MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANT *ESCHERICHIA COLI* ISOLATED FROM WILD AND DOMESTIC RATS TRAPPED IN AND AROUND NAIROBI, KENYA.

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

Dedicated to my parents Bernard Gakuya Nguru and Esther Wambui Gakuya.

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ABSTRACT

Drug-resistant micro-organisms belonging to the species *E. coli* remain a major health problem worldwide. They are of particular importance in developing countries where few therapeutic choices are available for treating infections. Few studies have addressed the epidemiological issues and the dynamics of antimicrobial resistance genes of *E. coli* causing disease in these countries. Antimicrobial resistance in rats as in other animals may have great impact on human health, particularly in an environment where humans and rats share the same ecosystem because of the zoonotic nature of *E. coli* infections. Furthermore, the tendency of rats to contaminate water sources and human food may lead to concentration of antimicrobial resistant *E. coli* with subsequent transmission to humans and other animals. This study was designed to determine if antimicrobial resistance occurs in *E. coli* isolated from rats living in the vicinity of human settlements. An attempt was made to determine if the resistance was encoded on plasmids and if it is transferable from one bacteria to another.

Rats were trapped from inside and outside the houses. *E. coli* and other members of the family enterobacteriaceae which included Proteus spp., Enterobacter spp., Citrobacter spp., Klebsiella spp., Morganella spp. and Salmonella spp., were isolated from contents of their gastro-intestinal tracts. Antimicrobial susceptibility tests were perfomed on *E. coli* isolates and plasmids isolated from the *E. coli* showing resistance. Transferable antimicrobial resistance was determined by the *in vitro* conjugation tests.

Rats were radomly trapped from the densely populated (slums) area of Kibera and the less densely populated areas of Kawangware and Kabete in Nairobi, Kenya. The significance of the three areas is that the densely populated areas have poor sewer systems and dumping sites hence there is high likelihood of contact between rats and human as opposed to the less densely populated areas. The proportions of *E. coli* isolated from rats trapped in Kibera and Kabete areas did not differ significantly (P=0.5658). The proportions did not also differ significantly depending on whether rats were trapped from inside or outside the houses (P=0.8696). Kawangware area was not used in the comparisons as no *E. coli* isolates were obtained.

Antimicrobial susceptibility to 11 antimicrobials was done using disc diffusion method. The antimicrobials tested were ampicillin (10 µg), co-amoxyclav (20:10 µg), sulphamethoxazole (10 µg), streptomycin (10 µg), tetracycline (10 µg), trimethoprim (5 µg), cefuroxime (30 µg), nalidixic acid (30 µg), gentamicin (10 µg), ceftazidime (30 µg) and ciprofloxacin (5 µg). Twelve (20%) of the 60 *E. coli* isolates tested were resistant to a single antimicrobial mainly ampicillin, streptomycin and sulphamethoxazole. Eight (13.3%) of the isolates showed multidrug resistance. Eight (13.3%) of the isolates were fully susceptible to all the 11 antimicrobials.

The minimum inhibitory concentrations (MICs) of the 60 *E. coli* isolates were also determined by the doubling agar dilution method. Fourteen (23.3%) of the isolates were resistant to ampicillin, 9 (15%) to streptomycin, 6 (6.6%) to co-trimoxazole, 2 (3.3%) to tetracycline and 1 (1.7%) to co-amoxyclav. The MIC_{50}

and MIC_{90} were exceptionally low except for ampicillin which were 16 and 64 µg, respectively. Exceptionally high MIC (128 µg\ml) was observed for only 2 (3.3%) isolates, one to ampicillin and the other to streptomycin.

There was no significant (P=0.2627) difference between the number of resistant *E. coli* isolates using the MIC or the disc susceptibility tests. A significant (P=0.0431) difference occurred between the number of resistant *E. coli* isolates from rats trapped in Kibera and Kabete areas but no significant (P=0.2884) difference occurred between resistant *E. coli* isolates from rats trapped inside and outside the houses.

Plasmid profile analysis grouped the 22 antimicrobial resistant *E. coli* into 5 plasmid groups depending on the number of plasmids each isolate carried. Sixteen (72.7%) of these isolates carried plasmids while 6 (27.3%) did not carry any plasmids. Plasmids of approximately 90-100 Mda, 55-65 Mda and 40-50 Mda were found at high frequency in resistant isolates and were thought to be responsible for the resistance.

In conjugation tests, resistance was only transferred from *E. coli* isolates resistant to ampicillin to *E. coli* K12. Five (35.7%) out of the 14 isolates resistant to ampicillin showed transferable resistance. Three (60%) out of 5 transconjugants carried plasmids while for the other 2 no plasmids were recovered.

In conclusion, rats may act as a pool of antimicrobial resistant *E. coli* and their plasmids. These antimicrobial resistant *E. coli* may be transmitted to humans and form their normal gastro-intestinal tract microflora with subsequent transfer of the resistance to other pathogenic micro-organisms. Further studies are recommended to determine if any relationships exist between plasmids carried by antimicrobial resistant *E. coli* isolates from rats, humans and other domestic animals.

CHAPTER ONE

1.0 INTRODUCTION

Escherichia coli is one of the predominant bacterial species that forms the normal flora of intestines in humans and other animals (Atlas, 1984). Most strains in this species are non-pathogenic but some have acquired a diarrheagenic ability and cause infection in humans (Honda, 1992). *E. coli* is an important cause of acute gastroenteritis which manifests with high morbidity and mortality in children (Senerwa *et al.*, 1989), urinary tract and wound infections (Holts *et al.*, 1994) and traveller's diarrhoea (Dupont *et al.*, 1982).

There has been a controversy over the natural ecology of *E. coli* and itsInfectious plasmids. Whether *E. coli* isolates and their plasmids are derived from humans and transmitted to animals or vice versa is still unclear. It is thought by some that animals may serve as reservoirs for *E. coli* strains found in humans and that the frequency of transmission to humans of *E. coli* strain containing antibiotic resistance plasmids could be as high as 78% within 2 days of exposure (Levy *et al.*, 1976).

