active efflux pumps has been cited as another mechanism of resistance, removing the antibiotic from bacterial cytoplasm before it can act on the target site. (Nikaido, 1998).

Bacteria has also been reported to destroy β-lactam antibiotics enzymatically. β-Lactamases reported present in some bacteria are capable of inactivating β-lactam antibiotics. Different microorganisms are reported to elaborate a number of distinct β-lactamases, although most bacteria produce only one form of the enzyme. The substrate specificities of some of these enzymes are relatively narrow, and these are often described as either penicillinases or

cephalosporinases. Other reported "extended spectrum" enzymes are less discriminant and are known to hydrolyze a variety of β-lactam antibiotics. β-Lactamases are grouped into four classes according to Bush classification: Class A β-lactamases include the extended-spectrum β-lactamases (ESBLs) and has been shown to degrade penicillins, cephalosporins, and, in some instances, carbapenems. Class A and D β-Lactamase enzymes are known to be inhibited by the commercially available β-lactamase inhibitors, such as clavulanate and tazobactam. Class B β-lactamases are reported to be Zn^{2+}-dependent enzymes that destroy all β-lactams except aztreonam, whereas class C β-lactamases are active against cephalosporins. Class D includes cloxacillin-degrading enzymes (Bush, 2001).

In Gram-negative bacteria, such as *E. coli,* β-lactamases are reported to be found in relatively small amounts and located in the periplasmic space. Since the enzymes of cell wall synthesis are on the outer surface of the inner membrane, these β-lactamases are strategically located for maximal protection of the microbe. β-Lactamases of Gram-negative bacteria are encoded either in chromosomes or in plasmids, and they may be constitutive or inducible. The plasmids can be transferred between bacteria by conjugation. These enzymes can hydrolyze penicillins, cephalosporins, or both (Davies, 1994).

2.4.3 Mechanisms of bacterial resistance to the cephalosporins

Resistance to the cephalosporins has been related to the inability of the antibiotic to reach its sites of action or to alterations in the penicillin-binding proteins (PBPs) that are targets of the cephalosporins and bacterial enzymes such as β-lactamases known to hydrolyze the β-lactam ring, thereby inactivating the cephalosporin activity (Spratt, 1994). The most prevalent reported mechanism of bacterial resistance to cephalosporins is destruction of the cephalosporins by

hydrolysis of the β-lactam ring. Although Gram-negative bacteria seem to produce less β-lactamase, the location of their enzyme in the periplasmic space may make it more effective in destroying cephalosporins as they diffuse to their targets on the inner membrane, as is the case for the penicillins. The cephalosporins have variable susceptibility to β-lactamase. Cefoxitin, cefuroxime, and the third-generation cephalosporins are reported to be more resistant to hydrolysis by the β-lactamases produced by Gram-negative bacteria than first-generation cephalosporins. Third-generation cephalosporins are susceptible to hydrolysis by inducible, chromosomally encoded (type I) β-lactamases. Cefuroxime has been reported to have similar antibacterial activity compared to loracarbef with broader activity against some *Citrobacter* and *Enterobacter* spp (Schaad *et al*., 1990).

2.4.4 Mechanism of bacterial resistance to the Tetracyclines

Bacterial resistance to tetracyclines has been reported to be plasmid-mediated and it is often inducible. There are three main reported resistance mechanisms, namely: (1) decreased accumulation of tetracycline as a result of either decreased antibiotic influx or acquisition of an energy-dependent efflux pathway; (2) production of a ribosomal protection protein that displaces tetracycline from its target, a "protection" that also may occur by mutation; and (3) enzymatic inactivation of tetracyclines. Cross resistance, or lack thereof, among tetracyclines depends on which mechanism is operative. For example, *S. aureus* strains that are tetracycline-resistant on the basis of efflux mediated by *tetK* has been known to be susceptible to minocycline. Tetracycline resistance due to a ribosomal protection mechanism (*tetM*) was reported to produce cross-resistance to doxycycline and minocycline because the target site protected is the same for all tetracyclines (Chambers, 2006).

The glycylcyclines are synthetic analogues of the tetracyclines, i.e the 9-tert-butyl-glyclyamido derivative of minocycline, tigecycline. The glycylcyclines has been reported to exhibit antibacterial activities against tetracycline-resistant organisms containing genes responsible for efflux mechanisms or ribosomal protection. The glycyclcyclines has also been reported to be active against other resistant pathogens including methicillin-resistant *S. aureus* and *S. epidermidis*, penicillin-resistant *S. pneumoniae*, and vancomycin-resistant enterococci (Chambers, 2006).

2.4.5 Bacterial Resistance to sulfamethoxazole/trimethoprim

Bacterial resistance to trimethoprim-sulfamethoxazole is a rapidly increasing problem, although resistance is lower than it is to either of the agents alone (Petri, 2006). Resistance often is due to the acquisition of a plasmid that codes for an altered dihydrofolate reductase. The development of resistance is a problem for the treatment of many different bacterial infections. In a survey of children with otitis media in Memphis, Tennessee, 29% of isolates were penicillin-resistant, and 25% of these also were resistant to trimethoprim-sulfamethoxazole (Centers for Disease Control and Prevention, 1994). In another survey, approximately 50% of *Shigella sonnei* isolates from The Netherlands was resistant (Voogd *et al.*, 1992). Emergence of trimethoprim-sulfamethoxazole-resistant *S. aureus* and Enterobacteriaceae is a special problem in AIDS patients receiving the drug for prophylaxis of *Pneumocystis jiroveci* (formerly called *Pneumocystis carinii*) pneumonia (Martin *et al.*, 1999).

2.5 Extended Spectrum Beta Lactamase Production

Production of ß-lactamase is a major means by which Gram-negative bacteria exhibit resistance to ß-lactam antibiotics. Extended-spectrum ß-lactamases (ESBLs) are a group of such enzymes that can hydrolyze a variety of ß-lactams including cephalosporins (e.g. ceftazidime, cefotaxime and ceftriaxone) and monobactam (aztreonam) in addition to penicillins. Most of them also have the ability to hydrolyze fourth-generation cephalosporins (e.g. cefepime and cefpirome).

As a result, bacteria that produce ESBLs become resistant to these ß-lactams (Todar, 2009). ESBLs do not hydrolyze cephamycins and carbapenems (Yohei *et al*., 2009).

Until recently, ESBL-producing organisms were viewed as hospital- acquired or healthcare associated pathogens, i.e. affecting patients who had typically been in hospitals or other health care facilities like nursing homes. In the last decade, however, these infections have increasingly been recognized in community patients who had no prior contact with the health care system. This new epidemic is driven mostly by *Escherichia coli* that produce CTX-M-type ESBLs (Yohei *et al*., 2009).

The global spread of ESBL-producing *E. coli* into the community has a potential to become a major public health problem in the near future.

2.5.1 Genetics of ESBLS in *E. coli*

E. coli and *Klebsiella pneumoniae* often carry genes for TEM-1, -2 and SHV-1 ß-lactamases. TEM-1, -2 and SHV-1 are so-called broad-spectrum (but not extended-spectrum) ß-lactamases that are capable of hydrolyzing penicillins but not cephalosporins. When certain mutations leading to substitutions of key amino acid residues are introduced, their kinetic properties are altered to allow for significant hydrolysis of cephalosporins, especially ceftazidime, and

monobactam. These TEM- and SHV-derived ESBLs were the commonly observed ESBL types in *E. coli* throughout the 1980s and 1990s (Bradford, 2001).

On the other hand, reports on a newer group of ESBLs, coined CTX-M for their preferential hydrolysis of cefotaxime over ceftazidime, started to emerge in *E. coli* in the late 1990s (Paterson *et al.*, 2005). Studies have indicated that the genes for CTX-M-type ESBLs were mobilized from the chromosomes of *Kluyvera spp.* to *E. coli* plasmids through transposon-mediated events(Humeniuk *et al.*, 2002; Poirel *et al*., 2002).

Unlike TEM and SHV-type ESBLs, CTX-M-type ESBLs do not have corresponding non-ESBL progenitors. Currently, five groups of CTX-M-type ESBLs have been identified in *E. coli* CTX-M-1, -2, -8,-9 and -25 groups. CTX-M-14 (belonging to the CTX-M-9 group) and CTX-M-15 (belonging to the CTX-M-1 group) are particularly associated with community-acquired isolates (Yohei *et al*., 2009).

2.5.2 Epidemiology of ESBL-producing *E. coli* in the community

Initial reports on ESBL-producing *E. coli* came largely from hospitalized patients. For example, a large surveillance study conducted in France in 1993 did not identify any community-acquired infections with ESBL-producing *E. coli* among over 2,000 cases collected (Goldstein *et al*., 1995). However, reports on community onset, ESBL-producing *E. coli* infections started to emerge in the late 1990s and early 2000s. While most studies were laboratory-based surveillance identifying community-onset cases, some of them represented true community-acquired infections (Bou *et al*., 2002; Cormican *et al* ., 1998; Goldstein, 2000).

Initially identified mostly from European countries, community-acquired ESBL-producing *E. coli* infections have now been reported from populated continents. The fact that these cases occur among outpatients, however, makes it difficult to estimate the magnitude of this problem

(Yohei *et al*., 2009). In a population-based study conducted in Canada between 2000 and 2002, the incidence of ESBL-producing *E. coli* infections was 5.5 cases per 100,000 population per year, 71% of which was community-onset (Pitout, 2004).

While earlier data suggested that CTX-M-14 was most commonly associated with community-acquired infections (Rodriguez, 2004; Pitout, 2005), more recent data indicate that a large part of this global epidemic is driven by a particular clone of *E. coli* that produces CTX-M-15 and defined by phylogenetic group B2, serotype O25:H4 and MLST sequence type 131(Coque *et al.,* 2008; Nicolas *et al*., 2008). Several studies have pointed out overseas travel, especially to the Indian subcontinent where the rate of CTX-M-15 production among *E. coli* is estimated to be very high (Enzor *et al*., 2006; Walsh *et al*., 2007), as a major risk factor for subsequent development of infection due to these organisms (Freeman *et al*., 2008; Laupland *et al*., 2008). . Alternatively, food animals, in particular poultry, are known to be sometimes contaminated with ESBL-producing *E. coli* and speculated to be one source of these organisms (Carattoli, 2008). High carriage rates of ESBL-producing *E. coli* by family members of a patient with this infection also lend support to this hypothesis (Rodriguez *et al.,*2004; Valverde *et al*., 2008).

While CTX-M-type ESBLs are most frequently encountered in *E. coli*, they have now spread to *K. pneumoniae* and other enterobacterial species worldwide as well (Rossolini *et al.*, 2008). Community-acquired, ESBLproducing *K. pneumoniae* infections are increasingly reported (Keynan *et al*., 2007).

Vertical transmission of ESBL-producing *E. coli* from a mother to a newborn causing bacteremia was reported (Lopez *et al*., 2008). Therefore, knowledge of the local epidemiology and risk factors for these infections is crucial in choosing appropriate empiric therapy for severe *E. coli* infections that originate in the community(Yohei *et al*., 2009).

In a complete Sequence and Molecular Epidemiology of IncK Epidemic Plasmid encoding $bla_{\text{CTX-M-14}}$ published by Cottell *et al.,* (2011) the complete sequence of a $bla_{\text{CTX-M-14}}$–carrying IncK plasmid, pCT, from an *E. coli* isolate from a cattle farm in the United Kingdom was reported. In order to amplify part of the IncK plasmid, pCT primer008[F] 5'CATTGTATCTATCTTGTGGG3' and Pct009[R] 5'GCATTCCAGAAGATGACGTT 3' was one of the primers used (Cottell *et al*., 2011).

Genes that confer resistance to antimicrobial drugs used in human or veterinary medicine are often mobilized on plasmids. One class of resistance genes encode extended-spectrum β-lactamases (ESBLs), which confer resistance against many β-lactam antimicrobial drugs, leading to treatment failures (Cottell *et al.,* 2011). Based on their report, one of the pCT primers was used in this study in order to prove if we have this plasmid present in this environment.

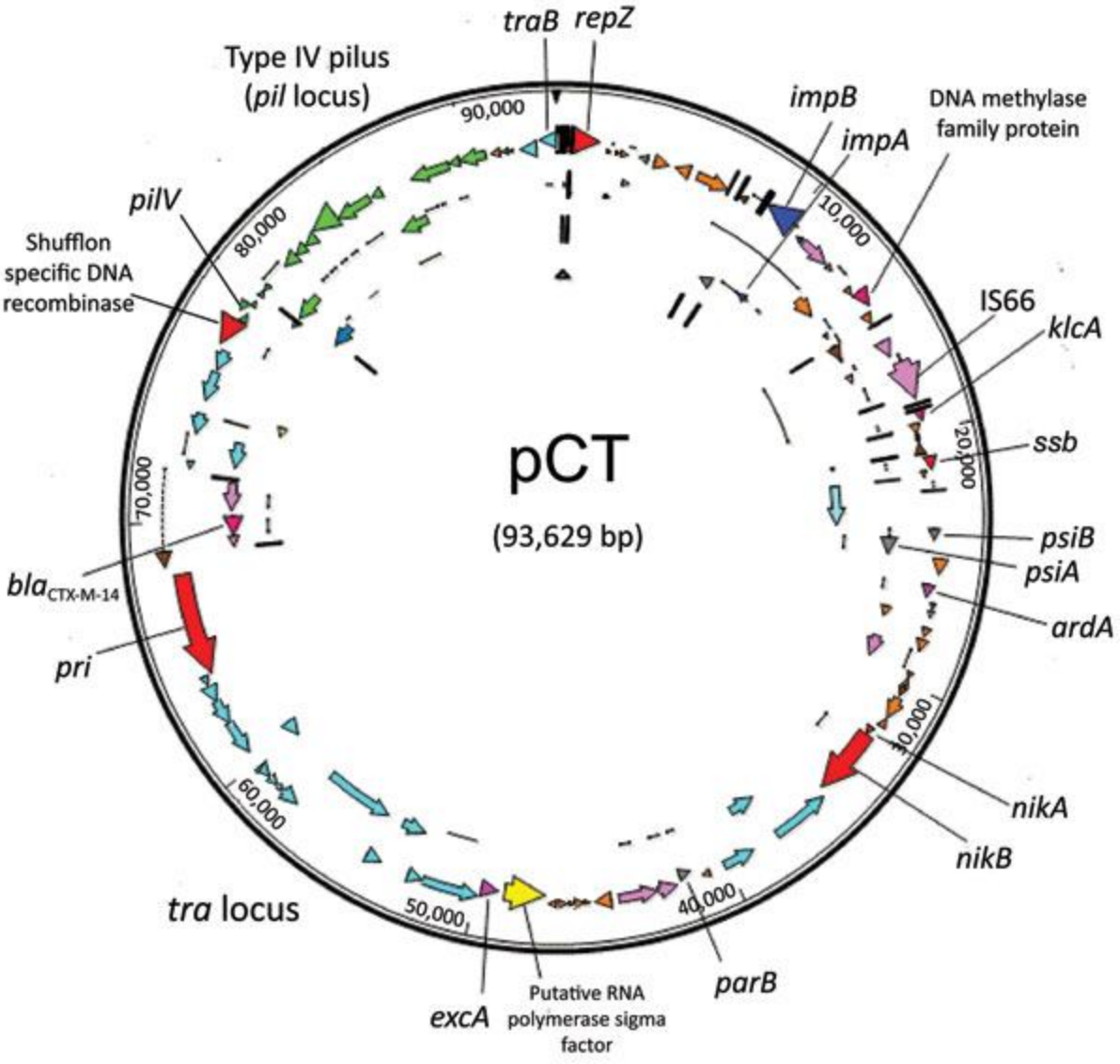


Figure 2. 1: Circular map of plasmid pCT.

Open reading frames are color coded as follows: brown, pseudogenes; orange, hypothetic proteins; light pink, insertion sequences; light blue, *tra* locus; green, *pil* locus; dark pink, antimicrobial drug resistance gene; yellow, putative sigma factor; red, replication-associated genes. Arrows show the direction of transcription. pCT, IncK plasmid (Adapted from Cottel , 2011).

2.6 Resistance to quinolones and fluoroquinolones

Resistance to first-generation quinolones (e.g. nalidixic acid) and fluoroquinolones [FQs] (e.g. norfloxacin, ciprofloxacin and levofloxacin) has been extensively reported for several years among human isolates which is related to their heavy usage in clinical practice. For instance, levels of FQ resistance among invasive *Escherichia coli* and *Klebsiella pneumonia* isolates in Europe has consistently increased over the past years and ranged in 2007 from 5% to 53% in *E.coli* and from 1% to 55% in *Klebsiella pneumonia* (Cattoir, 2009).

Resistance to quinolones in Gram-negative bacterial species most commonly results from the accumulation of mutations leading to specific alterations in type II topoisomerases that are molecular targets for these molecules: DNA gyrase (gyrA /gyrB genes) and topoisomerase IV (parC / parE genes) (Drlica and Zhao, 1997). It is known that a single mutation in gyrA is sufficient for high-level resistance to nalidixic acid whereas additional mutations are required for high-level FQ resistance. The chromosomal mechanism of resistance is the decrease of intracellular drug accumulation due to either decreased membrane permeability by porin loss or over expression of naturally-occurring efflux pump systems related to mutations in regulatory genes (Ruiz, 2003).

Although considered as impossible due to the plasmid curing effect of quinolones, plasmid-mediated quinolone resistance (PMQR) was first identified in 1998 from a *K. pneumoniae* isolate obtained from a urine culture collected in 1994 in Alabama, USA (Martinez *et al*., 1998).

Three different mechanisms have been identified to date: target protection by Qnr proteins, enzymatic inactivation by the aminoglycoside acetyltransferase AAC(6')-Ib-cr and active efflux by the QepA pump (Cattoir, 2009).

2.6.1 Qnr proteins

This first PMQR determinant which has been discovered is qnr (for quinolone resistance) located on a broad-host range conjugative plasmid conferring a MDR phenotype (Martinez *et al*., 1998).

Structurally, Qnr is a 218-amino acid protein belonging to the pentapeptide repeat proteins family which acts by protecting both DNA gyrase and topoisomerase IV from quinolone inhibition (Tran *et al*., 2002).

Qnr may also supplement quinolone resistance due to other resistance mechanisms, such as target modification, decreased permeability and efflux pump activation (Martinez *et al*., 2003).

Qnr may facilitate the recovery of resistant mutants with higher levels of quinolone resistance in vitro by increasing the mutant prevention concentration (MPC) by more than 10-fold (Robicsek, 2006).

Currently, five different types of Qnr-like proteins with several variants are reported: QnrA (6 variants), QnrB (20 variants), QnrC (1 variant), QnrD (1 variant), and QnrS (4 variants) sharing an overall amino acid identity from 39 to 64% (Jacoby *et al*., 2008).

Qnr determinants have been identified worldwide in many enterobacterial species, especially in *Enterobacter cloacae, K. pneumoniae, E. coli, and Salmonella spp.,* with an average prevalence between 1 and 5% depending on selection criteria of studied strains (Pitout, 2008).

Several lines of evidence indicate that the circulating qnr-like genes originate from the chromosome of water-dwelling environmental organisms, such as *Shewanella algae and Vibrio splendidus* which have been identified as the progenitors of qnrA- and qnrS-like genes, respectively (Poirel *et al*., 2005; Cattoir *et al*., 2007).

Aeromonas spp. which have been found harbouring plasmid-borne qnr genes and also present in the aquatic environment might have played an important role as a natural reservoir and an intermediate host for the qnr genes (Cattoir *et al.,* 2008).

To amplify a 448 bp fragment of the Quinolone Resistant Determining Region QRDR of *gyrB,* from nucleotides 995 to 1442, two 20-mer oligonucleotide primers, 5′-GCGCGTGAGATGACCCGCCGT-3′ and 5′-TGGCGGTAGAAGAAGGTCAG-3′ were used (Gomez *et al*., 2004). These specific primers were constructed based on the *E. coli* gene sequence data (GeneBank accession number D87842). This particular primer was used in this study to find out if the resistant genes are present in this environment.

2.7 Prevention of *E. coli* Infection

Control measures and good hygiene practices are important measures in the prevention of infections due to all types of *E. coli*. Measures given by Public Health, 2011 include:

- Washing hands thoroughly before eating or preparing food, after using the toilet, changing nappies or cleaning up others with diarrhoea and after contact with domestic or farm animals.
- Hygienic handling, preparation and storage of raw meat and other animal products and making sure that raw meat and the implements used to handle them do not come into contact with cooked meat or ready-to-eat food.
- Peeling raw fruit and vegetables and washing thoroughly before eating and avoiding unpasteurised milk (which can become contaminated from contact with cows udders) and unpasteurised fruit juices.
- Avoiding swallowing water when swimming in untreated or public water facilities.

- Having adequate fluid intake, especially water and avoiding contamination of the urinary tract by organisms from the anus by wiping 'front to back' after urinating (women).
- The risk of getting traveller's diarrhoea can be lowered by drinking only safe water (treated, boiled or sealed bottle), and by consuming ice known to be made from treated water. Food should be eaten freshly cooked and piping hot, salads should be avoided and fruit eaten only if it can be peeled (Public Health, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacteriological media

Media used were prepared according to Manufacturer's specification. They are:

- Nutrient Agar, Nutrient Broth, Mac Conkey Agar, Mac Conkey Broth, Eosin Methylene Blue Agar, Mueller Hinton Agar, Methyl Red Voges Proskauer and Peptone water obtained from Oxoid Ltd, England.
- Simon citrate Agar (BDH Chemicals Ltd, England)
- Luria-Bertani agar (BDH Chemicals Ltd, England)
-

3.1.2 Plasmid isolation kit and primers

- GeneJet™ Plasmid Miniprep kit, Dream Taq™ PCR Master mix (2X) obtained from Inqaba biochemical Industries Pty Ltd, South Africa.
- Primer 1 (F) 5'GCGCGTGAGATGACCCGCCGT 3'

  (R) 5' CTGGCGGTAGAAGAAGGTCAG 3'

  Gene accession number D87842.This primer was used to amplify a 448bp fragment of the gyrB gene.
- Primer 2 (F) 5' pCT 008 (F) CATTGTATCTATCTTGTGGG 3'

  (R) 5' pCT 009 (R) GCATTCCAGAAGATGACGTT3'

(An unestablished primer, adapted from Cottel *et al.,* 2011). Primer was used to amplify a 428bp fragment of $bla_{CTX\text{-}M\text{-}14}$ of ESBL gene.

3.1.3 Sensitivity Discs

Antibiotic sensitivity discs used in the study are: Amoxicillin (10µg), Ceftriazone (30µg) Ceftazidime (30µg), Gentimicin (30µg), Cefuroxime (30µg), Ciprofloxacin (10µg), Cotrimoxazole (25µg), Tetracycline (30µg), Ampicillin (10µg) obtained from Oxoid Ltd, England.

3.1.4 Reagents and Chemicals

They are : Acridine orange, Carbol fuschin, Crystal violet, Ethanol, Xylene solution, Creatinine, Pottasium hydroxide solution, Hydrochloric acid (conentrated) and Paradimethylaminobenzaldehyde, obtained from BDH chemical Ltd, England. Ethydium bromide, Iodine solution, EDTA and Glycerol obtained from Sigma chemical Ltd, England. Agarose gel obtained from Agarose from Schwarz/ Mann Biotech.

3.1.5 Equipments

- Agarose gel unit (HE 33; Hoefer, San Francisco CA, USA).
- UV Transilluminator (302 nm Vilber Lourmat).
- Optical microscope (Wild HeerbruggM11, Switzerland).
- Oven (Townson and Mercer Ltd. Croydon, England).
- Incubator (NAPCO Model 630 portland, Oregon,USA).
- Water bath (Model R76 New Brunswick Scientific, Edison N.J,USA).

3.1.6 Study area

This study was carried out in Zaria, Kaduna State. Nigeria. Zaria is between latitude 11°N and longitude 8°E, North-Western region of Nigeria. The study areas are:

- Hajia Gambo Sawaiba General hospital, Kofan – Gayan, Zaria – city, Zaria.
- Kaduna State Primary Health Care Centre, Samaru.
- Institute for Child Health. Ahmadu Bello University Ban – Zazzau, Zaria.

3.1.7 ETHICAL CLEARANCE

- Approvals for the study were obtained from the ethical committees of the different hospitals in Zaria, Nigeria.

3.1.8 CONSENT

- Informed consent in the language in which the patient or relative of patient understood were obtained before the study.

3.2 METHODS

3.2.1 Study population

Stool samples were collected from three hospitals in Zaria after ethical consent to carry out the study was granted from the hospital authorities. The hospitals are: Hajia Gambo Sawaiba General hospital, Kofan – Gayan, Zaria – city, Zaria, Kaduna State Primary Health Care Centre, Samaru and Institute for Child Health, Ahmadu Bello University Teaching Hospital, Ban – Zazzau, Zaria.

3.2.2 Sample Collection

Collection of samples was carried out from July 2010 to March 2011. Children (0-5years) who presented with diarrhoea with or without gastroenteritis within this period were recruited consecutively. The stool samples were collected into clean wide-mouth specimen containers and transported to the laboratory at ambient temperature (15°C to 30°C). In the laboratory, stool samples were inoculated into sterile nutrient broth and incubated at 37°C overnight. Overnight nutrient broth stool cultures were then sub-cultured on MacConkey agar and Eosin Methylene Blue Agar plates. The plates were further incubated at 37^0C for 18 hours for primary isolation.

3.3 IDENTIFICATION TEST

Lactose fermenting colonies from MacConkey and Eosin-Methylene Blue agar were subjected to microscopic examination to determine cell morphology and Gram stain reactions. Cultural characteristics and colonial morphology of the isolates were interpreted using a standard microbiological procedure manual (Cheesbrough, 2000).

Gram staining was carried out as described in standard microbiological procedure manual (Cheesbrough, 2000).

3.3.1 Biochemical Test

Gram negative bacilli were biochemically identified and confirmed as *Escherichia coli* using non-citrate utilization, indole formation, methyl red production, Voges Proskauer test and fermentation of sugars.

3.3.1.1 Indole test

Pure bacterial culture was grown in sterile peptone broth for 24 hours at 37°C. Following incubation, 5 drops of Kovac's reagent was added to the tubes. A positive indole test is indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent.

3.3.1.2 Methyl Red-Voges Proskauer test

This test uses MRVP broth. Bacteria isolates were grown in MRVP broth for 24 hours at 37°C After growth, the broth was separated into two different tubes, one for the Methyl Red (MR) test and one for the Voges-Proskauer (VP) test. The pH indicator Methyl Red was added to one tube and a red color indicated positive test. The VP test uses alpha-naphthol and potassium hydroxide to indicate a positive or negative test.

3.3.1.3 Citrate test

This test uses Simmon's citrate agar to determine the ability of a bacteria to use citrate as its sole carbon source. Bacteria colonies are picked up by a straight wire and inoculated into slope of

Simmons citrate agar and incubated overnight at 37 °C. If the organism has the ability to use citrate, the medium changes its color from green to blue.

3.3.1.4 Sugar fermentation test

This uses Phenol Red Broth to test for the fermentation of different sugars. Phenol Red Broth is a general purpose fermentation media that includes the pH indicator Phenol Red and a series of tubes each with a different sugar. A wire loop was aseptically used to inoculate the test organism into the broth containing the test sugar and inverted Durham tubes. This was incubated at 37°C for 18 hours. A bright yellow colour indicated the production of enough acid products from fermentation of the sugar. Production of gas was determined with a Durham tube, a small inverted vial filled with the broth. Gas production during fermentation of the sugar, was seen trapped at the top of the Durham tube and appeared as a bubble.

3.4 SUSCEPTIBILITY TESTING

The susceptibility of *Escherichia coli* isolates to nine (9) commonly prescribed antibiotics was determined according to Wolf, (1975) as modified by CLSI (1995) viz:

An overnight culture of each isolate was prepared in nutrient broth and incubated at 37^0C for 18 hours. Dry sterile plates of prepared Mueller Hinton's agar were flooded with standardized inoculum (10^6 cfu/ml) of 18 hours culture of *E. coli* isolate. After inoculation, Mueller Hinton's agar plates were allowed to dry for 15 minutes in properly disinfected incubator, before placing the various antibiotic sensitivity discs aseptically on the dried Mueller Hinton agar plates. The plates were allowed to stay for one hour before they were incubated at 37^0C for 18-24 hours. After incubation, the plates were examined for zones of inhibition which were measured in millimeters. The result was interpreted using the interpretation criteria published by CLSI

(2007). Isolates were reported as Sensitive (S), moderately sensitive or Intermediate (I) and resistant (R) to the various antibiotics, depending on the sizes of the zones of inhibition.

3.5 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (MIC)

The Minimum Inhibitory Concentrations (MIC) determination of four (4) test antibiotics namely: Amoxicillin, Cefuroxime, Tetracycline and Ciprofloxacin were carried out using agar dilution method as described by Lennette *et al*. (1990) with modifications viz:

Freshly prepared stock solution of the various antibiotics were each prepared and sterilised using Millipore membrane filter (0.22µ) aseptically. Graded concentrations of the various antibiotics in 5ml volume were prepared in triplicates using sterilized distilled water, and then aseptically mixed with 5 ml volume of double strength sterile Mueller Hinton's agar and allowed to set. 5ml volume of sterilized distilled water mixed with 5ml double strength Mueller Hinton agar was set up as positive control. Eighteen hours broth culture of the test isolates were standardized to an inoculum density of 10^6cfu/ml (Wood and Washington, 1995). The dried Mueller Hinton agar surfaces were aseptically inoculated with test organisms in triplicates at equidistant spacing. The plates were allowed to stay for one hour (1 hour) and then incubated at 37°C for 18 hours. After incubation, the plates were then examined for the presence or absence of growth. The lowest antibiotic concentration at which there was no visible growth was taken as the minimum inhibitory concentration (MIC).

3.6 CONJUGATION STUDIES

The transfer of resistance traits by the resistant isolates of *Escherichia coli* to ciprofloxacin sensitive *Proteus mirabilis* was investigated using the methods described by Onaolapo (1986) with some modifications.

The minimum inhibitory concentrations (MIC) of the test antibiotics against the sensitive *Proteus mirabilis* were determined as described under section 3.5 above. The resistant isolates of *E. coli* were each grown in sterile nutrient broth (5ml) each at 37°C for 18 hours. The ciprofloxacin sensitive *Proteus mirabilis* was subcultured into the sterile nutrient broth and incubated at 37°C for 18 hours. The overnight cultures of the potential donor (R^+) *E. coli* resistant isolates and the recipients (R^-) i.e. sensitive *Proteus mirabilis* was grown in a ratio of 10:1 respectively in 5ml volume of sterile nutrient broth and incubated in a static incubator at 37°C for 18 hours. One loopful of recombinants (transconjugants) from the admixtures (*E. coli* and *Proteus mirabilis*) bottles were subcultured in triplicate on MacConkey agar plates incorporated with the antibiotic of MIC strength (4 µg/ml of ciprofloxacin in double strength MacConkey agar) against sensitive *Proteus mirabilis* and incubated at 37°C for 24 hours. The plates were examined for the presence or absence of cultural characteristics of *Proteus mirabilis* and lactose fermenting properties. Original culture of *Proteus miralibis* and *E. coli* separately was diluted in1:100 ratio and a drop was spread on MacConkey agar containing ciprofloxacin 4µg/ml for positive control. The colonies of *Proteus mirabilis* observed were aseptically picked and transferred into nutrient agar slant and subcultured, after which the MICs of transconjugants were determined as described by Lennette *et al*. (1990).

3.7 CURING TRANSCONJUGANTS

The curing of transconjugants *Proteus mirabilis* (Rf plasmid) was carried out by treating the *Proteus mirabilis* transconjugants with acridine orange dye as described by Onaolapo (1986), viz: Each of the transconjugants (PR) was grown overnight on sterile nutrient broth and incubated at 37°C for 18 hours in a static incubator. The overnight culture of *Proteus mirabilis*

transconjugants were standardized (10^6 cfu/ml). A stock solution of acridine orange in sterile distilled water (10,000µg/ml), was prepared and 1.0ml of the solution was dispensed into test tubes containing sterile nutrients broth (2ml). The contents of each tube was vortexed and mixed properly, mixtures were labeled and allowed to settle.

Twenty microlitre (20µl) of standardized *Proteus mirabilis* transconjugants (10^6cfu/ml) was inoculated into mixed solution of acridine orange and sterile nutrient broth and incubated at 37°C for 18 hours.The growth from the overnight culture of *Proteus mirabilis* transconjugants were sub cultured. The colonies obtained from curing transconjugants experiment were assessed for their antibiotic sensitivity using the MIC method. This was an attempt to determine whether the resistant pattern had changed or not.

3.8 ISOLATION OF PLASMID DNA

Isolation of DNA plasmid and its analysis was carried out at the centre for Biotechnology research and training institute of the University. The transconjugant strains and resistant isolates were subjected to plasmid DNA isolation following the protocol of Gene Jet™ Plasmid Miniprep kit:-

One loopful of the resistant isolates and transconjugants were inoculated into 10ml Luria Bertani medium for purification of low copy plasmid and incubated for 18 hours. The pelleted cells from the overnight culture were resuspended in 250µl of the resuspension solution and transferred to a micro-centrifuge tube. Bacteria suspension were then completely resuspended by vortexing and pipetting up and down until no cell clumps remained.

250µl of the lysis solution was added and mixed thoroughly by inverting the tube 4 – 6 times until the solution was viscous and slightly clear. 350µl of the neutralization solution was added

and mixed immediately and thoroughly by inverting the tube 4 – 6 times. Neutralized bacterial lysate was then centrifuged for 5 minutes to pellet cell debris and chromosomal DNA at 10,000 rpm.