The extensive use of antibiotics for the treatment of bacterial infections in humans and animals selects for resistant micro-organisms which may in turn transfer resistance to other bacteria thereby enhancing their spread (Levy *et al.*, 1988). The transfer of resistant bacteria has been shown to occur among different animal species, between humans and from animals to humans and vice versa (Rolland *et al.*, 1985; Marshall *et al.*, 1990). Antibiotic resistant *E. coli* may be passed from animals to humans through contact with faecal material or

faecal contaminated water. Normal *E. coli* flora acquire resistance plasmids from the ingested resistant *E. coli* strains. Eventually the antimicrobial resistant normal *E. coli* flora disseminate resistance plasmids to pathogenic bacteria (Espinasse, 1993).

Although use of antimicrobials for treatment and prophylaxis in human and veterinary medicine has resulted in spectacular improvement of infections (Espinasse, 1993), their indiscriminate use has provided a selective advantage to resistant bacteria and accelerated spread of the genetic elements coding for resistance (Mazodier and Davies, 1991). Multi-drug resistant *E. coli* has already been implicated as an important cause of childhood diarrhoea in Kenya (Senerwa *et al.*, 1989, 1991; Chunge *et al.*, 1992).

Antibiotic resistance in animals may have great impact on human health, especially in an environment where human and animals share the same ecosystem (Kruse and Sorum, 1994). Rats contaminate water sources and with their tendency to invade houses with subsequent contamination of food and utensils (Centre for Disease Control, 1988), may act as an important vehicle of transmission of antibiotic resistant *E. coli* and their plasmids.

Justification of the study.

Rats and humans live in close proximity in houses. Rats invade houses, eat and contaminate stored food, cooked food and water. The contamination can occur through faeces. On eating these foods, humans are exposed to *E. coli* which are

normal microflora of the rat gut. In farms, rats contaminate vegetables and other food crops with their faeces (Oboegbulem and Okoronkwo, 1990). If these are eaten raw or poorly cooked there is a possibility of transmission of *E. coli* and other bacteria through faeces of these animals to humans.

Rats acquire antibiotic resistance to *E. coli* through eating human foods containing antibiotic residues, eating human and animal faeces as well as fecal contaminated foods in sewer systems. These antibiotic resistant *E. coli* become part of the normal gut microflora and can be transmitted to humans (Espinasse, 1993). In humans these micro-organisms become part of the normal gut microflora and the resistance can be transferred to pathogenic *E. coli*, *Salmonella*, *Shigella*, *Yersinia* and other pathogenic micro-organisms which could be present in the gut.

During the day or when there are people in the house, rats may leave the house and hide in toilets and sewer systems where they get soiled with human waste. This waste can contaminate foods, hence rats can act as direct vehicles of antibiotic resistant *E. coli* from one human individual to another.

The areas where the study was conducted are densely populated with very poor sewer systems, and toilets are very close to the dwelling places. Contact of rats with human waste, foods, other animal waste and dumped refuse is highly possible. The present study was therefore designed to investigate if rats carry antibiotic resistant *E. coli* and their plasmids and if they could transmit them to

humans.

Specific objectives of the study.

1. To determine if antimicrobial resistance occurs in *E. coli* isolated from rats.
2. To determine both the antimicrobial susceptibility profiles and minimum inhibitory concentrations (MICs) of the *E. coli* isolates.
3. To determine if the antimicrobial resistance is encoded on plasmids.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Rats

Rats belong to the omnivorous rodents of worldwide distribution.

Taxonomically, their classifications have been a subject of several reviews (Kingdom, 1974; Oboegbulem and Okoronkwo, 1990; Karim, 1991; Dubey and Frenkel, 1998). According to Kingdom (1974), rats belong to the genus *Rattus* which is comprised of five species. These are the *Rattus mus, Rattus praomys, Rattus rattus, Rattus zerotomys and Rattus novergicus*. Kingdom (1974), further classified *R. rattus* into several subspecies. These include *Rattus r. rattus*, *Rattus r. frugivoras*, *Rattus r. wroughtoni*, *Rattus r. kijabius*, *Rattus r. rufescens*, *Rattus r. alexandrinus* and *Rattus r. diardii*.

Dubey and Frenkel (1998) classified rats into two genera, namely, *Rattus* and *Hydromys*. These authors indicated that the genus *Rattus* has the following species: *R. rattus, R. norvegicus, R. assimilitis, R. conatus, R. alexandrinus, R. exulans, R. villosissimus* and *Hydromys* having one species, *H. chrysogaster*.

Generally, rats belonging to the various species differ in sizes, fur characteristics and colours. The most common are the black rats, the *Rattus rattus* and brown rats, the *Rattus norvergicus*. In East Africa, *R. rattus* occurs everywhere while, *R. norvegicus* occurs in the coastal areas (Kingdom, 1974).

other related rodents include African great cane rat (*Thryonomys swinderinus*) (Oboegbulem and Okoronkwo, 1990), naked mole-rat (*Heterocephalus glaber*)

(Karim, 1991) and wild mice (*Mus musculus*)(Kingdom, 1974). The naked mole-rat (*H. glaber*) is a hairless rodent which lives in subterranean areas. They are also found in the arid regions of Kenya, Somalia and Ethiopia. They feed on roots and tubers (Karim, 1991).

2.2 Public Health importance of rats

Rats are of great importance as reservoirs of various bacterial microorganisms which cause diseases in humans and animals and further, they cause huge losses to stored foods, crops and property (Kayihura, 1982). Their tendency to invade houses in search of food support the idea that domestic and wild rats play major roles in the epidemiology of diseases in both human and animals (Centre for Disease Control, 1988). Rats have been implicated as reservoirs of various *Salmonella* species which cause salmonellosis (Kayihura, 1982), *Yersinia enterocolitica* which cause enteric yersiniosis (Egorov *et al.*, 1997), *Yersinia pestis* which cause plague (Cleri *et al.*, 1997) and *Streptococcus moniliformis* which cause Harverhill and Rat bite fevers (Wullenweber, 1995).

Other bacteria isolated from rats include *E. coli* (Moine *et al.*, 1987), *Yersinia pseudotuberculosis* (Fukushima and Gomyoda, 1991), *Pasteurella* species (Moine, *et al.*, 1987), *Bacillus piliformis* (Hansen, 1990), *Corynebacteria diphtheria* (Okewole *et al.*, 1990), *Treponema hyodysenteriae* (Blaha, 1983), *Clostridia* species (Leet, 1989), *Erysipelothrix rhusiopathiae* (Feinstein and Eld, 1989), *Leptospira* species (Zepeda *et al.*, 1986), *Campylobacter jejuni* (Meanger and Marshall, 1989), *Staphyloccus xylosus* and *Proteus mirabilis*

(Detmer *et al.*, 1991), and *Pseudomonas aeruginosa* and *Enterococcus durans* (Dietrich, 1987).

Salmonella species remain the most widely studied bacterial micro-organisms in rats with reports from Kenya (Kayihura, 1982), Nigeria (Oboegbulem and Okoronkwo, 1990), Malaysia (Joseph *et al.*, 1984), India (Singh *et al.*, 1980), Algeria (Mered *et al.*, 1980), U.S.A. (Ikeda *et al.*, 1986 ; Nathaniel et al., 1989) and Egypt (Abdi El-Ghan, 1977).

2.3 Overview of the family *enterobacteriaceae*

Members of the family *enterobactericeae* are facultative gram-negative rods that ferment glucose (Atlas, 1984). They are also oxidase negative and reduce nitrates to nitrites (except for some strains of the genera *Enterobacter*, *Erwinia*, *Klebsiella* and *Yersinia*). They may be motile by peritrichous flagella (except *Tatumella*) or are nonmotile (Holts *et al.*, 1994). Micro-organisms in this family form a major component of the normal intestinal flora of human and animals but are relatively uncommon in the normal flora of other body sites (Honda, 1992) . *Enterobactericeae* are important causes of nosocomial infections and account for over 50% of cases of septicemia in many hospitals (Kelly *et al.*, 1985). Although many of the *enterobactericeae* have been implicated as causes of diarrhoea, only members of the genera *Escherichia*, *Salmonella*, *Shigella* and *Yersinia* are clearly established as specific enteric pathogens (Holts *et al.*, 1994).

E. coli and related coliform bacteria predominate among the aerobic commensal

flora in human and animal gastro-intestinal tracts. *E. coli* also causes urinary tract infections (Boyd, 1984; Tsakris *et al.*, 1993). It has also been isolated from wounds (Holts *et al.*, 1994). Among the enterobacteria, *E. coli* is the major pathogen which affects different age-groups. It is the most important pathogen in the young and old individuals of poor economic and social status (Kariuki, 1996). In developed countries, *E. coli* rarely causes gastro-enteritis except for travellers' diarrhoea (Dupont *et al.*, 1982). In the tropics, however, *E.coli* is an important cause of acute gastro-enteritis which has high morbidity and mortality rates in children (Senerwa *et al.*, 1989). Thus, it is the one reviewed in the subsequent subsections and the main subject of this study.

2.4 *Escherichia coli*

2.4.1 History

In an attempt to identify the causative agent(s) of infantile diarrhoea, a German paediatrician Theodor Escherich isolated *Bacteria coli commune* (Sussman, 1985). This micro-organism is what is known as *Escherichia coli* today. The earliest indication of a specific virulence factor associated with *E. coli* was reported by De *et al.* (1956). They showed that certain strains of *E. coli* were associated with diarrhoeal diseases which caused secretion of fluids and electrolytes into the ileal loops of rabbits.

Although *E. coli* forms a part of the normal microflora of the gut of humans and animals it has been found to be potentially pathogenic for both humans and animals (Dupont *et al*, 1971; Mundell *et al.*, 1976; Field, 1979).

2.4.2 Morphology and biochemical characteristics

Escherichia coli belongs to the family enterobacteriaceae and is the only member of the genus *Escherichia*. Morphologically, *E. coli* is a short gram-negative, non-spore forming and usually peritrichous and fimbriate bacillus (Holts *et al.*, 1994). *E. coli* is a facultative anaerobe and most strains frequently ferment lactose although this fermentation may be delayed or even be absent in some cases (Holts *et al.*, 1994).

The major biochemical characteristics associated with *E. coli* are shown in Table 1

Table 1: Major biochemical characteristics of *Escherichia coli*

Optimum growth temperature	37°C
Catalase	+
Oxidase	-
D-galactosidase	-
Gas from glucose at 37□C	+
KCN (Growth on)	-
Mucate (acid)	-
Nitrate	+
G+c moles %	50-51
Carbohydrates	
(acid from)	
Adonitol	-
Arabinose	+
Dulcitol	d*
Esculin	d*
Inositol	-
Lactose	+ or X*
Maltose	+
Mannitol	+
Salicin	d*
Sorbitol	+
Sucrose	d*
Trehalose	+
Xylose	d*
Other carbon sources	
Citrate	-
Malonate	-
d-Tartrate	+*
Methyl red reaction	+
Voges-Proskauer reaction	-
Protein utilization	
Arginine	d*
Gelatin hydrolysis	-
H_2S from triple sugar iron medium	-
Indole production	+
Lysine decarboxylation	+
Ornithine	d*
Urea	-
Glutamic acid	-
Phenylalanine	-

d* refers to different reactions by different serotypes. X* refers to late and irregular positive (mutative).

SOURCE: **Holts *et al.*, 1994 in Bergey's manual of determinative bacteriology (9th ed.)**

2.4.3. Serology

The most useful way of classifying *E. coli* is serology based on antigenic properties of various structures of the bacterium. The serological classification has been reviewed by Kauffman (1966) and forms the basis of its typing.

Antigens used in the serological classification of *E. coli* include the O or somatic antigen, which denote the polysaccharide moeity of the cell wall, the K or capsular antigen, generally an acidic polysaccharide, and the H or flagellar antigen which are proteinaceous in nature. Currently 171 different O antigens (designated 01 to 171), 55 H antigens (H1 to H55) and 133 K antigens have been identified with the recognition and establishment of new antigens being co-ordinated by the WHO Centre for Reference and Research on *E. coli* in Copenhagen (Guinee *et al.*, 1980).