Supernatants were transferred to the supplied Gene Jet^{TM} spin column by decanting. Supernatant was then centrifuged for 1 minute and flow through were discarded then columns were placed back into the same collection tube. 500µl of the wash solution (diluted with ethanol prior to first use) was added to the Gene Jet^{TM} spin column and centrifuged for 30 seconds and the flow – through was then discarded and column placed back into collection tube. Wash procedure above was repeated using 500µl of wash solution. Flow through was discarded and column was centrifuged for 1 minute to remove residual wash solution.

Gene Jet^{TM} spin column were transferred into fresh 1.5ml microcentrifuge tube. 50µl of Elution Buffer was added to the center of Gene Jet^{TM} spin column membrane to elute the plasmid DNA then incubated for 2 minutes at room temperature and centrifuge for 2 minutes. The step above was repeated to recover residual DNA from membrane. Column was discarded and purified plasmid DNA were stored at -20°C.

3.8.1 Agarose Gel Electrophoresis

3.8.1.1 Protocol

A 1% agarose gel was used to resolve the plasmid fragment. The agarose gel was prepared by combining lg of agarose in ten times concentration of Tris acetate ethylene diamine tetra acetate (10ml 10x TAE) buffer and 90 ml distilled water in a 250ml beaker flask and heating in a microwave for 2 minutes until the agarose was dissolved.

Two and half milliliter (2.5ml) ethidium bromide (5.0 mg/ml) was added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and the casting combs were inserted. It was then allowed to set for 30 minutes.

The casting combs were then carefully removed after the gel had completely solidified, one time concentration (1X) TAE electrophoresis buffer was then added to the reservoir until the buffer just covered the agarose gel.

3.8.1.2 Loading Plasmid DNA Samples

Five microlitres (5µl) of gel tracking dye (bromophenol blue) was added to 15µl of each plasmid DNA isolates with gentle mixing. 20µl of the mixture was then loaded onto the wells of the gel, the mini horizontal electrophoresis gel set up was then covered and the electrodes connected. Electrophoresis was carried out at 100mV for 45 minutes.

At the completion of the electrophoresis, the gel was removed from the buffer and the gel was viewed under a long wave UV light box i.e. Transilluminator (302 nm).

The band pattern of the DNA fragments were then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system.

3.9 POLYMERASE CHAIN REACTION AMPLIFICATION OF TARGET GENES

Polymerase chain reaction (PCR) amplification of target genes from isolated plasmid DNA was carried out. Gene specific primers were used in PCR to amplify specific fragments from genes coding for resistance of fluoroquinolones and $bla_{CTX\text{-}M}$ ESBL as described by the manufacturer (Iquaba Biotec).

3.9.1 Protocol

Dream Taq^{TM} PCR master mix (2x) was vortexed and centrifuged for 30 seconds at 10,000rpm after thawing. A thin – walled PCR tube was placed on ice and the following components were added for each 50µl reaction. viz:

25µl of Dream Taq^{TM} PCR master mix (2x) was added in the PCR tube then 1.0µl of the forward primer's was added followed by the addition of 1.0 µl of the reverse primer. Thereafter, 10.0µl of template DNA (plasmid DNA) was added and finally the nuclease – free water (13 µl) was added in the PCR tube to make up a total volume of 50µl. The samples were vortexed gently and spun down. Polymerase chain reaction was performed using the thermal cycling conditions on table 3.1.

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three . The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik *et al,* 1990).

- *Initialization step*: This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Sharkey *et al,* 1994).

- *Denaturation step*: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- *Annealing step*: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

- *Extension/elongation step*: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C (Chien *et al*, 1976 : Lawyer *et al,* 1993), and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

- *Final elongation*: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

- *Final hold*: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Table 3.1: Primers used in the study

Gene	Primers	Sequence, 5'→ 3'	Target	Product size bp	References
$bla_{CTX\text{-}M\text{-}14}$	pCT(008)F	CATTGTATCTATCTTGTGGG	pCTpCT 008-pCT009 region	428	Cottel *et al.,* 2011
	pCT(009)R	GCATTCCAGAAGATGACGTT	pCTpCT 008-pCT009 region	428	
gyrB	Primer 1 F	GCGCGTGAGATGACCCGCCGT	QRDR region of 995 to 1442	448	Gomez *et al.,* 2004
	Primer 1 R	CTGGCGGTAGAAGAAGGTCAG	QRDR region of 995 to 1442	448	
		Gene bank accession number D87842			

Table 3. 2: PCR conditions for Primers 1 and 2

Step	Temperature °C	Time	Number of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	
Annealing	52 (primer 1) 50 (primer 2)	30 seconds	35 cycles
Extension	72	1minute/kb	
Final extension	72	15 minutes	1

Total time for PCR – 1 hour 53 minutes (primer 1)

Total time for PCR – I hour 49 minutes (primer 2)

3.9.2 Agarose Gel Electrophoresis of PCR Products

1% Agarose gel was used to resolve the PCR target genes from isolated plasmid DNA with their primers and documented. Protocol is as documented in 3.8.1.1 and 3.8.1.2 on pages 55-56.

3.10 Statistical Analysis

Data was entered in Microsoft excel 2007 and then transferred to SPSS version 15.0 for analysis. Students t-test and analysis of variance (ANOVA) were used to compare the results.

CHAPTER FOUR

RESULTS

4.1 DISTRIBUTION OF *E.COLI* ISOLATES

One hundred and fourteen samples of diarrhoeic stools were collected from patients in the three selected locations for study. They are:-

- Institute of child health, Ban-zazzau, in affiliation with Ahmadu Bello University Teaching Hospital, Zaria,Nigeria
- Primary Health care Centre, Samaru,Nigeria.
- Gambo Sawaiba General Hospital, Kofan-Gaya, Zaria City, Nigeria.

Samples were collected within the period of July, 2010 to March, 2011. A total of 10 samples were collected from the Institute of Child Health, Ban-zazzau, 43 samples were collected from Samaru Primary Health care centre and 61 samples were collected from the General Hospital, Kofan-Gaya. Isolation rates were 70%, 74.4% and 50.8% respectively.

Out of the total of 114 patients studied, 54 (47.4%) were male and 60 (52.6%) were female (2weeks-5years).

A total of seventy *Escherichia coli* isolates were identified. Isolates from female patients were 37 (52.79%) while 33 (47.1%) were from male patients.

Higher proportion of *E. coli* positive stools were observed with samples from the Primary Health Centre Samaru and Kofar Gaya General Hospital, compared with proportion from institute of child health, Ban-zazzau. The total isolation rate of *E. coli* from all the stool samples observed from this study was 61.4% (70/114).

4.2 ANTIBIOTIC SUSCEPTIBILITY PROFILES

The susceptibility pattern of 70 isolates of *E. coli* to antibiotics was determined using the disc diffusion method. Antibiotics commonly prescribed in the environment were used. Isolates which were intermediate and sensitive were considered susceptible on the CLSI (2007) interpretative chart.

Amoxicillin, ampicillin and tetracycline showed the least activity with 0% susceptibility, then sulphamethoxazole/trimethoprim showed susceptibility of 2.9%, cefuroxime (14.3%), ceftazidime (11.4%) and ceftriaxone (41.4%) showed low antibacterial activity as observed in this study. Ciprofloxacin and gentamicin displayed high level of antibacterial activities of 60.0% and 64.3% respectively against the the test *E. coli* isolates. (Fig 4.1).

The minimum inhibitory concentrations were determined and the peak plasma levels (obtained from the complete drug reference, Martindale, (2009)) for each of the antibiotics tested was used as the break point to determine *E. coli* isolates resistance. Amoxicillin was observed to be ineffective against all the *E. coli* isolates while ciprofloxacin gave the highest antibacterial activity of 65.7%. Tetracycline showed antibacterial activity of 24.3%. Cefuroxime showed low level antibacterial activity against test bacteria with only 15.7% of the *E. coli* isolates sensitive to it.

The minimum inhibitory concentration result (Table 4.3) showed that 67.6% and 37.8% of 37 test bacteria isolates from female patients stool samples were resistant to Tetracycline and Ciprofloxacin as compared to 54.6% and 30.3% of 33 test bacteria isolates from male patients stool samples that were resistant to Tetracycline and ciprofloxacin respectively in this study. Although no statistical significance exist among them.

Table 4. 1: Percentage of *E. coli* isolation from the three study centres.

Hospital	No of samples collected	No and % of *E. coli* isolated	Female	Male
Kofan Gayan	61	31 (50.8)	20	11
Samaru	43	32 (70.0)	13	19
Ban-zazzau	10	7 (74.4)	4	3
Total	114	70 (61.4)	37	33

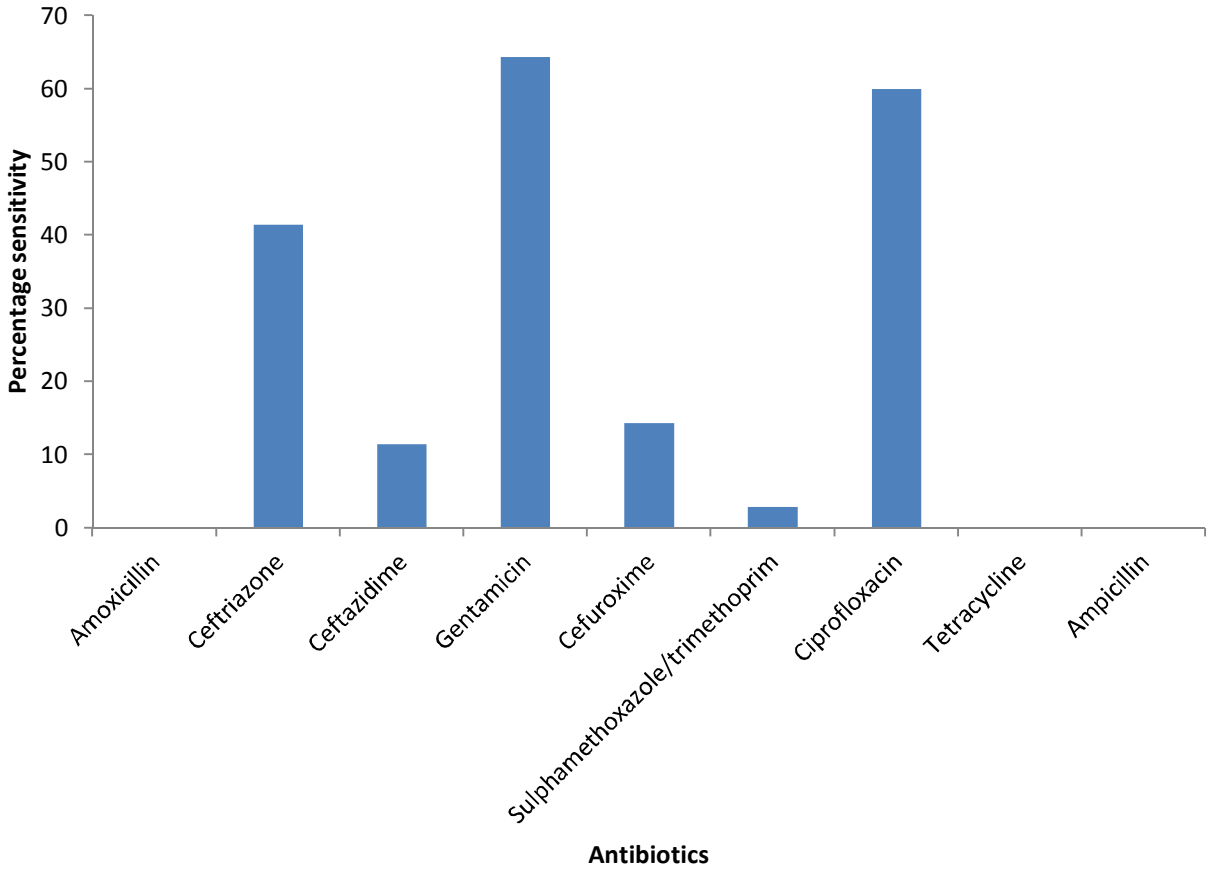


Figure 4. 1: Percentage distribution of *E. coli* isolates sensitive to the test antibiotics.

Table 4. 2: Percentage resistance of *E. coli* isolates using minimum inhibitory concentration values of selected antibiotics

S/N	Antibiotics (concentrations of MIC)	% resistance
1	Amoxicillin (1000µg / ml)	100%
2	Tetracycline (10µg /ml)	75.7%
3	Ciprofloxacin (3µg /ml)	34.3%
4	Cefuroxime Na (54µg /ml)	84.3%

The peak plasma levels were used as the break points to determine resistance among the test *E. coli* isolates (n=70).

Peak plasma level for amoxicillin = 5µg / ml

Peak plasma level for tetracycline= 4-5µg /ml

Peak plasma level for ciprofloxacin = 2.5µg /ml

Peak plasma level of Cefuroxime Na = 27µg/ml

Table 4. 3: Antibiotic resistance profile of *E. coli* isolates from stools of diarrhoeic male and female children under five years in Zaria, Nigeria.

S/N	Antibiotics	Female (n=37) (Mean value)	male (n=33) (Mean value)	t-value	p-value
1	Amoxicillin	37(7.0)	33(6.8)	0.691	0.492
2	Ceftriaxone	23(10.2)	18(10.1)	0.113	0.911
3	Ceftazidime	33 (10.1)	29 (10.1)	0.101	0.920
4	Gentamicin	11 (9.6)	13 (10.3)	1.387	0.178
5	Cefuroxime	36 (10.4)	24 (10.4)	0.025	0.980
6	Cotrimoxazole	36 (7.5)	31 (7.4)	0.254	0.801
7	Ciprofloxacin	19 (11.3)	9 (10.6)	1.107	0.279
8	Tetracycline	37 (9.0)	33 (9.4)	0.960	0.340
9	Ampicillin	37 (7.4)	33 (6.9)	0.869	0.388

Zone of inhibition interpretation as susceptible according to CLSI, 2007.

Students T-test was used to compare the resistance between males and females, no statistically significant differences exist as p-values are all greater than 0.05.

Table 4. 4: Susceptibility profile of 70 *E. coli* isolates obtained in Zaria, Nigeria using the test antibiotic peak plasma level as breakpoint.

S/N	ANTIBIOTIC	PEAK PLASMA LEVEL BREAKPOINTS (µg/ml)	FEMALE (N-37) (Mean value)	MALE (N=33) (Mean value)	t-value	p-value
1	Amoxicillin	>5.0	37 (1000)	33 (1000)	0	0
2	Tetracycline	>5.0	25 (32.4)	18 (4.7)	0.957	0.342
3	Ciprofloxacin	>3.0	14 (16.9)	10 (9.6)	1.038	0.303
4	Cefuroxime Na	>27.0	30 (226.1)	29 (188.8)	0.587	0.559

Peak plasma levels used as breakpoint were obtained from Martindale, 2009.

Students T-test was used to compare theresistances, using MIC values. No statistical difference exist between the values as p-values are greater than 0.05.

Table 4. 5: Antibiotic resistance profile of *E. coli* isolates using zone of inhibition from the three hospitals

S/N	Antibiotics	Kofan Gaya n=31 (Mean value)	Ban-zazzau n=7 (Mean value)	Primary health n=32 (Mean value)	F-value	p-value
1	Amoxicillin	31 (7.3)	7 (6.3)	32 (6.7)	2.089	0.132
2	Ceftriazone	17 (10.2)	1 (11.0)	23 (9.9)	0.399	0.674
3	Ceftazidime	27 (10.4)	3 (9.7)	32 (9.9)	0.933	0.399
4	Gentimicin	11 (9.8)	2 (11.0)	12 (10.0)	0.757	0.480
5	Cefuroxime	29 (10.5)	4 (10.4)	27 (10.3)	0.106	0.899
6	Cotrimoxazole	31 (7.6)	5 (6.2)	32 (7.5)	1.999	0.144
7	Ciprofloxacin	16 (10.8)	1 (13.0)	11 (11.2)	0.953	0.400
8	Tetracycline	31 (9.3)	7 (9.4)	32 (9.1)	0.171	0.843
9	Ampicillin	31 (7.5)	7 (5.9)	32 (7.2)	1.559	0.218

Zone of inhibition interpretation is shown in Appendix.

No statistical difference exist between the values from the three study sites, as the p-values are all greater than 0.05.

Table 4. 6: Resistance pattern of *E. coli* isolates using test antibiotics peak plasma levels in three hospitals.

Antibiotics	µg/ml Peak plasma level	Resistance %		
		K/Gayan N=31	Ban zazzau N=7	Samaru n=32
Amoxicillin	>5.0	31 (100.0)	7 (100.0)	32 (100.0)
Tetracycline	>5.0	20 (64.5)	5 (71.4)	18 (56.3)
Ciprofloxacin	>3.0	12 (38.7)	1 (14.3)	7 (21.9)
Cefuroxime Na	>27.0	29 (93.6)	7 (100.0)	23 (71.9)

Test antibiotics susceptibility break points against gram negative bacteria were according to Martindale (2009).