Less than half of the isolates obtained from humans have been specifically serotyped for two antigens, and only a third can be O:K:H serotyped (Caughant *et al.*, 1985). The proportions are even lower for isolates from animals (Bettelhein, 1978). Additionally, serotype antigens like some other phenotypic characters, may be lost during laboratory culture (Orskov and Orskov, 1983

2.4.4 Ecology

The primary habitat of *E. coli* is the gastro-intestinal tract of mammals and birds. This habitat has enabled the extensive use of *E. coli* as an indicator micro-organism of fecal contamination of water and foods (Atlas, 1984).

E. coli may show opportunistic pathogenicity by causing enteritis, urinary tract infections and neonatal meningitis and mastitis in cows (MacDonald *et al.*, 1970: Sarff *et al.*, 1975: Kariuki, 1996). The most important of these diseases is the *E. coli* associated enteric disease in children and young animals.

2.4.5 Enteropathogenicity

The different pathogenicity mechanisms involved in enteric infections have been classified in human as follows; (Honda, 1992)

1. Enteropathogenic *E. coli* (EPEC)
2. Enterotoxigenic *E. coli* (ETEC)
3. Enterohemorrhagic *E. coli* (EHEC)
4. Enteroinvasive *E. coli* (EIEC)
5. Enteroadherent *E. coli* (EAEC)
6. Enteroaggregative (EAggEC)

2.4.5.1 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* belongs to the O serogroups or serotypes O:H, and are capable of causing diarrhoea without producing enterotoxins (heat-labile or heat-stable) and are not invasive (Levine *et al.*, 1983). EPEC is responsible for many cases of infantile gastrointestinal disease with an appreciable mortality even in the developed world (Rowe, 1979). About 15 to 20 of the known *E. coli* serogroups (O, K and H) have been designated EPEC with the most prevalent serogroups worldwide being 011, 055, 026, 0119, 0127 and 0128 (Rowe, 1979).

2.4.5.2 Enterotoxigenic E. coli (ETEC

Enterotoxigenic *E. coli* cause diarrhoea by elaborating heat-labile toxin (LT) and heat-stable toxin (ST) (Clements and Finkelstein, 1979). Heat-labile enterotoxin (LT) is a protein, which has a molecular weight ranging from 72,000 to 150,000 daltons. It consists of antigenic determinants common to subunit A and B of cholera toxin (Clements and Finkelstein, 1979). The LT from human ETEC is closely related to, but distinct from, that found in porcine ETEC strains (Honda *et al.*, 1981).

Heat-stable enterotoxin (ST) from both human and animal ETEC strains have been purified and characterised (Staples *et al.*, 1980). Different purified preparations of ST appear to have different molecular weights ranging from 2000 to 5000 daltons. Burgess *et al.* (1978) described two *E. coli* ST activities, STA and STB. The STA is methanol-soluble, active in infant mouse assay and is found in ETEC of human and animal origins. The STB is methanol-insoluble, inactive in infant mouse assay but active in ligated pig and rabbit intestinal loop assays. The STB has only been identified in ETEC pathogenic for pigs (Kapitany *et al.*, 1979). No ETEC of human origin has been shown to produce STB.

The production of both LT and ST is encoded by transferable DNA plasmids with specific plasmids governing production of LT alone, LT and ST and ST alone (Wachsmuth *et al.*, 1976).

ETEC also possesses accessory virulence factors. The best characterised of these

factors are the adherence or colonization factors which permit attachment of ETEC to the mucosa of small intestines (Nagy *et al.*, 1977), permitting the release of enterotoxin close to the reactive sites. Overall, the disease caused by ETEC is clinically indistinguishable from clinical cholera (Mundell *et al.*, 1976 and Field, 1979).

2.4.5.3. Enteroheamorrhagic *E. coli* (EHEC)

Since 1983 reports of outbreaks of haemorrhagic colitis associated with *E. coli* 0157:H7 from the United States, it has been recognized with increasing frequency (Riley *et al.*, 1983). The microorganism produces cytotoxins referred to as verocytotoxin (VT) or shiga-like toxin which appears to play a major role in the pathogenesis of haemorrhagic colitis (Pai *et al.*, 1986). There is significant incrimination of VT-producing *E. coli* (VTEC) as cause of Hemolytic Uraemic syndrome (HUS) (Spika *et al.*, 1986).

2.4.5.4 Enteroinvasive *E. coli* (EIEC)

Ogawa *et al.* (1968) isolated *E. coli* of 0114:K, 043:K, 0136:K, 0124:K and 028 a,c:K73 O:K types and showed that these microorganisms possess invasive properties characteristic of virulence factors found in shigella strains. The EIEC does not produce enterotoxins, but invade the colonic mucosal cells. This invasive property was demonstrated by the guinea pig keratoconjuctivitis test (Sereny, 1955).

2.4.5.5 Enteroadherent *E. coli* (EAEC)

E. coli which do not belong to typical EPEC serotypes but adhere to Hep-2 cells were discovered in a diarrheric patient in 1985 and were named entero-adherent *E. coli* (EAEC). There are two types of adherence for EAEC. i.e. localized and diffuse (Mathewson et al., 1985), but detailed analysis of the properties has not yet been made.

2.4.5.6 Enteroaggregative (EAggEC)

These diarrheagenic bacteria are known to show characteristic "stacked brick" adherence not only to Hep-2 cells but to a glass surface (Vial, et al., 1988). These organisms are often isolated from patients with prolonged diarrhea persisting longer than 2 weeks.

2.5. Types of bacterial DNA used as genetic markers in epidemiological studies

2.5.1. Plasmids

Plasmids are double-stranded circular pieces of extrachromosomal DNA that can replicate and are independently inherited (Sambrook *et al.*, 1989). Plasmids carry genes conferring virulence or pathogenicity as well as antimicrobial resistance. Other characteristics that may be mediated by plasmids include tolerance to heat and metallic ions such as mercury (O'Brien et al., 1993). The presence of plasmids in bacteria is non-essential for their survival except in the face of a hostile external environment such as antibiotics. This factor contributes to the stability of plasmid-mediated resistance in bacteria. The presence of different

types of antimicrobial resistance genes on plasmids and their potential for transmission suggest that plasmids are major vectors in the dissemination of resistance genes through bacterial populations (Kariuki, 1996).

There are different types of plasmids that serve different functions (Atlas, 1984; Boyd, 1984). These include F (fertility) plasmids, Col plasmids, R (resistance) and Mercury and other heavy metals ion-resistant plasmids.

2.5.1.1 F (fertility) plasmids

The F plasmid code for mating behaviour in *E. coli*. The strains of *E. coli* which have the F plasmids are donor strains and those that lack the F plasmid are the recipient strains (Atlas, 1984). In addition the F plasmid can be integrated into the host chromosome. In this state they are called episomes (Boyd, 1984).

2.5.1.2. The Col plasmids

The Col plasmids are carcinogenic plasmids that carry the genes for a protein referred to as bacteriocin which is released into the environment and is lethal to related species of bacteria (Boyd, 1984). Most of the bacteriocins are produced by Gram-negative bacteria such as *E. coli* and their bacteriocins are referred to as colicins (Boyd, 1984). The Col plasmids may also carry the information necessary for bacterial conjugation (Atlas, 1984)

2.5.1.3. R (resistance) plasmids

The R plasmids carry genes that code for antibiotic resistance in many

microorganisms (Akiba *et al.*, 1960; Boyd, 1984; Atlas, 1984). The enzymes coded by genes of R plasmids are able to degrade antibiotics, thus conferring resistance on bacterial strains that possess them (Boyd, 1984). Some R plasmids also carry genes for conjugation (Atlas, 1984). The R plasmids can be passed from one bacterial species to another, such as from *E. coli* to pathogenic strains of *Shigella* or *Salmonella* (O'Brien *et al.*, 1982).

2.5.1.4 Mercury and other heavy metals ion-resistant plasmids

These plasmids confer resistance to certain heavy metals such as mercury, arsenic, cadmium and lead. They are most common in *Staphylococcus aureas* and *Pseudomonas* species (Akiba *et al.*, 1960).

2.5.2 Transposons

Transposons are short DNA sequences capable of inserting themselves into various locations on the bacterial chromosomes as well as into plasmids and bacteriophage DNA (Murray, 1982). Transposons cannot replicate independently, but they carry genes that are necessary for the process of transposition into a functional replicon (Chopra, 1990). The significance of transposons in dissemination of antimicrobial resistance in bacteria was reviewed by Kopecko *et al.* (1976). Transposons are more diverse in their interaction than plasmids. They have been known to transpose one or more resistance genes within one genus and even across genera of bacteria (Davies, 1994).

2.5.3 Integrons

The integron is a type of mobile genetic element found either as part of the transposons or independently on several groups of broad-host plasmids, coding for a site specific integration system (Davies, 1994).

Integrons make it possible for resistance genes from different locations on bacterial genomes to come together on mobile elements that could be incorporated into genetic replicons such as plasmids. Thus, resistance genes of diverse localities in bacteria (i.e. plasmid or transposon) may spread across bacteria on integrons (Davies, 1994). Several studies (Stokes and Hall, 1989; Bissonette and Ray, 1991) have shown that integrons are widespread in isolates of members of the family Enterobacteriaceae.

2.5.4 Insertion sequences

Insertion sequences are large (1 to 1.5 kb) nucleotide repeats that usually flank most transposons (Speer *et al.*, 1992). These transposons have been known to encode transposable antimicrobial resistance genes that have their origin either on the chromosome or on plasmid of a bacterium. Together with conjugative transposons, they play a major role in the dissemination of chromosome-mediated characteristics. In both prokaryotic and eucaryotic organisms, insertion sequences are known to play an important role in enhancing evolution by aiding the rearrangements of chromosomal DNA material.

2.6. Antimicrobial resistance

The increasing prevalence of bacterial resistance to commonly prescribed antimicrobials, especially in developing countries posess a major concern in the management of infections (Shanahan *et al.*, 1993; Nijsten *et al.*, 1996a). This has mainly resulted from extensive use, and often misuse of antimicrobials in both human and veterinary medicine (Kayser, 1993; Mitsuhashi, 1993). The finding that plasmids can spread between different species and genera has led to the concept of "epidemic plasmids". In one instance, a gentamicin resistance plasmid was found in eight states in the United States of America and two continents in multiple genera (O'Brien *et al.*, 1985).

2.6.1 The origin of antimicrobial resistance genes

Resistance genes originated as a protective mechanism for antibiotic producing micro-organisms (Benvensite and Davies, 1973). Thus, resistance genes existed even before the discovery and commercial purification of antibiotics. This is exemplified by the presence of aminoglycoside-modifying enzymes in aminoglycoside-producing strain of *Streptomyces* spp (Thompson and Gray, 1983) that are closely homologous to modifying enzymes found in bacteria resistant to aminoglycosides.

The origin of the enzymes that confers resistance to synthetic drugs trimethoprim and sulphonamide remain a complete mystery. It is possible that mutation in chromosomal genes encoding drug sensitive enzymes could have resulted in drug

resistance, although it is difficult to obtain a high level of resistance in the laboratory (Forster, 1983).

Although the role of R-plasmids as mediators of resistance was recognised as early as 1960 (Akiba *et al.*, 1960), it had been thought for sometime that R-plasmid mediated antibiotic resistance determinant may have evolved before antibiotic era (Forster, 1983). The origin of R-plasmids and the development of resistance was first reviewed by Watanabe (1971) and subsequently expanded to include the role of transposons and integrons as mediators of resistance (Kopecko *et al.*, 1976; Maltinez, 1990).