4.3 CONJUGATION STUDIES

Conjugation studies was carried out to determine the existence of transferable antibiotic resistance determinants using ciprofloxacin resistant *E. coli* isolates as donor. The studies showed that out of the twenty (20) donor *E. coli* isolates with ciprofloxacin resistant factor, only twelve (12) were observed to transfer their resistance factor. The increase in the minimum inhibitory concentration of the recipient *Proteus mirabilis* (0.375µg/ml) to that of ciprofloxacin (6-100µg/ml) after conjugation showed that the phenotypic resistance factor of the donor bacterium had been incorporated. (Table 4.7).

4.4 PLASMID CURING

Curing of the transconjugants with acridine orange showed changes in the antibiotic sensitivity pattern of all the tested transconjugants. The MIC values of ciprofloxacin against the tested cured transconjugants decreased significantly when compared to the values obtained before curing .(Table 4.8).

Out of the seventy *E.coli* isolates, twenty- seven were observed to be resistant to ciprofloxacin. Twelve out of the twenty donor *E.coli* isolates used were found to transfer plasmids to the recipient *Proteus mirabilis*.(Table 4.7 & 4.8).

Table 4. 7: Minimum inhibitory concentration of ciprofloxacin before and after conjugation.

S/N	Isolates code	MIC of ciprofloxacin before conjugation (µg/ml)	Recipient *Proteus mirabilis* characteristics	MIC of ciprofloxacin after conjugation (µg/ml)
1	S36	50.0	+	100
2	S 30	50.0	+	50
3	K 57	100.0	+	12
4	K53	100.0	+	100
5	K10a[1]	3.0	_	_
6	K52	12.0	_	_
7	S18	12.0	_	_
8	S29	12.0	+	20
9	K37	50.0	+	100
10	K1	100.0	_	_
11	K49	100.0	+	100
12	K48	3.0	_	_
13	K56	100.0	_	_
14	K6b	50.0	+	50
15	K47	6.0	+	6
16	K59	20.0	+	20
17	S27	50.0	+	50
18	S16	100.0	_	_
19	K58	20.0	+	20
20	S21	3.0	_	_

MIC Range 3-100 µg/ml. MIC of recipient *Proteus mirabilis* before conjugation = 0.375µg/ml

+=isolates which showed *Proteus mirabilis* characteristics.

_= isolates which showed no characteristics for *Proteus mirabilis*.

Table 4. 8: Transconjugants *Proteus mirabillis* MIC and phenotypic resistant pattern at transfer temperature of 37ºC.

Donor isolates lab no	Transconjugants MIC (μg/ml)	Donor phenotypic resistance profile
S36	100	Aml, Caz, Cn, Sxt, Te, Amp.
S30	50	Aml. Caz,Cxm,Sxt,Te,Amp.
K57	12	Aml,Cro,Caz,Cxm,Sxt,Te,Amp.
K53	100	Aml,Cro,Caz,Cxm,Sxt,Cip,Te,Amp.
S29	20	Aml, Cro,Caz, Cxm,Sxt,Te,Amp.
K37	100	Aml,Caz,Cxm,Sxt,Te,Amp
K49	100	Aml, Cro, Caz, Cxm, Sxt, Cip, Te, Amp.
K6b	50	Aml, Cro, Caz, Cn, Cxm,Sxt,Cip,Te,Amp.
K59	20	Aml,Cro,Caz,Cn,Cxm,Sxt,Cip,Te,Amp.
S27	50	Aml, Cro,CAz,Cxm,Sxt,Cip,Te,Amp.
K58	20	Aml,Cro,Caz, Cxm, Sxt, Te,Amp.
K47	6	Aml, Cro, Caz, Cxm, Sxt, Te, Amp.

Aml=Amoxicillin	Cro = Ceftriaxone	Sxt = Sulfamethoxazole/Trimethoprim
Caz = Ceftazidine	Cxm = Cefuroxime	Te = Tetracycline
Cn = Gentamicin	Cip = Ciprofloxacin	Amp = Ampicillin

Table 4. 9: Minimum inhibitory concentrations of ciprofloxacin against the cured transconjugants (*Proteus mirabilis*).

S/No	Isolates No.	MIC before curing	MIC after curing
1	S_{36}	100	0.375
2	S_{30}	50	0.375
3	K_{57}	12	0.75
4	K_{53}	100	0.375
5	S_{29}	20	0.75
6	K_{37}	100	6
7	K_{49}	100	6
8	K_{6b}	50	0.75
9	K_{47}	6	1.5
10	K_{59}	20	0.375
11	S_{27}	50	1.5
12	K_{58}	20	0.375

4.5 MULTIPLE ANTIBIOTIC RESISTANCE (MAR)

The multiple antibiotic resistance pattern of the *Escherichia coli* isolated from diarrhoeic stools of children is as shown in Table 4.10.

The multiple antibiotic resistance (MAR) was calculated based on the ratio of the number of antibiotics to which the isolates were resistant to, out of a total of 6 classes of antibiotics used. The result shows that 69 of the test *E. coli* isolates showed multiple antibiotic resistance with 17 different phenotypic resistant patterns (Table 4.10). Sixteen isolates out of seventy showed 100% resistance to the nine antibiotics tested. Eighteen showed multiple antibiotics resistance index of 0.8 with nine phenotypic patterns as shown in Table 4.10. Thirty-four isolates had a MARI of 0.7 with six phenotypic patterns. One isolate showed MARI of 0.5 with one phenotypic pattern. All the seventy isolates were consistently resistant to amoxicillin, tetracycline and ampicillin.

Table 4. 10: Profile of multiple antibiotic resistance of *E. coli* isolates.

Phenotypic resistance pattern	No of antibiotic resistance	Classes of antibiotic resistance	No of isolates
Aml, Cro, Caz, Cn, Cxm, Sxt, Cip, Te, Amp	9	6	16
Aml, Cro, Caz, Cxm, Sxt, Cip, Te, Amp	8	5	6
Aml, Cro, Caz, Cn, Cxm, Sxt, Te, Amp	8	5	2
Aml, Caz, Cxm, Sxt, Cn, Te, Amp	7	5	1
Aml, Cro, Caz, Cxm, Sxt, Te, Amp	7	4	16
Aml, Caz, Cxm, Sxt, Cip, Te, Amp	7	5	2
Aml, Cn, Cxm, Sxt, Cro, Te, Amp	7	5	1
Aml, Cn, Sxt, Cip, Te, Amp	6	5	1
Aml, Caz, Cxm, Sxt, Te, Amp	6	4	14
Aml, Caz, Cn, Sxt, Te, Amp	6	5	3
Aml, Caz, Sxt, Cip, Te, Amp	6	5	1
Aml, Cxm, Sxt, Cip, Te, Amp	6	5	1
Aml, Cxm, Sxt, Te, Amp	5	4	1
Aml, Sxt, Cip, Te, Amp	5	4	1
Aml, Caz, Sxt, Te, Amp	5	4	1
Aml, Cn, Sxt, Te, Amp	5	4	1
Aml, Cxm, Te, Amp	4	3	1
Aml, Te, Amp	3	2	1

Aml=Amoxicillin	Cro = Ceftriaxone	Sxt = Sulfamethoxazole/Trimethoprim
Caz = Ceftazidine	Cxm = Cefuroxime	Te = Tetracycline
Cn = Gentamicin	Cip = Ciprofloxacin	Amp = Ampicillin

Table 4. 11: Multiple antibiotic resistance index of *E. coli* isolates.

MAR INDEX	NO OF ISOLATES	PERCENTAGE OF ISOLATES
0.3	1	1.4
0.5	1	1.4
0.7	34	48.6
0.8	18	25.7
1.0	16	22.9
Total =	70	

The multiple antibiotic resistance index (MAR I) of the isolates shows that 69 isolates were resistant to three (3) or more antibiotics.

MARI >/ 0.3 indicates that the isolate originated from an environment where antibiotics were frequently being used.

4.6 PLASMID DNA ANALYSIS

Plates 4.1-4.3 shows the plasmid DNAs obtained from the multiple antibiotic resistant *E. coli* isolates on agarose gel electrophoresis. Plasmid analysis of *E. coli* isolates yielded five different plasmid profiles (2800, 3100, 3200, >4000 and >5000bp) (Table 4.12). From the agarose gel electrophoresis of the plasmid DNAs isolated, eleven plasmid bands were observed in this study. Plate 4.1 shows six resistant isolates harbouring nine plasmids, plate 4.2 shows an isolate harbouring one plasmid band and plate 4.3 also shows an isolate harbouring one plasmid band. Plates 4.4-4.8 shows polymerase chain reaction products of gene-specific primers 1 and 2. Primer 1 codes for part of IncK plasmid of the bla_{CTX-M} of ESBLs while primer 2 codes for gyrB gene of QRDR for fluoroquinolone resistance. Three resistant isolates were found to carry the bla_{CTX-M} of the ESBL genes (lane 12 in plate 4.4 and lanes 13 and 15 in plate 4.6), while nineteen resistant isolates were observed to carry the gyrB genes (lanes 2,4,6,8,10,12 and 14 in plate 4.6 ,lanes 2 and 5 in plate 4.7 and lanes 3,4,5,7,9,10,11,13,14,15 in plate 4.8). Seven isolates out of the resistant *E.coli* donor cells for conjugation studies which showed no plasmid transfer were observed to harbour plasmid band and they showed the presence of resistant genes on PCR (Table 4.13). It was also observed that the transconjugant *Proteus mirabilis* displayed one or two plasmid bands in them and they were also observed to carry resistant genes on PCR. The order of occurrence of these plasmid base pairs was >5000bp (4), 2800bp (3), >4000bp (2), 3200bp (1)=3100bp (1) (Table4.13).

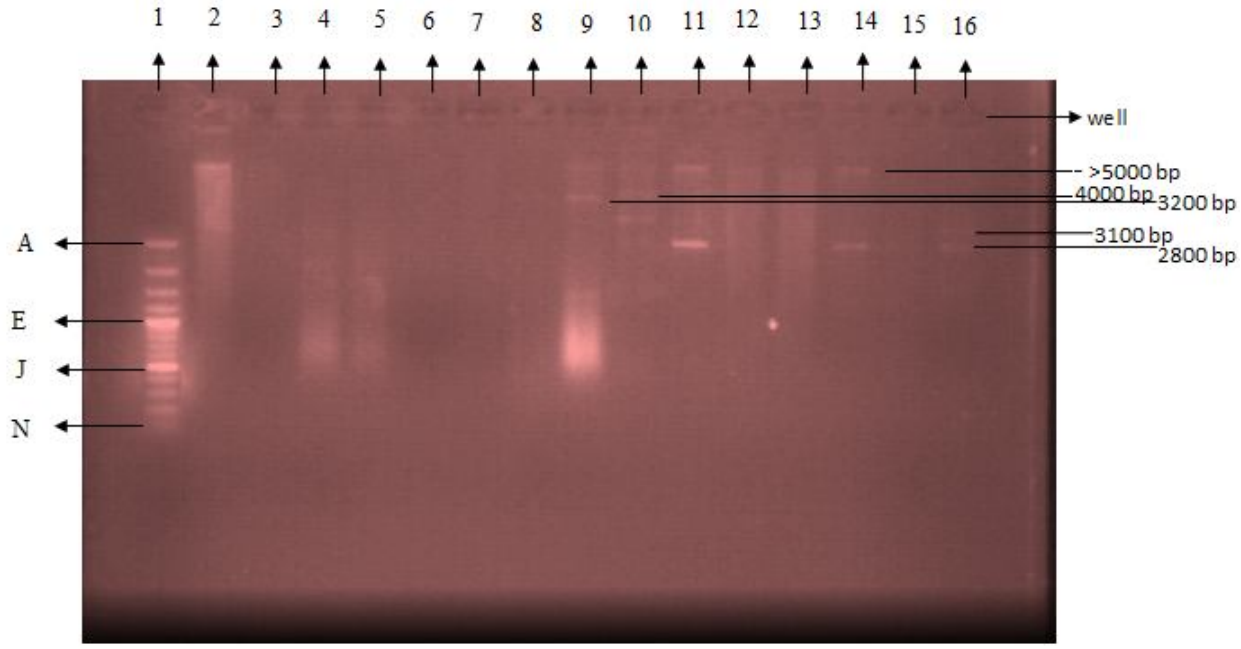


Plate 4. 1: 1% Agarose gel electrophoresis (100V, 45 min) of plasmid DNAs from multiple antibiotics resistant E. coli and transconjugant isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).

Lane 2- 6: Resistant *E. coli* isolates ($R_{16\text{-}R20}$)

Lane 7-16: Transconjugant ($T_{1\text{-}T10}$).

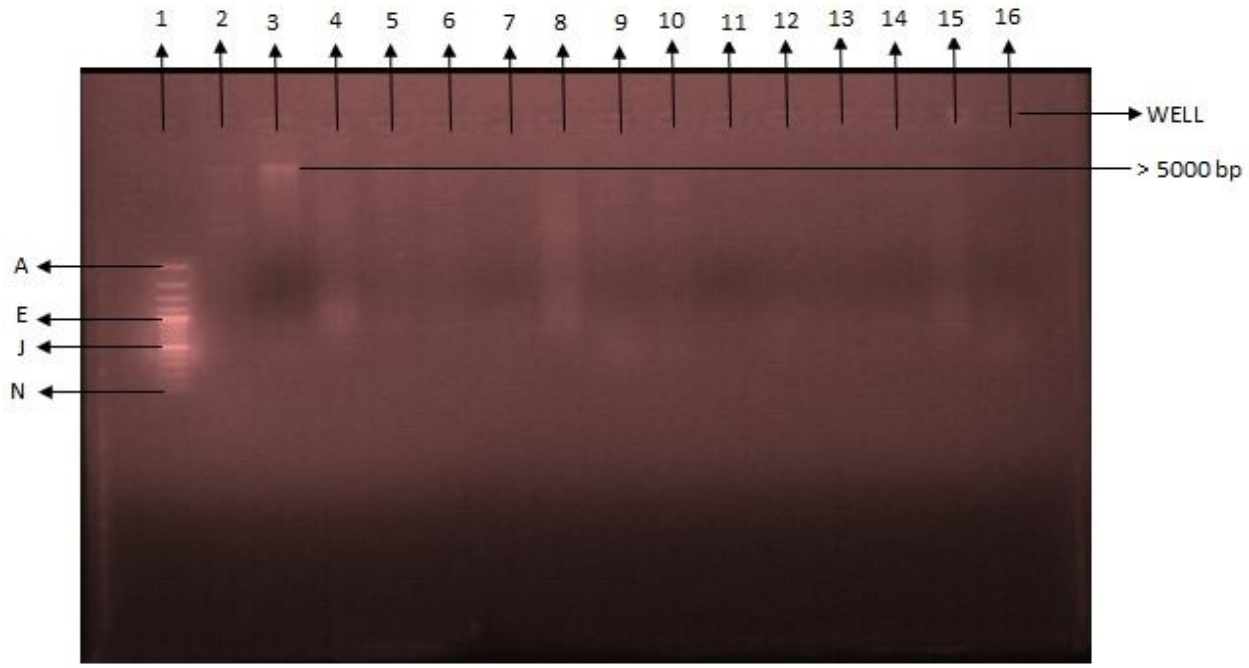
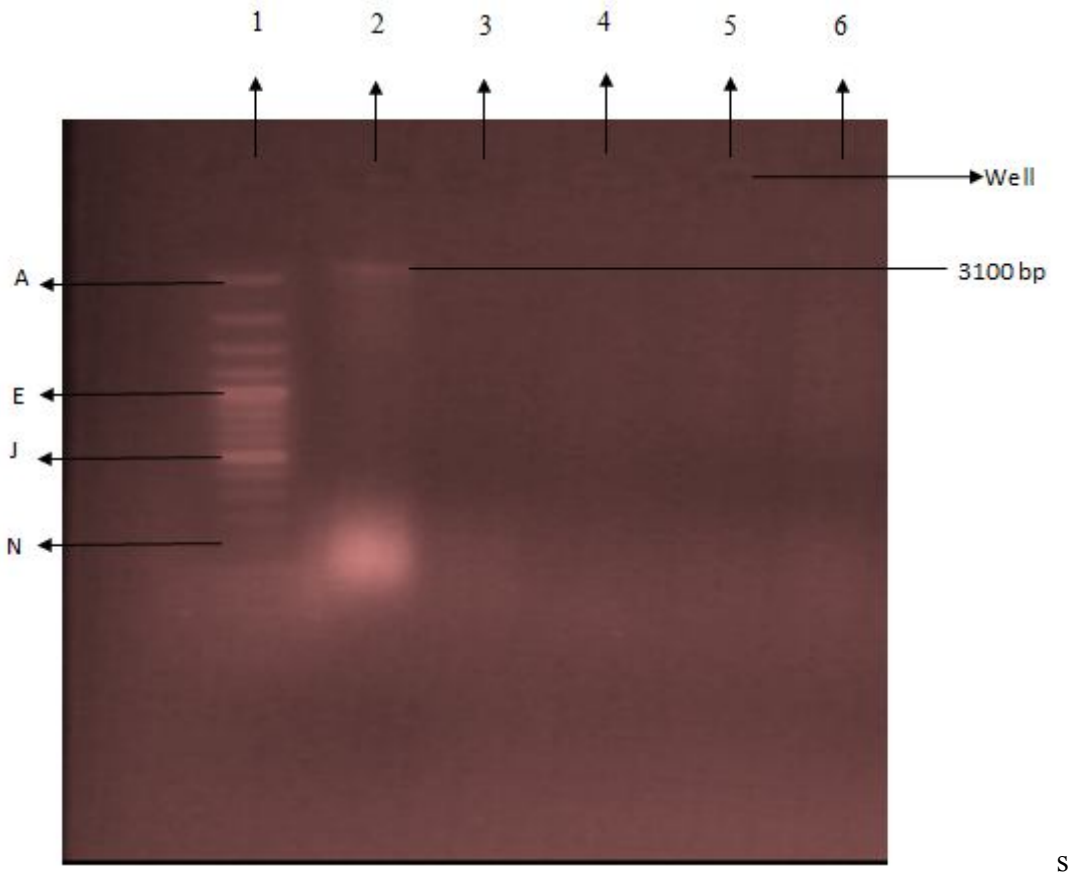


Plate 4. 2: 1% Agarose Gel Electrophoresis (100V, 45 min) of plasmid DNAs from multiple antibiotics resistant *E. coli* isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).

Lane 2-16: Resistant *E. coli* isolate (R_1-R_{15})



S

Plate 4. 3: 1% Agarose Gel Electrophoresis (100V, 45 mins) of Plasmid DNAs isolate from Transconjugants, Cured and recipient isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 150

0, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).

Lane 2-3: transconjugant (T_{11-T12}) ; Lane 4-5: Cured transconjugant (T_{6-T7})

Lane 6: Conjugation recipeint (*Proteus mirabilis*)

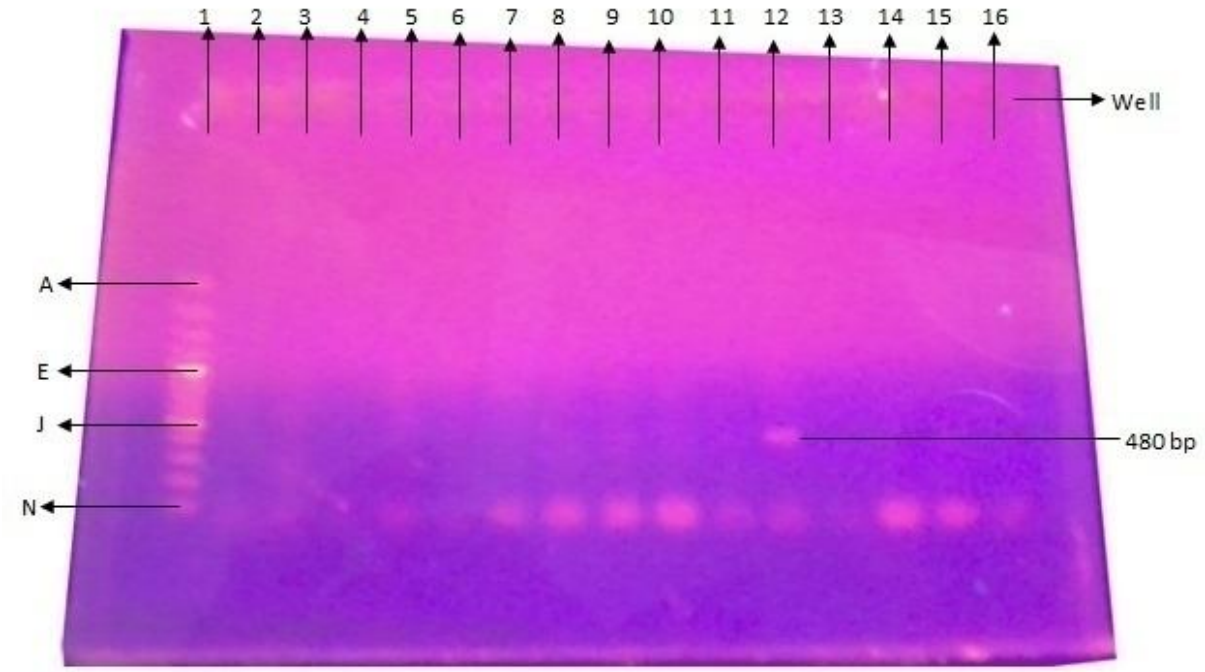


Plate 4. 4: Polymerase chain reaction products with primer 2 on 1% agarose gel electrophoresis (100v, 45 min) of plasmid DNAs from multiple antibiotic resistant and tranconjugant isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).
Lane 2 to 4: Transconjugants ($T_1 - T_3$) with primer 2 respectively.
Lane 5 to 16: Resistant *E. coli* isolates (R_1 to R12) with primer 2.

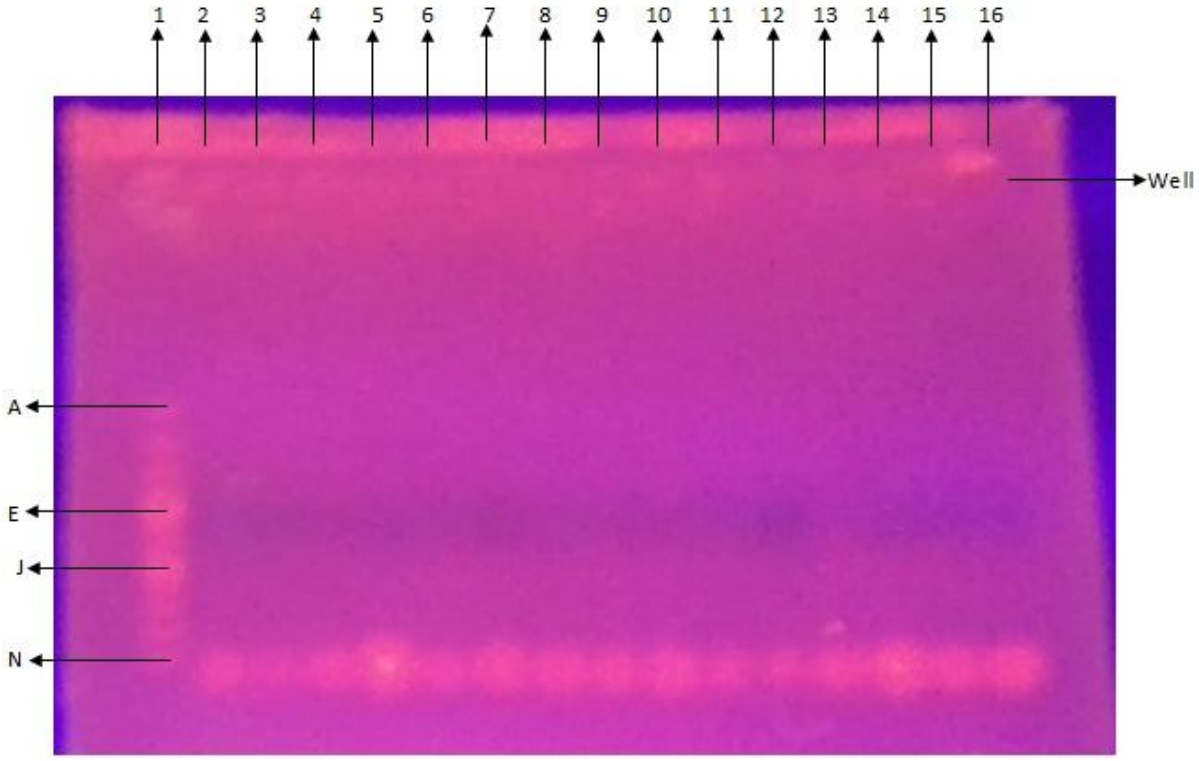


Plate 4. 5: Polymerase chain reaction products with primer 2 on 1% agarose gel electrophoresis (100v, 45 min) of plasmid DNAs from multiple antibiotic resistant and tranconjugant isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).
Lane 2 – 5: Transconjugants ($T_{4,}$ T_5, T_8 and$T_{12\ respectively}$) with primer 2.
Lane 6 – 13: Resistant *E. coli* isolates (R_{13} – R_{20}) with primer 2.
Lane 14: Recipient isolate (*Proteus mirabilis*) with primer 2.
Lane 15-16: Transconjugant ($T_{6\text{-}T7}$) with primer 2.

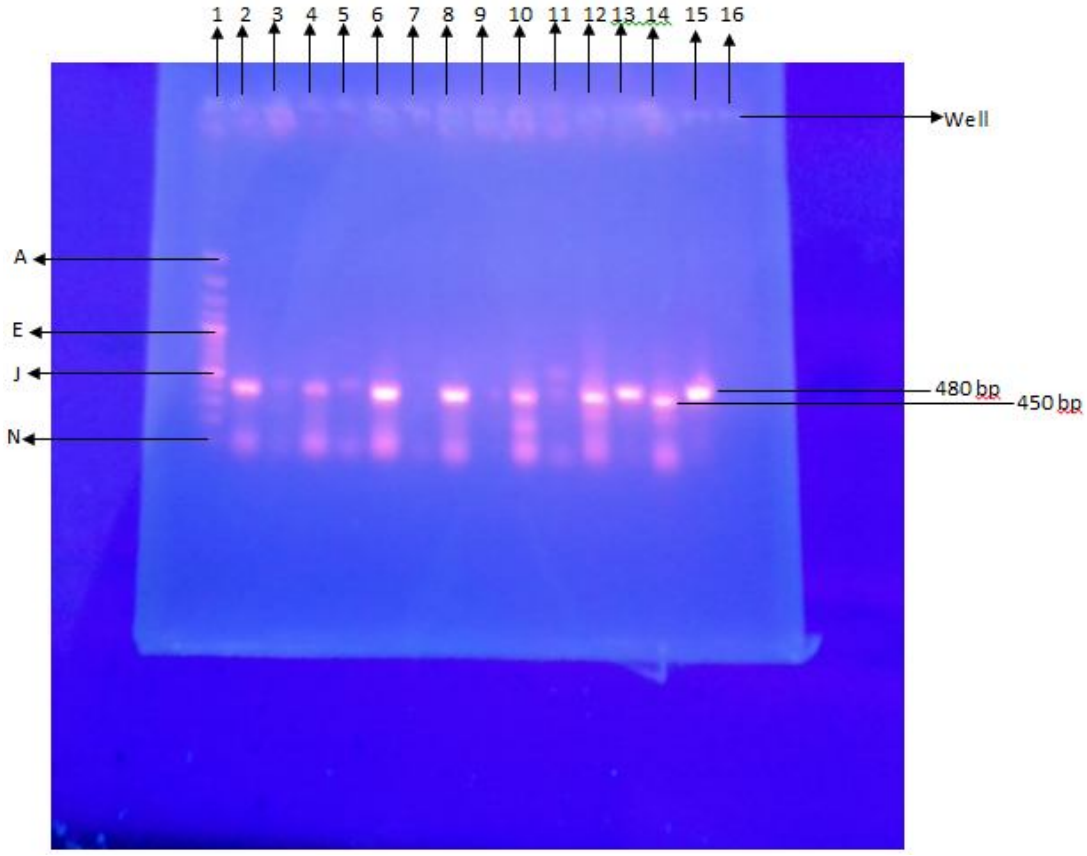


Plate 4. 6: Polymerase chain reaction products with primer 1 and 2 in alternate positions on 1% agarose gel electrophoresis (100v, 45 min) of plasmid DNAs from multiple antibiotic resistant *E. coli* and transconjugant isolates in Zaria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).

Lane 2 and 3: Resistant *E. coli* (R_{17}) with primer 1 and 2 respectively.

Lane 4 and 5: Transconjugant (T_9) with primer 1 and 2 respectively.

Lane 6 and 7: Transconjugant (T_{10}) with primer 1 and 2 respectively.

Lane 8 and 9: Transconjugant (T_{11}) with primer 1 and 2 respectively.

Lane 10 and 11:Resistant *E. coli* isolate (R_{14}) with primer 1 and 2 respectively

Lane 12 and 13:Resistant *E. coli* isolate (R_{15}) with primer 1 and 2 respectively

Lane 14 and 15:Resistant *E. coli* isolate (R_{16}) with primer 1 and 2 respectively

Lane 16: Empty

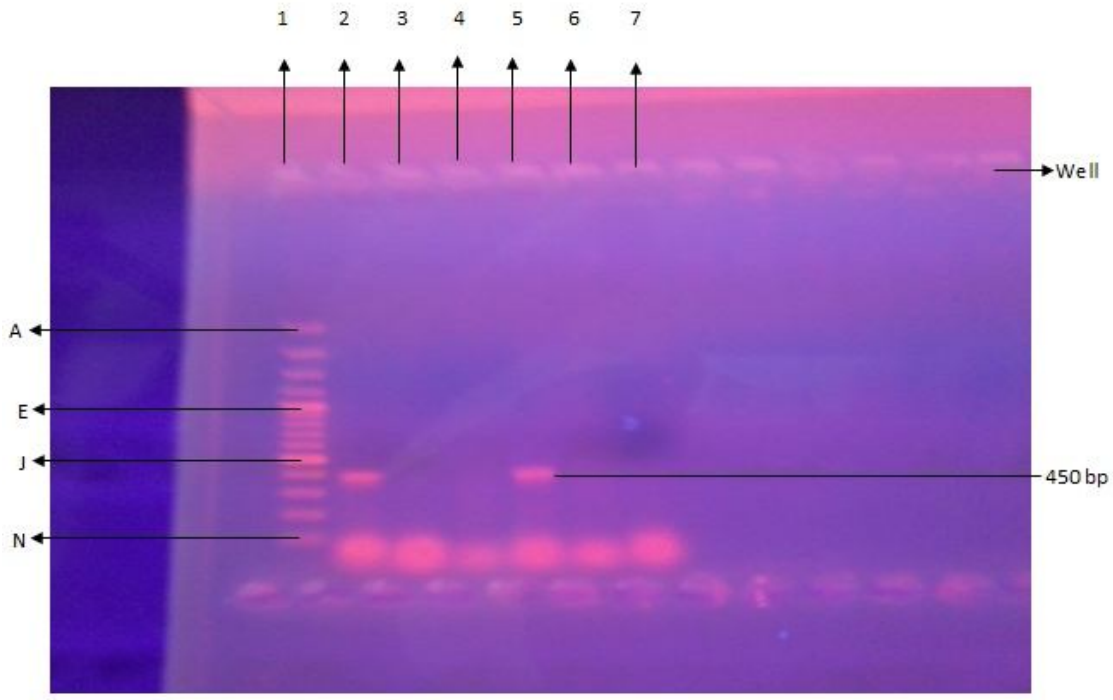
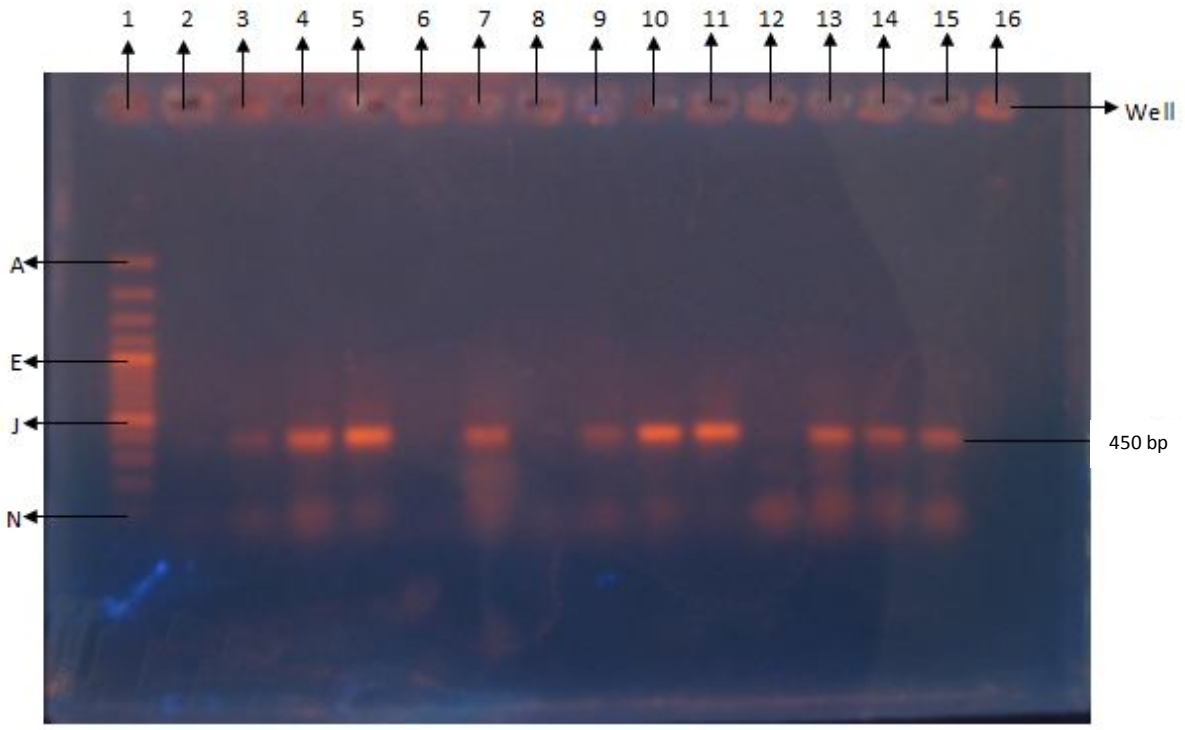


Plate 4. 7: Polymerase chain reaction products with primer 1 on 1% agarose gel electrophoresis (100v, 45 min) of plasmid DNAs from multiple antibiotic resistant *E. coli* and transconjugant isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).