The widespread use of antimicrobial agents has accelerated the spread of genetic elements coding for resistance by giving a selective advantage to resistant bacteria (Mazodier and Davis, 1991). Resistance to antimicrobial agents is complicated by the fact that resistance genes may be located on plasmids, bacterial chromosomes and on transposons which are capable of integrating into plasmids and into the chromosomes (Clowes, 1972).

2.6.2 Types of antimicrobial resistance

The mechanisms of antimicrobial resistance can be classified as innate (natural) or acquired (Courvalin, 1996).

2.6.2.1 Natural antibiotic resistance

Some bacteria are naturally resistant to certain antimicrobial agents. Well studied

examples include the resistance of *Mycobacteria* and *Pseudomonas* spp. to most antibiotics, Gram-positive bacteria to nalidixic acid (Chopra, 1990) and most enterobacteria to macrolides(Courvalin, 1996). This form of resistance can be explained by the presence of a cell wall impermeable to the antimicrobials, or metabolic pathways that antimicrobials cannot influence. Normally, natural resistance is chromosomally-mediated and is thus predictable (Fling and Richards, 1983).

2.6.2.2 Acquired antibiotic resistance

Acquired resistance refers to the emergence of resistant strains within a bacterial population (Kallings, 1982). This form of resistance is the most important. Acquired resistance can be indigenous in bacteria, i.e., occurs through mutations. It can also be exogenous, whereby the bacteria acquires exogenous DNA from related or unrelated bacteria by transformation, transduction or conjugation (Courvalin, 1996).

2.6.3 Genetic basis of acquired antimicrobial resistance

Genes that code for resistance to antimicrobial agents may emerge from spontaneous mutation or may be acquired from other bacteria (Courvalin, 1996). These genes are located on the chromosome or plasmid or on both (Forster, 1983). Acquired resistance in bacteria results from R-plasmid transfer by conjugation, transduction or transformation. The distinction between plasmid encoded and chromosomally encoded resistance can become blurred because of the dynamic movements of transposons between plasmids and the chromosome

(Forster, 1983).

2.6.3.1 Mutations

Spontaneous mutations occur in a bacterial population at a frequency of 10^{-5} to 10^{-9} per generation (Kallings, 1982). Statistically, the probability of this type of resistance emerging in bacteria is minor but becomes significant when bacteria are exposed to selective antibiotic pressure (Doss, 1994), and the presence of other bacteria that facilitate the dissemination of resistance (Courvalin, 1996). Mutations are stably inherited by the progeny, they confer resistance to all members of a family of antibiotics and can be deleterious to the host bacterium hence are only transiently required (Courvalin, 1996).

Mutation in chromosomal DNA, without affecting the ability of bacteria to survive in the natural environment, would be expected to occur over a long period of time (Doss, 1994). The accumulation of chromosomal mutations would seem to be an unsatisfactory explanation to the rapid emergence of multiresistant bacteria (Forster, 1983).

2.6.3.2 Transferable resistance

Plasmid-mediated resistance may spread by three main routes; transformation, transduction and conjugation.

(a) Transformation.

Certain recipient bacteria can acquire high molecular weight DNA from the

surrounding medium thus transforming their genetic constitution (Stewart, 1989). The resistant strain can spread from person to person carrying its plasmid with it. This route was exemplified by an outbreak described by Green *et al.* (1973) in which a multi-resistant *Pseudomonas aureginosa* strain spread to infect several patients in a leukaemiac ward. The isolates from the patients and some from the environment were shown to be the same by several epidemiological typing methods, and all carried a similar plasmid which encoded resistance to carbencillin and aminoglycosides. The plasmid itself was non-transmissible and thus could only spread in the bacterial host population.

Transformation has been reported among pneumococci, haemophilus and neisseria spp. These bacteria are capable of excreting short, transforming DNA located on resistance plasmid or transposons in the slime capsule of certain recipient bacteria. If the bacteria are closely related, they may transfer DNA by homologous recombination into chromosomal locations of recipient bacteria (Courvalin, 1996). In many bacteria such as staphylococci, enterobacteria and pseudomonads, transformation is a less important mode of transfer of resistance.

(b) Transduction.

In transduction, the genetic material is carried from the donor cell by bacteriophages which infect the recipient cells (Kokjohn, 1989). For transduction to occur, transducing phage particles need to be produced from donor strains through infection with a lytic bacterial phage or by induction of a prophage. Calcium ions are required for the attachment of phage particles to cell surfaces,

and so transduction, as well as lytic phage activity can be inhibited by the presence of chelating agents such as citrate ions (Levin, 1985). The best examples are the transfer of plasmid-mediated penicillinase production in staphylococci and resistance to tetracycline and chloramphenicol in some enterobacteria (Murray, 1991).

(c) Conjugation.

Conjugation refers to the mating between donor cell (which has fertility factor,F^+) and a recipient cell (deficient of fertility factor, F^-). Conjugation is a phenomenon that occurs mainly among Gram-negative bacteria, but certain Gram-positive bacteria such as streptococci may also acquire resistance by conjugation (Murray, 1992). Among the three genetic mechanisms of acquiring antimicrobial resistance, conjugation is by far the most important in bacteria. Conjugation occurs *in vivo* and can cause epidemic spread of plasmid-mediated resistance on a worldwide scale, crossing borders between species and genera of bacteria (Davies, 1994).

Resistance genes can spread by means of conjugal transfer to another bacterial host of the same or different species. This may occur within a single host or in the environmental reservoir. The dissemination of a single resistance plasmid in this manner was described by O'Brien *et al.*(1982) when a plasmid encoding gentamicin-modifying enzyme was identified in several species of enterobacteriaceae.

The transfer genes, located together with the resistance genes on the plasmid, create a highly efficient mechanism for dissemination of antimicrobial resistance. Nearly 60-90% of resistance determinants in bacteria reside on plasmids. The transfer of R-plasmids is therefore the most important phenomenon in the spread of antimicrobial resistance (Kayser, 1993).