Lane 2: Transconjugant (T_8) with primer 1; Lane 3: Transconjugant (T_7) with primer 1.

Lane 4-7 : Resistant *E. coli* isolate (R13,R18,R19 and R20 respectively) with primer 1



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Plate 4. 8: Polymerase chain reaction products with primer 1 on 1% agarose gel electrophoresis (100v, 45 min) of plasmid DNAs from multiple antibiotic resistant *E. coli* and transconjugant isolates in Zaria, Nigeria.

Lane 1: 100bp plus DNA ladder showing plasmid sizes of 3000(A), 2000, 1500, 1200, 1000 (E), 900, 800, 700, 600, 500 (J), 400, 300, 200, 100 (N).
Lane 2: Resistant *E. coli* isolate (R_1) with primer 1.
Lane 3 -11: Resistant *E. coli* isolates ($R_{2,R3,R4,R5,R8,R9,R10,R11\ and}$ $R_{12\ respectively}$) with primer 1.

Lane 12-16: Transconjugant ($T_{1\text{-}T5}$) with primer 1.

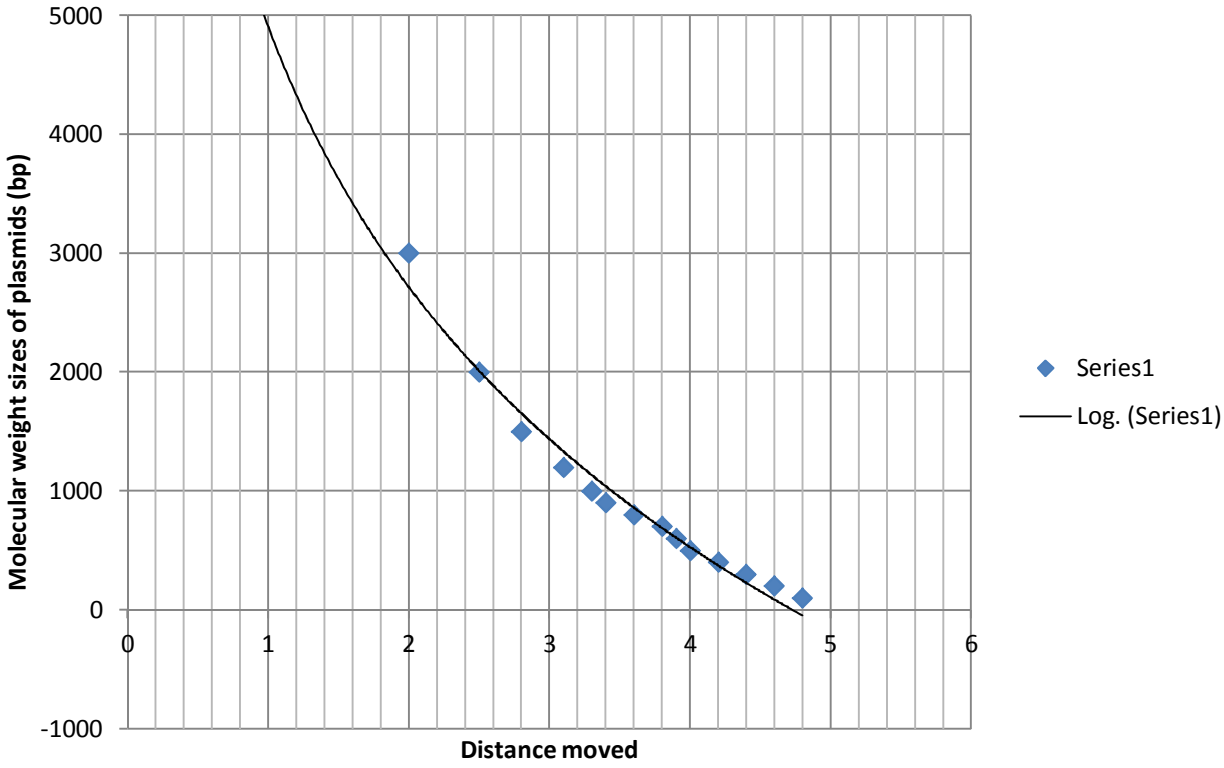


Figure 4. 2: Logarithmic graph of the molecular weight sizes of marker against the distance travelled in centimetres by the plasmid DNA.(Agarose)

Table 4. 12: Profile of resistant isolates and transconjugants harbouring plasmids.

Isolate no.	*E. coli* isolate	No. of plasmids	Estimated molecular sizes of plasmids (bp)
S_{18}	R_2	1	Higher than (>) 5000
K_{53}	R_{16}	1	Higher than (>) 5000
S_{36}	T_3	1	Higher than (>) 4000
K_{6b}	T_4	2	>4000 and 3200
S_{27}	T_5	2	> 5000, 2800
K_{49}	T_8	2	> 5000, 2800
K_{57}	T_{10}	1	2800
K_{58}	T_{11}	1	3,100
Total	8	11	

From the agarose gel electrophoresis of plasmid DNAs from multiple antibiotic resistant *E. coli* and transconjugant isolates carried out. Eleven plasmid bands were detected and some isolates were found to carry more than one plasmid band.(Table 4.12).

Table 4. 13: Profile of resistant isolates and transconjugants harbouring plasmids with their phenotypic resistant patterns.

Lab No.	Resistant isolate	Corresponding transconjugants	No. of plasmids	Estimated molecular weight (bp)	Phenotypic resistant patterns
S_{18}	R_2	□	2	>5000, 450	AML, CAZ, CN, CXM, TE, AMP
S_{36}	R_6	T_3	2	>4000, 450	AML, CAZ, CN, SXT, TE, AMP.
S_{27}	R_{10}	T_5	3	>5000, 2800, 450	AML, CAZ, CRO, CXM, SXT, CIP, TE, AMP.
S_{21}	R_3	□	1	450	AML, CRO, CAZ, CN, CXM, SXT, CIP, TE, AMP.
S_{29}	R_4	□	1	450	AML, CRO, CAZ, CXM, SXT, TE, AMP.
S_{30}		T_2	1	450	AML, CAZ, CXM, SXT, TE, AMP.
K_{6b}	R_8	T_4	5	>4000, 3200, 480, 450, 450	AML, CRO, CAZ, CN, CXM, SXT, CIP, TE, AMP.
K_{57}	R_{18}	T_{10}	3	2800, 450, 450	AML, CRO, CAZ, CXM, SXT, TE, AMP.
K_{58}		T_{11}	2	3100, 450	AML, CRO, CAZ, CXM, SXT, TE, AMP.
K_{52}	R_{15}	□	2	480, 450	AML, CRO, CAZ, CXM, CN, SXT, TE, AMP, CIP.
K_{53}	R_{16}	T_9	4	>5000, 480, 450, 450	AML, CRO, CAZ, CXM, SXT, CIP, TE, AMP.
K_{49}	R_{14}	T_8	4	>5000, 2800, 450, 450	AML, CRO, CAZ, CXM, SXT, CIP, TE, AMP.
K_{56}	R_{17}	□	1	450	AML, CRO, CAZ, CN, CXM, SXT, CIP, TE, AMP.
K_{37}	R_{11}	□	1	450	AML, CAZ, CXM, SXT, TE, AMP.
K_{47}	R_{12}	□	1	450	AML, CRO, CAZ, CXM, SXT, TE, AMP.
Total	13	8	33		

KEY

bp = base pair

Plasmid DNA prevalence analysis			
450 bp	19x	3100 bp	1x
480 bp	3x	3200 bp	1x
2800 bp	3x	≥4000 bp	2x
		≥5000 bp	4x

CHAPTER FIVE

5.0 DISCUSSION

5.1 ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY RESULTS OF *E. coli.*

The results obtained from the isolation rates of *E. coli* from the three study sites, shows that there is a high prevalence of *E. coli* infection among children which correlates with works documented by previous workers (Ogunsanya et al, 1994; Akinyemi et al, 1998; O'Ryan, et al, 2005; Bryce, et al, 2005; Afset, 2007 and Ifeanyi et al, 2010).

The results of antibiotic susceptibility in this study also correlates with the reports of previous studies that documented high resistant *E. coli* isolates to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazole.(Aibinu *et al*, 2004: Ngwai *et al.* 2005; Nwanze et al, 2010). It has been reported that broad spectrum antibiotics are sometimes given in place of narrow spectrum antibiotics as a substitute for culture and sensitivity tests, with the consequent risk of selection of antibiotic-resistant mutants.(Iruka *et al* .,1999). This situation is made worse by patients not completing their course of medication, probably as a result of ignorance or poor financial level (Iruka *et al*.,1999). This may be the reason for high level of resistance and low sensitivities of the *E. coli i*solates observed in this study. *E. coli* isolates resistance to multiple antibiotics was common in this study. This study is in contrast to the work carried out by Nkang and co workers (2009) who carried out a study in Calabar, Nigeria and observed that *E. coli* was susceptible to antibiotics like ciprofloxacin, gentamicin, tetracycline, sulphadoxine/trimethoprim, amoxicillin and ampicillin among others with 90.9% susceptibilty percentage .

Sixteen isolates (16/70) (22.9%) were found to be resistant to all the antibiotics used. This shows that there are more antibiotic resistant *E. coli* in the study which could probably have evolved

from an environment where antibiotics are being abused or misused. If this current trend continues, the emergence of resistant *E. coli* strains to other useful antibiotics may probably follow and a grave situation may soon arise when there will be no antibiotics to treat life threatening bacterial infections caused by these resistant *E. coli* strains.

Observations from the results of the minimum inhibitory concentrations of the test antibiotics against resistant *E. coli* isolates showed that amoxicillin was the least active while ciprofloxacin gave the highest antibacterial activity against the tested *E. coli* isolates. Also Cefuroxime showed low level antibacterial activity against test *E. coli* isolates with only 15.7% of the bacteria sensitive (Table 4.2), this is possibly due to the fact that cefuroxime is orally available and so can be easily abused or misused.

The study of antimicrobial resistance has been a major driving force in the understanding of many genetic and biochemical process in bacterial cells. The high level of isolation of beta-lactam resistant *E. coli* may probably suggest frequent abuse of beta-lactam antibiotics in this locality.

Barrett *et al.,* (1999) reported *E. coli* isolates from children were 98.9% sensitive to ciprofloxacin . In this study, 60% and 64.3% of the *E. coli* isolates were sensitive to ciprofloxacin and gentamicin respectively. This also correlates with the work reported by Nwanze and co workers, (2010) and Chigor and co workers, (2010). The observed moderate level of gentamicin activity in this study may be due to the fact that it is a parenteral antibiotic and also the moderate level of ciprofloxacin activity is possibly due to fact that quinolones are not used to treat children below 12 years. Also from this study, *E. coli* isolates from male children were found to be more sensitive to ciprofloxacin than gentamicin while the *E. coli*

isolates from the female children were more sensitive to gentamicin than ciprofloxacin (Table 4.3). The reason for this variation is not clear although no statistical difference exist among them.

Decrease in antibacterial activity of fluoroquinolones has been reported to increase in many countries and emergence of resistance of bacterial isolates to fluoroquinolones has become prevalent. Consistent stepwise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) by Bolon and co-workers, (2004). Ciprofloxacin resistance in Portugal was 25.8% and 24.3% in Italy while in Germany and Netherlands it was reported to be 15.2% and 6.8% respectively (Oteo *et al*., 2005). In this study, the percentage of ciprofloxacin resistance observed was 40%. This therefore suggests that there is an increase in the prevalence of resistant isolates of *E. coli* against ciprofloxacin and this agrees with what was reported by other workers (Bolon *et al* ., 2004; Oteo *et al*., 2005)

Seventeen different phenotypic *E.coli* resistant patterns were observed in this study with 98.6% of the *E. coli* isolates showing multiple antibiotic resistance to at least three classes of antibiotics. This observation could suggest that the resistant isolates in this study may probably originate from the environment where antibiotics are often used (Krumperman, 1983; Paul *et al.*, 1997; Ngwai *et al.,* 2005), or it is possible that the resistance in the study is innate.

5.2 R-PLASMID INVESTIGATION

Twenty resistant isolates from the antibiotic susceptibility testing results were used for conjugation studies, of which thirteen were from Kofan-Gaya and seven from the Primary health care centre,Samaru . The result of the conjugation studies showed that some of the antibiotic resistance genes were likely plasmid-encoded and transmissible. The MIC of ciprofloxacin was done before and after

conjugation in other to ascertain if there was plasmid transfer between the donor and recipient organisms. The increase in the MIC value of ciprofloxacin after conjugation shows that the recipient had taken the characteristics of the donor resistant *E. coli* donor isolates.

The curing of transconjugants showed a reduced MIC value for ciprofloxacin against the same transconjugants. The MIC of ciprofloxacin against the cured transconjugant decreased as compared to the values obtained before the curing. This shows that the curing of the plasmid from the transconjugants with the acridine orange dye has affected the change in MIC values observed (Table 4.9).

Plasmid analysis on agarose gel electrophoresis of 35 isolates revealed a total of 11 plasmid bands on agarose gel electrophoresis (Plates 4.1-4.3) and 22 plasmid genes on PCR with primers 1 and 2 (Plates 4.4 to 4.8). The bands did not occur randomly, this observation disagrees with a previous work carried out by Faruque *et al.*(1993) who analyzed 69 clinical isolates of *E. coli* and found 29 different plasmid bands. In the work documented, none of the plasmids were found to be common in all the strains indicating that none of these plasmids carried information for characteristics essential for the bacteria to survive in the environment and within the host. In other words, there were no functional constraints on these plasmids to be stably maintained in the host. This was in agreement with the assumptions that plasmids usually carry accessory characteristics (Hardy, 1986). In contrast to this report, in this study, some of the plasmid bands were found to be common in other resistant isolates (Plates 4.1,4.2 and 4.3) .

The plasmid analysis of *E. coli* isolates yielded five different plasmid profiles with estimated molecular weights of 2800bp, 3100bp, 3200bp ,>4000bp and >5000bp (Table 4.12). The highest molecular base pair plasmid profile which corresponds to >5000bp was shared by four resistant

isolates. From this study, it was observed that all the isolates which carried this plasmid band showed phenotypic resistance to amoxicillin, ceftazidime, cefuroxime, ampicillin and tetracycline (Table 4.13). So we can say that this plasmid possibly codes for resistance to the beta-lactam antibiotics, cephalosporins and tetracycline. Two resistant isolates shared the plasmid profile of >4000bp with phenotypic resistant pattern to amoxicillin, ceftazidime, cefuroxime, ampicillin, tetracycline, sulphamethoxazole/trimethoprim and gentimicin. Three isolates shared the plasmid profile of 2800bp which showed phenotypic resistance to amoxicillin, ceftazidime, cefuroxime, ampicillin, tetracycline, sulphadoxine/trimethoprim, ceftriazone and ciprofloxacin. One isolate each shared plasmid profile of 3200bp and 3100bp which showed phenotypic resistance to amoxicillin, ceftazidime, cefuroxime, ampicillin, tetracycline, sulphadoxine/trimethoprim, ceftriazone, ciprofloxacin and gentamicin for 3200bp, then amoxicillin, ceftazidime, cefuroxime, ampicillin, tetracycline, sulphadoxine/trimethoprim and ceftriazone for 3100bp.

The plasmid profiles observed in this study (Table 4.13), indicates a notable correlation between antibiotic resistance and plasmid presence. It was observed that all the eight isolates which were found to have plasmid bands showed close antibiotic resistant patterns to about four antibiotics, and five out of the eight isolates carried one plasmid band in them while the remainig three isolates carried two plasmid bands each making a total of eleven plasmid bands (Table 4.12). This report is similar to what was observed by Smith *et al.,* (2003) who reported plasmid range sizes from 0.564kb to >23kb in Lagos, Nigeria. It was also observed from this study that 5 resistant isolates out of eight were found to come from the General Hospital Kofan- Gayan, Zaria, Nigeria giving a percentage of 16.1% (5/31) of isolates harbouring plasmids, while three

resistant isolates (3/32), 9.4% were found to harbour plasmids from the Primary Health Care Centre, Samaru.

5.3 PRESENCE OF bla_{CTX-M} ESBL GENES

The designed primer (pCT008-009) adapted from Cottell *et al.,* (2011), was used on the plasmid DNAs of 29 resistant *E. coli* isolates for the detection of IncK epidemiologic plasmid of bla_{CTX-M} of ESBL encoding gene for amplification. PCR detection using this gene specific primers showed that three resistant *E. coli* isolates harboured the bla_{CTX-M} genes with molecular weight sizes of about 480bp. The finding of bla_{CTX-M} plasmid in this study is also consistent with the work done by Cottel *et al*., (2011). This suggests that the genes found are plasmid borne. It was observed in this study that the three resistant *E.coli* isolates which were observed to harbour these genes were from the General Hospital, Kofan-Gayan. According to Bonnet, 2004; Perez *et al* ., 2007 and Yohei, 2009, the CTX-M type of beta –lactamases which have become important, originated from beta-lactamases found in environmental species of the genus kluyvera, and this enzyme has been reported to hydrolyse cefotaxime and ceftriazone but is weakly active against ceftazidime . In this study, the resistant isolates which harboured plasmids coding for this resistance were found to be resistant to ceftriazone and ceftazidime. This study therefore agrees with the report of the other workers. (Bonnet, 2004; Perez *et al*., 2007 and Yohei, 2009).

In addition, according to Livermor *et al*., 2007, Yohei, 2009, and Cottell *et al*., 2011 the bla_{CTX-M} gene is considered the most prevalent ESBL-encoding gene worldwide and is replacing TEM and SHV types as the predominant ESBL in many European countries . The finding in this study may soon become wide spread in this locality if the pattern of resistance persist. The finding of this study also agrees with what was reported that the emergerce of *Escherichia coli* and

Klebsiella pneumonia resistant to ceftazidime and other cephalosporins seriously compromise the efficacy of these life saving antibiotics.(Perez *et al.*, 2007).

5.4 PRESENCE OF gyrB RESISTANT GENE OF THE QRDR

Fluoroquinolone resistant genes were detected by PCR using gene specific primer pairs (gene accession number D87842). This established primer (Primer 2) was used on the plasmid DNA of 28 resistant *E. coli* isolates for the amplification of a 448bp fragment of the QRDR of gyrB. gyrB genes were detected in 19 isolates with estimated molecular weight sizes of about 450bp. This also suggests that the genes for this resistance are plasmid borne and is consistent with the reports of other workers (Park *et al.,* 1998; Gomez *et al.,* 2004; Cattoir, 2009). These genes are known to be among the four genes responsible for quinolone resistance. The presence of this gene in nineteen *E. coli i*solates, indicates prevalence of the quinolone resistant plasmid genes in this study. This probably explains the reason for the observed increase prevalence of resistance to ciprofloxacin in this study. Also, thirteen out of the nineteen isolates found to harbour these resistant genes observed in this study are from the General Hospital, Kofan- Gaya and six from Primary Health Care Centre, Samaru.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

This study was carried out to evaluate the prevalence of *E. coli* in the stool of diarrhoeic children below five years in Zaria, Nigeria and to determine their antibiotic susceptibility profiles. A farther step was taken to study the plasmid profile, possible correlation between plasmid band on agarose gel electrophoresis and antibiotic sensitivity pattern and finally to find out if the IncK epidemiologic plasmid encoding gene of bla_{CTX-M} of ESBL and gryB encoding genes in quinolones were present in the resistant plasmid DNAs isolated.

The overall prevalence of *E. coli* from the study was 61.4%. The highest isolation rate of *E. coli* from the stool samples collected from children below five years was from the Primary Health Care Centre, Samaru. Zaria (74.4%), followed by the Institute for Child Care, Ahmadu Bello University Teaching Hospital, Ban-zazzau with 70.0% and Kofan- Gaya had a percentage isolation rate of 50.8%.

The results of the antibiotics susceptibility testing showed that most of the *E.coli* isolates were resistant to six antibiotics such as Amoxicillin, Ampicillin, Tetracycline, Sulphadoxine/ Trimethoprim, Ceftazidime and Cefuroxime. However, gentamicin showed the highest antibacterial activity against test *E. coli* isolates followed by ciprofloxacin and then ceftriaxone. There was a high prevalence of multidrug antibiotic resistance in this study.

Eleven (11) plasmid bands were observed in the resistant *E. coli* isolates studied. Their sizes ranged from 2800 to > 5000 bp. The genes for resistance to various antibiotics were probably distributed among plasmids of various sizes as well as the chromosomes. The detection of IncK

epidemiologic plasmid encoding gene of the bla_{CTX-M} for ESBL and gyrB encoding genes for resistance to ESBLs and quinolones respectively shows that the resistance determinants are plasmid mediated and they are found in our environment. The resistant genes observed in this study were from the General Hospital, Kofan- Gayan and Primary Health Care Centre, Samaru.

6.2 CONCLUSIONS

From the findings of the study, it may be concluded that:

(a) There was a high prevalence of *E. coli* infection among diarrhoeic children in Zaria, Nigeria.

(b) The studied *E. coli* isolates had 100% resistance to Amoxicillin, Ampicillin and Tetracycline.

(c) The *E. coli* isolates were more susceptible to Gentamicin, Ciprofloxacin and Ceftriaxone.

(d) There was a high level of multiple antibiotic resistance among the isolates observed in this study.

(e) Eleven out of a total of thirty-five resistant isolates were found to harbour plasmid bands with sizes ranging from 2800 to >5000 bp.

(f) Three *E. coli* isolates were found to harbour resistant plasmids encoding IncK epidemiologic gene of bla_{CTX-M} for ESBL genes with molecular weight of 480 bp. All the three *E. coli* isolates carrying this gene were from the same centre, General Hospital, Kofan- Gaya, Zaria. Nigeria.

(g) Nineteen *E. coli* isolates were found to harbour resistance plasmid carrying gryB gene for quinolone resistance with molecular weight of 450 bp. Thirteen of these isolates were from the General Hospital, Kofan- Gayan and six from Primary Health Care Centre, Samaru. Zaria. Nigeria.

(h) Some of the multiple antibiotic resistant *E. coli* isolates were observed to be plasmid-mediated.

6.3 RECOMMENDATIONS

- Judicious use of antimicrobials i.e. using the appropriate antibiotics at the appropriate dosage and for the appropriate duration through the appropriate route of administration is an important means of reducing the selective pressure that helps resistant organisms emerge.

- There is the need for controlling the spread of multidrug-resistant organisms by providing sufficient personnel and resources for infection control in all healthcare facilities.
- Since antimicrobial resistant patterns are constantly evolving, and present global public health problems, there is the need for constant antimicrobial susceptibility surveillance. This will help clinicians provide safe and effective empiric therapies.
- There is also the need for serotyping of *E. coli* so as to be sure of the strains being isolated and the need for more molecular studies for the characterization of resistant genes and for further guide in treatment therapy.
- New antibacterial agents with different mechanisms of action are needed.
- It is difficult to out-smart organisms that have had several billion years to learn how to adapt to hostile environments, such as those containing antimicrobial agents. Yet, with sufficient efforts to use antimicrobial agents wisely both in the human and veterinary fields, in order to prevent the emergence of resistant organisms, and strict attention to infection control guidelines to limit the spread of resistant organisms when they develop, we should be able to stay at least one step ahead of the next resistant plague.

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APPENDIX

Table 1: Interpretative chart according to CLSI (2007).

S/no	Antibiotics	Zones of inhibition (mm)		
		Intermediate	Sensitive	Resistance
1.	Amoxicillin	14 – 17	≥ 18	13
2.	Ceftriazone	14 – 20	≥ 21	13
3.	Ceftazidime	15 – 17	≥ 18	14
4.	Gentamicin	13 – 14	≥ 15	12
5.	Cefuroxime	15 – 22	≥ 23	14
6	Sulphadoxine/ Trimethoprim	11 – 15	≥ 16	10
7.	Ciprofloxacin	16 – 20	≥ 21	15
8.	Tetracycline	12-14	≥ 15	11
9.	Ampicillin	14 – 16	≥ 17	13

ANTIBIOTIC SENSITIVITY RESULTS FOR 70 *E. coli* ISOLATES.

S/NO	ISOLATE CODE	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP	NO. OF RESISTANCE
1F	KG_1	R	R	R	R	R	R	R	R	R	9
2F	G_4	R	I	S	S	R	R	S	R	R	5
3F	$KG5_a$	R	R	R	I	R	R	R	R	R	8
4F	$KG5_b$	R	R	R	R	R	R	R	R	R	5
5F	$KG6_a$	R	R	R	R	R	R	R	R	R	9
6F	6_b	R	R	R	R	R	R	R	R	R	9
7F	7_a	R	R	R	R	R	R	R	R	R	9
8M	8_a^1	R	S	S	S	S	R	R	R	R	5
9M	8_a	R	S	S	R	S	R	R	R	R	6
10M	9	R	I	R	R	R	R	S	R	R	7
11F	10_a^1	R	R	R	R	R	R	R	R	R	9
12F	11_a	R	R	R	R	R	R	R	R	R	9
13M	K_{45}	R	R	R	S	R	R	R	R	R	8
14F	K_{37}	R	I	R	S	R	R	S	R	R	6
15F	K_{36}	R	R	R	S	R	R	S	R	R	7
16F	K_{26}	R	S	R	S	R	R	S	R	R	6
17M	S_{21}	R	R	R	R	R	R	R	R	R	9
18F	S_{20}	R	S	R	S	R	R	S	R	R	6
19M	S_{19}	R	R	R	R	R	R	R	R	R	9
20M	S_{18}	R	S	R	S	R	R	S	R	R	6

ANTIBIOTIC SENSITIVITY RESULTS FOR 70 *E. coli* ISOLATES.

S/NO	ISOLATE CODE	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP	NO. OF RESISTANCE
21M	S_{17}	R	R	R	S	R	R	S	R	R	7
22F	S_{16}	R	R	R	R	R	R	R	R	R	9
23M	S_{15}	R	R	R	S	R	R	S	R	R	7
24F	S_{12}	R	R	R	R	R	R	R	R	R	9
25M	S_{11}	R	R	R	R	R	R	S	R	R	8
26M	S_{10}	R	R	R	R	R	R	R	R	R	9
27F	S_{5}	R	S	R	S	R	R	S	R	R	6
28M	S_{1}	R	R	R	S	R	R	S	R	R	7
29F	S_{8}	R	R	R	S	R	R	S	R	R	7
30M	S_{14}	R	R	R	S	R	R	S	R	R	7
31F	S_{23}	R	R	R	R	R	R	R	R	R	9
32M	S_{24}	R	R	R	S	R	R	S	R	R	7
33M	S_{26}	R	R	R	S	R	R	S	R	R	7
34F	S_{27}	R	R	R	S	R	R	R	R	R	8
35M	S_{28}	R	R	R	S	R	S	R	R	1	6
36F	S_{29}	R	R	R	S	R	R	S	R	R	7
37F	S_{30}	R	I	R	S	R	R	S	R	R	6
38F	K_{38}	R	R	R	S	R	R	S	R	R	7
39F	K_{47}	R	R	R	S	R	R	S	R	R	7
40M	K_{48}	R	R	R	S	R	R	S	R	R	7

ANTIBIOTIC SENSITIVITY RESULTS FOR 70 *E. coli* ISOLATES.

S/NO	ISOLATE CODE	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP	NO. OF RESISTANCE
41M	S_{33}	R	I	R	S	R	R	R	R	R	7
42F	K_{49}	R	R	R	I	R	R	R	R	R	8
43M	K_{51}	R	R	R	R	R	R	S	R	R	8
44F	K_{52}	R	R	R	R	R	R	R	R	R	9
45F	K_{53}	R	R	R	S	R	R	R	R	R	8
46M	K_{54}	R	I	R	S	R	R	S	R	R	6
47F	K_{55}	R	S	R	S	R	R	S	R	R	6
48M	K_{56}	R	R	R	R	R	R	R	R	R	9
49M	K_{57}	R	R	R	S	R	R	S	R	R	7
50M	K_{58}	R	R	R	S	R	R	S	R	R	7
51F	K_{59}	R	R	R	R	R	R	R	R	R	9
52M	K_{60}	R	I	R	S	R	R	S	R	R	6
53M	K_{61}	R	S	R	S	R	R	S	R	R	6
54M	S_{34}	R	I	R	R	S	R	S	R	R	6
55M	S_{35}	R	I	R	R	I	R	S	R	R	6
56M	S_{36}	R	I	R	R	I	R	S	R	R	6
57M	S_{37}	R	I	R	S	I	R	S	R	R	5
58M	S_{38}	R	R	R	R	R	R	R	R	R	9
59F	S_{39}	R	I	R	S	S	R	R	R	R	6

ANTIBIOTIC SENSITIVITY RESULTS FOR 70 *E. coli* ISOLATES.

S/NO	ISOLATE CODE	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP	NO. OF RESISTANCE
60M	S_{40}	R	I	R	I	R	R	S	R	R	6
61F	S_{41}	R	I	R	S	R	R	S	R	R	6
62F	S_{42}	R	R	R	R	R	R	R	R	R	9
63F	S_{43}	R	R	R	S	R	R	S	R	R	7
64F	B_1	R	S	S	S	R	S	S	R	R	4
65F	B_2	R	S	R	S	R	R	R	R	R	7
66M	B_3	R	S	S	S	S	S	S	R	R	8
67M	B4	R	S	S	R	S	R	S	R	R	5
68M	B_8	R	I	R	S	R	R	S	R	R	6
69F	B_9	R	R	I	R	R	R	S	R	R	7
70F	B_{10}	R	I	R	S	R	R	S	R	R	6

MIC of ciprofloxacin against cured transcojugants from conjugation studies.

Ppl = 2.5-3µg/ml

		A 100	**B 50**	**C 20**	**D 12**	**E 6**	**F 3**	**D 1.5**	**H 0.75**	**T 0.375**	**J 0.1875**	**MIC for recipient**
1	$^{C}S_{16}$	--	--	--	--	--	--	--	--	--	++	0.375 µg/ml
2	$^{C}S_{18}$	--	--	--	--	--	--	--	--	++	++	
3	$^{C}S_{21}$	--	--	--	--	--	--	--	--	--	++	
4	$^{C}S_{29}$	--	--	--	--	--	--	--	++	++	++	
5	$^{C}S_{30}$	--	--	--	--	--	--	—	++	++	++	
6	$^{C}S_{36}$	--	--	--	--	--	--	--	--	++	++	
7	$^{C}K_{1}$	--	--	--	--	--	--	--	--	++	++	
8	$^{C}K_{6b}$	--	--	--	--	--	--	--	++	++	++	
9	$^{C}K_{10a}{}^{1}$	--	--	--	--	--	--	--	--	++	++	
10	$^{C}S_{27}$	--	--	--	--	--	--	++	++	++	++	
11	$^{C}K_{37}$	--	--	--	--	++	++	++	++	++	++	
12	$^{C}K_{47}$	--	--	--	--	--	--	++	++	++	++	
13	$^{C}K_{48}$	--	--	--	++	++	++	++	++	++	++	
14	$^{C}K_{49}$	--	--	--	--	++	++	++	++	++	++	
15	$^{C}K_{52}$	--	--	--	--	--	--	++	++	++	++	
16	$^{C}K_{53}$	--	--	--	--	--	--	--	--	--	--	
17	$^{C}K_{56}$	--	--	--	--	--	--	++	++	++	++	
18	$^{C}K_{57}$	--	--	--	--	--	--	++	++	++	++	
19	$^{C}K_{58}$	--	--	--	--	--	--	--	--	++	++	
20	$^{C}K_{59}$	--	--	--	--	--	--	--	--	++	++	

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	8.0 ± 0	Nil
4	Nil	18.0 ± 0	22.0 ± 0	23.5 ± 0.71	14.0 ± 0	Nil	29.0 ±1.41	Nil	Nil
5a	Nil	12.0 ± 0	Nil	13.0 ± 0	Nil	Nil	Nil	Nil	Nil
5b	Nil	14.5 ± 0.71	17.5 ±0.71	20.0 ± 0	12.5 ± 0.71	Nil	Nil	Nil	Nil
6a	Nil	10.0 ± 0	Nil	9.0 ± 0	Nil	Nil	11.0 ± 0	Nil	Nil
6b	Nil	10.0 ± 0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
7a	Nil	10.5 ± 0.71	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8a	Nil	29.0 ± 1.41	30.0 ± 0	20.5 ± 0.71	20.0 ± 0	Nil	12.0 ± 0	Nil	Nil
8a	Nil	23.0 ± 1.41	27.0 ± 1.41	Nil	23.5 ± 0.71	Nil	Nil	Nil	Nil
9	Nil	15.0 ± 0	Nil	Nil	12.0 ± 0	Nil	23.5 ± 0.71	Nil	Nil
10a	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
11a	Nil	Nil	Nil	13.0 ± 0	Nil	Nil	Nil	Nil	Nil
45	Nil	11.5 ± 0.71	Nil	16.5 ± 2.12	Nil	Nil	10.0 ± 0	Nil	Nil