For conjugation to occur, pili or fimbriae projecting from the donor cell must be formed and this occurs only in F+ cells. The F-factor is principally carried on plasmid DNA, which may be transferable or non-transferable. During mating, the sex pilus on the donor cell extends to form contact with a receptor site on the surface of the recipient cell. Duplicated genetic material from the donor cell, usually extrachromosomal DNA, then passes along the pilus into the recipient cell (Courvalin, 1994). Streptococcus spp. however, do not form sex pili. Instead, a certain sex "hormone" is produced which stimulates production of aggregation factors facilitating cell-to-cell contact, and subsequent transfer of resistance genes to the recipient bacterial cell (Kallings, 1982).

2.6.4 Biomechanisms of acquired antimicrobial resistance Error! Bookmark not defined.

Bacteria become resistant to antimicrobial agents by one or more of the four biomechanisms; altering the target site of antibiotic, modifying the antibiotic so that it is no longer active, preventing the antibiotic from entering the cell and specifying an enzyme which provides a substitute for the bacteria-specific

enzymes which is the target of the antibiotic (Hardy, 1981; Davies, 1994).

2.6.4.1 Alteration of the target site of antibiotic

Resistance due to altered sites has been encountered for streptomycin, erythromycin and the ß-lactams. Resistance to streptomycin occurs due to mutation leading to altered ribosomes. Methylation of ribosomal RNA inhibits binding of erythromycin while altered penicillin binding proteins (PBP's) may lead to resistance to ß-lactams (Murray, 1989). An example of altered PBP's that result in resistance to ß-lactams is found in *Streptococcus pneumoniae* which does not produce its own ß-lactamases, but through stepwise mutations in the PBP's leads to resistance (Spratt, 1994).

2.6.4.2 Modifications of antibiotics

Drug modification causing inactivation of the drug is probably the most common mechanism of resistance to antimicrobial agents (Murray, 1991). These include resistances caused by ß-lactamase, chloramphenicol acetyltransferases and aminoglycoside modifying enzymes (Amyes and Gemmel, 1992). It has also been reported in resistance to macrolides, lincosamides, streptogramins and tetracyclines (Salyers *et al.*, 1990). Enzymes that inactivate antimicrobial resistance are usually plasmid or transposon mediated (Faragasan *et al.*, 1997).

2.6.4.3 Entry prevention of antibiotic into cells

This mechanism is most expressed in Gram-negative bacteria resistant to vancomycin and nafcillin, and that of enterococci to low level of aminoglycosides

(Murray, 1991). Decreased permeability of the cell wall may be explained by mutation of the porin gene (e.g. Omp C and Omp F in *E. coli*) that leads to altered non-specific aqueous diffusion channels normally used for entry of antimicrobials into the bacterial cell (Neu, 1992). This mechanism of resistance has also been reported to be the basis of cross resistance to chemically unrelated drugs such as resistance to tetracycline and chloramphenicol by *E. coli* (Chopra, 1990).

Decreased drug accumulation in a bacterial cell may also occur by an active energy-dependent efflux of the antimicrobial agents. The resistance to tetracycline, quinolones, macrolides and chloramphenicol is thought to occur through this mechanism (Neu, 1992). Resistance due to active efflux of the antimicrobial agent can either be acquired or may arise from mutations (Chopra, 1990).

2.6.4.4 Specifying an enzyme which provides a substitute for the bacteria specific enzyme which is the target of the antibiotic.

In this mechanism of resistance, the bacteria produce a novel enzyme that is not susceptible to conventional antimicrobial agents (Amyes and Gemmel, 1992). An example is the transferable resistance of Gram-negative bacteria to trimethoprim and the sulphonamide. Resistance to trimethoprim in enterobacteria is caused by a mutation that leads to the overproduction of the target enzyme, thus requiring a high inhibitor concentration (Spratt, 1994).

2.6.5 Resistance to ß-lactam antibiotics

Gram-negative bacteria are resistant to ß-lactam antibiotics through three mechanisms; production of ß-lactamases, reduced outer membrane permeability and diminished ability for the penicillin-binding proteins. The production of ß-lactamases is the most important in clinical isolates of Gram-negative bacteria (Sanders, 1992).

ß-lactamases are either found extracellularly in Gram-positive bacteria and in the periplasmic space in Gram-negative bacteria (Du Bois et al., 1995).

Resistance to ß-lactam antibiotics that result from production of ß-lactamases coded on plasmids in *enterobacteriaceae* is clinically important due to the ease with which plasmids are exchanged within species and even between different genera of bacteria. One of the most important enterobacterium that plays a major role in dissemination of plasmid mediated ß-lactamases is *E. coli* (Sanders, 1992). This bacterium does not harbour any ß-lactamases that normally confer resistance to ß-lactam antibiotics but resistance arises due to acquisition of new genes or to mutations affecting the expression of its constitutive chromosomal ß-lactamases (Sanders, 1992).

2.6.6 Cross-resistance between antimicrobial agents

In certain circumstances the application of one antibiotic may co-select for resistance to other related or non-related antibiotics. For example, London *et al.* (1994) observed an increase in prevalence of amoxycillin and tetracycline

resistance in faecal flora of patients treated with amoxycillin. Similarly, treatment of patients with doxycycline resulted in an increased prevalence of resistance to both tetracyclines and amoxycillin.

Cross-resistance to different antibiotics is a common phenomenon. A bacterium can become simultaneously resistant to several ß-lactams by the acquisition of a single ß-lactamase (Fisher, 1985). This is referred to as positive cross-resistance since the pleiotropic effect of the ß-lactamase is to increase resistance to these ß-lactams. Similarly, negative cross-resistance can develop if a resistance gene has a pleiotropic effect of increasing resistance to one antibiotic while decreasing resistance to a second one. For example, resistances to isoniazid and ethionamide in *Mycobacterium tuberculosis* are negatively correlated, as are resistances to rifampicin and novobiocin in a *Microspora* species and resistances to tetracycline and fusaric acid in both *Salmonella typhimurium* and *E. coli* (Maloy and Nunn, 1981; Gado *et al.*, 1982; Bochner *et al.*, 1980 and Canetti, 1965).