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
K37	Nil	15.0 ± 0	Nil	20.5 ± 0.71	Nil	Nil	29.0 ± 1.41	Nil	Nil
K36	Nil	Nil	Nil	19.0 ± 2.82	Nil	Nil	22.5 ± 0.71	Nil	Nil
K26	Nil	22.0 ± 5.65	10.5 ± 0.71	16.5 ± 2.12	Nil	Nil	30.0 ± 0	Nil	Nil
S_{21}	Nil	Nil	Nil	11.5 ± 0.71	Nil	Nil	Nil	Nil	Nil
S_{20}	Nil	23.0 ± 1.41	Nil	19.5 ± 0.71	Nil	Nil	29.0 ± 0.71	Nil	Nil
S_{19}	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
S_{18}	Nil	24.5 ± 0.71	Nil	18.0 ± 2.82	Nil	Nil	23.5 ± 0.71	Nil	Nil
S_{17}	Nil	Nil	Nil	16.5 ± 0.71	Nil	Nil	29.0 ± 0.71	Nil	Nil
S_{16}	Nil	Nil	Nil	11.0 ± 0	Nil	Nil	Nil	Nil	Nil
S_{15}	Nil	Nil	Nil	19.5 ± 0.71	Nil	Nil	21.0 ± 1.41	Nil	Nil
S_{12}	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
S_{11}	Nil	Nil	Nil	11.0 ± 0	Nil	Nil	22.5 ± 3.53	Nil	Nil
S_{10}	Nil	Nil	Nil	10.0 ± 0	Nil	Nil	Nil	Nil	Nil

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
S_5	Nil	28.0 ± 2.82	Nil	20.5 ± 0.71	Nil	Nil	30.0 ± 0	Nil	Nil
S_1	Nil	Nil	Nil	20.0 ± 0	Nil	Nil	25.0 ± 0	Nil	Nil
S_8	Nil	Nil	Nil	18.5 ± 0.71	Nil	Nil	25.0 ± 0	Nil	Nil
S_{14}	Nil	Nil	Nil	20.5 ± 2.12	Nil	Nil	25.5 ± 0.71	Nil	Nil
S_{23}	Nil	13.5 ± 0.71	Nil	10.5 ± 0.71	Nil	Nil	Nil	Nil	Nil
S_{24}	Nil	10.0 ± 1.41	Nil	20.0 ± 1.41	Nil	Nil	27.0 ± 1.41	Nil	Nil
S_{26}	Nil	9.0 ± 0	7.5 ± 0.71	20.0 ± 0	Nil	Nil	24.0 ± 1.1	Nil	Nil
S_{27}	Nil	8.5 ± 0.71	Nil	14.5 ± 0.71	Nil	Nil	11.5 ± 2.12	Nil	Nil
S_{28}	Nil	9.5 ± 2.12	Nil	16.5 ± 2.12	Nil	Nil	29.0 ± 1.41	Nil	Nil
S_{29}	Nil	11.0 ± 0	Nil	19.0 ± 1.41	Nil	7.0 ± 0	27.5 ± 2.12	Nil	Nil
S_{30}	Nil	18.5 ± 2.12	Nil	18.5 ± 0.71	Nil	6.0 ± .0	25.0 ± 1.41	Nil	Nil
K_{38}	Nil	12.5 ± 2.12	Nil	16.5 ± 0.71	Nil	Nil	20.0 ± 2.82	8.5 ± 0.71	Nil

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
$K_{4.7}$	NIL	NIL	Nil	20.5 ± 0.71	NIL	NIL	25.0 ± 1.41	NIL	NIL
$K_{4.8}$	Nil	10.0 ± 0	Nil	22.5 ± 2.12	Nil	Nil	27.0 ± 1.41	Nil	Nil
S_{33}	Nil	16.0 ± 1.41	Nil	15.0 ± 1.41	Nil	Nil	Nil	Nil	Nil
K_{49}	Nil	Nil	Nil	13.5 ± 0.71	Nil	Nil	Nil	Nil	Nil
K_{51}	Nil	Nil	Nil	11.0 ± 0	Nil	Nil	25.5 ± 0.71	Nil	Nil
K_{52}	Nil	Nil	Nil	10.0 ± 0	Nil	Nil	Nil	Nil	Nil
K_{53}	Nil	Nil	Nil	15.0 ± 1.41	Nil	Nil	Nil	Nil	Nil
K_{54}	Nil	14.5 ± 0.71	Nil	18.0 ± 0	Nil	Nil	25.0 ± 0	Nil	Nil
K_{55}	Nil	24.0 ± 1.41	Nil	21.0 ± 0	Nil	Nil	18.0 ± 0	Nil	Nil
K_{56}	Nil	Nil	Nil	9.5 ± 0.71	Nil	Nil	Nil	Nil	Nil
K_{57}	Nil	9.0 ± 0	Nil	19.5 ± 2.12	Nil	Nil	25.5 ± 0.71	Nil	Nil
K_{58}	Nil	Nil	Nil	21.0 ± 0	Nil	Nil	28.5 ± 2.12	8.5 ± 0.71	Nil

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
K_{59}	Nil	Nil	Nil	9.0 ± 0	Nil	Nil	Nil	Nil	Nil
K_{60}	Nil	15.0 ± 0	Nil	20.0 ± 0	Nil	Nil	26.0 ± 0	6.5 ± 0.71	Nil
K_{61}	Nil	26.0 ± 1.41	Nil	20.5 ± 0.71	Nil	Nil	26.5 ± 0.71	Nil	Nil
S_{34}	Nil	17.5 ± 2.12	Nil	10.0 ± 0	18.5 ± 0.71	Nil	26.5 ± 0.71	9.0 ± 0	Nil
S_{35}	Nil	20.0 ± 0	Nil	11.0 ± 0	15.5 ± 0.71	Nil	30.0 ± 0	7.5 ± 0.71	Nil
S_{36}	Nil	17.0 ± 1.41	Nil	10.5 ± 0.71	15.0 ± 1.41	Nil	27.0 ± 1.41	Nil	Nil
S_{37}	Nil	17.5 ± 0.71	9.0 ± 0	19.0 ± 0	16.5 ± 0.71	Nil	28.0 ± 0	Nil	Nil
S_{38}	Nil	11.0 ± 0	8.5 ± 0.71	10.0 ± 0	Nil	Nil	Nil	Nil	Nil
S_{39}	Nil	17.0 ± 0	Nil	17.5 ± 0.75	18.0 ± 0	Nil	12.0 ± 0	Nil	Nil
S_{40}	Nil	18.0 ± 0	Nil	14.0 ± 1.41	Nil	Nil	25.5 ± 0.71	Nil	Nil
S_{41}	Nil	17.0 ± 0	Nil	17.0 ± 0	Nil	Nil	26.0 ± 0	Nil	Nil
S_{42}	Nil	13.5 ± 0.71	Nil	11.5 ± 0.71	10.0 ± 0	Nil	15.0 ± 0	Nil	Nil

Zones of inhibition produced by 70 E. coli isolates for susceptibility tests.

	AML	CRO	CAZ	CN	CXM	SXT	CIP	TE	AMP
S_{43}	Nil	17.0 ± 1.41	Nil	20.5 ± 0.71	Nil	Nil	26.5 ± 0.71	Nil	Nil
B_1	Nil	21.0 ± 1.41	23.5 ± 3.53	25.0 ± 0	Nil	17.0 ± 1.41	38.0 ± 0	13.0 ± 0	Nil
B_2	Nil	21.0 ± 0	Nil	16.5 ± 2.12	Nil	Nil	Nil	Nil	Nil
B_3	Nil	29.0 ± 1.41	25.5 ± 0.71	21.0 ± 1.41	17.5 ± 3.53	15.5 ± 0.71	30.0 ± 0	Nil	Nil
B_4	Nil	30.5 ± 0.71	26.0 ± 0	12.0 ± 0	20.5 ± 0.71	Nil	29.0 ± 1.41	Nil	Nil
B_8	Nil	16.0 ± 1.41	Nil	18.0 ± 0	Nil	Nil	26.0 ± 0	Nil	Nil
B_9	Nil	11.0 ± 0	17.0 ± 1.41	Nil	Nil	Nil	22.0 ± 1.41	Nil	Nil
B_{10}	Nil	20.0 ± 0	Nil	20.0 ± 0	Nil	Nil	29.5.± 0.71	Nil	Nil

Distances travelled by the Plasmid DNA marker on Agarose Gel during Electrophoresis at 100V for 45mins

Distance moved in cm	Molecular weight of plasmid maker
2	3000
2.5	2000
2.8	1500
3.1	1200
3.3	1000
3.4	900
3.6	800
3.8	700
3.9	600
4	500
4.2	400
4.4	300
4.6	200
4.8	100

T-Test to compare resistance between males and females

Group Statistics

	sex	N	Mean	Std. Deviation	Std. Error Mean
Amoxiline	female	36	7.00	1.493	.249
	male	33	6.76	1.415	.246
Cetriaxone	female	20	10.15	1.268	.284
	male	20	10.10	1.518	.340
cefradine	female	33	10.09	1.466	.255
	male	31	10.13	1.544	.277
Gentamicin	female	12	9.58	1.564	.452
	male	14	10.29	.994	.266
Cefroxine	female	36	10.42	1.105	.184
	male	27	10.41	1.845	.355
Cotrimoxazole	female	36	7.50	1.464	.244
	male	32	7.41	1.583	.280
Ciproflaxine	female	18	11.28	1.638	.386
	male	9	10.56	1.509	.503
tetracycline	female	37	9.03	1.708	.281
	male	33	9.42	1.751	.305
Ampiciline	female	37	7.41	2.362	.388
	male	33	6.94	2.091	.364

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amoxiline	Equal variances assumed	.060	.808	.691	67	.492	.242	.351	-.458	.943
	Equal variances not assumed			.692	66.919	.491	.242	.350	-.456	.941
Cetriaxone	Equal variances assumed	.019	.891	.113	38	.911	.050	.442	-.845	.945
	Equal variances not assumed			.113	36.830	.911	.050	.442	-.846	.946
cefradine	Equal variances assumed	.001	.973	-.101	62	.920	-.038	.376	-.790	.714
	Equal variances not assumed			-.101	61.186	.920	-.038	.377	-.791	.715
Gentamicin	Equal variances assumed	1.548	.225	-1.387	24	.178	-.702	.506	-1.748	.343
	Equal variances not assumed			-1.340	18.103	.197	-.702	.524	-1.803	.398
Cefroxine	Equal variances assumed	11.382	.001	.025	61	.980	.009	.373	-.738	.756
	Equal variances not assumed			.023	39.737	.982	.009	.400	-.799	.818
Cotrimoxazole	Equal variances assumed	.003	.955	.254	66	.801	.094	.370	-.644	.832
	Equal variances not assumed			.252	63.523	.801	.094	.371	-.648	.836
Ciproflaxine	Equal variances assumed	.128	.724	1.107	25	.279	.722	.652	-.621	2.066
	Equal variances not assumed			1.139	17.364	.270	.722	.634	-.614	2.058
tetracycline	Equal variances assumed	.553	.460	-.960	68	.340	-.397	.414	-1.223	.428
	Equal variances not assumed			-.959	66.676	.341	-.397	.414	-1.224	.430
Ampiciline	Equal variances assumed	1.954	.167	.869	68	.388	.466	.536	-.604	1.536
	Equal variances not assumed			.876	67.998	.384	.466	.532	-.596	1.528

T-Test to compare resistance between males and females (MIC)

Group Statistics

	sex	N	Mean	Std. Deviation	Std. Error Mean
MIC Amoxicilline	female	37	1000.00	.000[a]	.000
	male	33	1000.00	.000[a]	.000
MIC Tetracycline	female	37	32.39	163.536	26.885
	male	32	4.69	2.599	.459
MIC Ciprofloxacine	female	37	16.93	32.400	5.326
	male	32	9.56	25.578	4.522
MIC Cetronoxine	female	37	226.12	263.292	43.285
	male	33	188.80	267.612	46.585

a. t cannot be computed because the standard deviations of both groups are 0.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MIC Tetracycline	Equal variances assumed	3.388	.070	.957	67	.342	27.704	28.942	-30.063	85.472
	Equal variances not assumed			1.030	36.021	.310	27.704	26.889	-26.828	82.237
MIC Ciprofloxacine	Equal variances assumed	2.837	.097	1.038	67	.303	7.374	7.107	-6.812	21.560
	Equal variances not assumed			1.055	66.485	.295	7.374	6.987	-6.574	21.322
MIC Cetronoxine	Equal variances assumed	.073	.788	.587	68	.559	37.317	63.531	-89.456	164.091
	Equal variances not assumed			.587	66.829	.559	37.317	63.591	-89.616	164.251

Oneway ANOVA to compare resistance in the 3 study sites.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Amoxiline	K	29	7.28	1.556	.289	6.68	7.87	5	10
	S	33	6.67	1.407	.245	6.17	7.17	4	10
	B	7	6.29	.756	.286	5.59	6.98	5	7
	Total	69	6.88	1.451	.175	6.54	7.23	4	10
Cetriaxone	K	22	10.23	1.232	.263	9.68	10.77	8	12
	S	17	9.94	1.600	.388	9.12	10.76	5	11
	B	1	11.00	.	.	.	.	11	11
	Total	40	10.13	1.381	.218	9.68	10.57	5	12
cefradine	K	28	10.39	1.343	.254	9.87	10.91	8	12
	S	33	9.91	1.608	.280	9.34	10.48	7	12
	B	3	9.67	1.528	.882	5.87	13.46	8	11
	Total	64	10.11	1.492	.186	9.74	10.48	7	12
Gentamicin	K	13	9.77	1.235	.343	9.02	10.52	8	12
	S	11	10.00	1.414	.426	9.05	10.95	6	11
	B	2	11.00	1.414	1.000	-1.71	23.71	10	12
	Total	26	9.96	1.311	.257	9.43	10.49	6	12
Cefroxine	K	30	10.50	1.656	.302	9.88	11.12	7	14
	S	28	10.32	1.307	.247	9.81	10.83	7	12
	B	5	10.40	1.140	.510	8.98	11.82	9	12
	Total	63	10.41	1.455	.183	10.05	10.78	7	14
Cotrimoxazole	K	30	7.63	1.159	.212	7.20	8.07	5	10
	S	33	7.48	1.805	.314	6.84	8.12	4	12
	B	5	6.20	.447	.200	5.64	6.76	6	7
	Total	68	7.46	1.510	.183	7.09	7.82	4	12
Ciproflaxine	K	16	10.81	1.797	.449	9.85	11.77	6	13
	S	10	11.20	1.229	.389	10.32	12.08	9	13
	B	1	13.00	.	.	.	.	13	13
	Total	27	11.04	1.605	.309	10.40	11.67	6	13
tetracycline	K	30	9.30	1.557	.284	8.72	9.88	6	12
	S	33	9.09	1.756	.306	8.47	9.71	6	12
	B	7	9.43	2.440	.922	7.17	11.68	6	13
	Total	70	9.21	1.727	.206	8.80	9.63	6	13
Ampiciline	K	30	7.50	2.193	.400	6.68	8.32	3	11
	S	33	7.18	2.417	.421	6.32	8.04	3	12
	B	7	5.86	.690	.261	5.22	6.50	5	7
	Total	70	7.19	2.235	.267	6.65	7.72	3	12

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Amoxiline	Between Groups	8.517	2	4.259	2.089	.132
	Within Groups	134.555	66	2.039		
	Total	143.072	68			
Cetriaxone	Between Groups	1.570	2	.785	.399	.674
	Within Groups	72.805	37	1.968		
	Total	74.375	39			
cefradine	Between Groups	4.162	2	2.081	.933	.399
	Within Groups	136.073	61	2.231		
	Total	140.234	63			
Gentamicin	Between Groups	2.654	2	1.327	.757	.480
	Within Groups	40.308	23	1.753		
	Total	42.962	25			
Cefroxine	Between Groups	.463	2	.231	.106	.899
	Within Groups	130.807	60	2.180		
	Total	131.270	62			
Cotrimoxazole	Between Groups	8.859	2	4.429	1.999	.144
	Within Groups	144.009	65	2.216		
	Total	152.868	67			
Ciproflaxine	Between Groups	4.925	2	2.463	.953	.400
	Within Groups	62.038	24	2.585		
	Total	66.963	26			
tetracycline	Between Groups	1.044	2	.522	.171	.843
	Within Groups	204.742	67	3.056		
	Total	205.786	69			
Ampiciline	Between Groups	15.319	2	7.660	1.559	.218
	Within Groups	329.266	67	4.914		
	Total	344.586	69			