2.6.7 Epidemiology of antimicrobial resistance

There is convincing evidence that both subtherapeutic and therapeutic doses of antibiotics cause increased antibiotic resistance in the intestinal flora of animals (Linton, 1977). Resistance may be engendered not only to the antibiotic used but also to other antibiotics (Julia and Chan, 1987), because resistance genes are often linked.

The epidemiology of resistance reflects the spread of bacterial species themselves

(Levin, 1985). Kariuki *et al.* (1997) performed Mean Inhibitory concentration (MIC) of 11 antibiotics for 97 isolates of *S. typhimurium* and found that fifteen (16%) were fully sensitive. Nineteen (20%) were resistant to one antibiotic usually streptomycin or tetracycline. Forty-six (47%) were resistant to four or more antibiotics, commonly amoxycillin, chloramphenicol, co-trimoxazole, streptomycin and tetracycline, with MIC_{90} > 64 micrograms\ml. Thirty-four (35%) were resistant to cefuroxime. None was resistant to ceftazidime or ciprofloxacin. He further reported that an approximate 100kb plasmid, in addition to other smaller plasmids (3-10kb) were present in all resistant isolates. Fifty-four out of 82 (66%) resistant *S. typhimurium* transferred at least one resistance on the 100kb conjugative plasmid, the commonest being ApAmTmSuCmTe (Ampicillin, Amoxycillin, Trimethoprim, Sulfamethoxazole, Ceftazidime and Tetracycline). Cefuroxime resistance was non-transferable.

The increasing prevalence of antibiotic resistant non-typhi *Salmonella* strains create serious problems in human and animal hygiene, especially the zoonotic serovars (Tekorando et al., 1983). For example, in a study carried out in the USA by Tacket *et al.* (1985) involving an outbreak of *Salmonellosis* due to consumption of raw milk, multidrug resistant strains were isolated. The resistance, especially to chloramphenicol, reduced the number of therapeutic choices for the control of this epidemic. A single plasmid of 105 MDa was responsible for the multidrug resistance which transferred to *E. coli* at 25°C.

Espinasse (1993) observed that enterobacteria in healthy people were mainly

derived from foodstuff of animal and plant origins. He also observed that *E. coli* and other enterobacteria may also be passed from animal to humans by contact with faecal material or faecally-contaminated water. This author concluded that the interplay between human microflora and ingested resistant *E. coli* strains allow for acquisition of resistance by normal *E. coli* flora. Antimicrobial resistant *E. coli* flora may then act as a reservoir for dissemination of resistance plasmid to the pathogenic bacteria.

Antimicrobial resistant *E. coli* isolated from clinical sources continues to pose therapeutic problems worldwide. Commonly available and inexpensive orally administered antimicrobials have become ineffective for the treatment of serious infections caused by *E. coli* and other micro-organisms (London *et al.*, 1994). In the United States for example, several outbreaks of *E. coli* 0157:H7 infections have been associated with a high prevalence of resistance to commonly used orally administered antibiotics such as co-trimoxazole, streptomycin and tetracycline (Kim *et al.*, 1994). Similar multi-drug resistant phenotypes have been reported among EPEC strains causing outbreaks of infantile diarrhoea in several parts of the United States (Moyenuddin *et al.*, 1989).

In many developing countries, for example, Bangladesh (Faruque *et al.*, 1993), Sudan and Zaire (Shears *et al.*, 1988), and Kenya (Bebora *et al.*, 1994), strains of *E. coli* isolated from children with diarrhoea were found to be multiply resistant to almost all the commonly available antibiotics that are used for treating other infections as well. In Kenya, Senerwa *et al.* (1991) also reported a

high prevalence (50% to 80%) of hospital EPEC strains from children with diarrhoea which were resistant to ampicillin, co-trimoxazole, tetracyclines and chloramphenicol.

Other studies in Hong Kong (Ling *et al.*, 1994) have also reported resistance of all clinical isolates of *E. coli* to ampicillin and 41% resistance to co-amoxyclav. In Greece, a high prevalence of *E. coli* resistant to trimethoprim has been reported among patients with urinary tract infections (Tsakris *et al.*, 1993). Resistances of *E. coli* strains to fluoroquinolones have been reported in Germany (Lehn, *et al.*, 1996), while Johnson *et al.* (1994) observed a high prevalence of gentamicin resistance among clinical isolates of *E. coli* in a London hospital, U.K.

2.6.8 Analytical procedures of antimicrobial resistance

2.6.8.1 Disc susceptibility test

One of the method for antibiotic susceptibility testing of bacteria is the use of Standardized Disk Diffusion Method (Bauer *et al.*, 1966). This method measures the ability of drugs to inhibit the growth of micro-organisms. It involves use of discs impregnated with specific antibiotics. These discs are placed on agar plates inoculated with test material and incubated overnight (Washington, 1985).

The sizes of zones of growth inhibition determine the level of resistance. These zonal sizes vary with the molecular characteristics of different drugs. Thus, zonal sizes of one drug cannot be compared to the zonal sizes of another drug acting

on the same micro-organism. However, for any one drug, the zonal sizes can be compared to a standard, provided media, innoculum sizes and other conditions are carefully standardized. It is then possible to list for each drug a minimum zone size which denotes susceptibility (Jawetz *et al.*, 1970).

Tablets may also be used in place of discs. Each tablet is made aseptically and contains a standard concentration of the desired drug (Bou Cassals, 1980). Hill (1966) compared impregnated paper discs with tablets and found a larger safety margin in the interpretation of results with tablets than with paper discs.

The single disc method used in most clinical laboratories has been shown to correlate well with results of dilution techniques. The method is suitable for most rapidly growing pathogens (Bou Cassals, 1980).

2.6.8.2. Minimum inhibitory concentration (MIC) dilution test

Agar or broth Minimum Inhibitory Concentration (M.I.C.) tests are often considered to be the standard reference method for the evaluation of antibiotic resistance according to Waterworth (1980). The terms broth (or tubes) and agar (or plates) are actually misnomers because it is the antimicrobial agent that is being diluted. Nevertheless, it should be remembered that dilution methods are commonly performed with serial two-fold dilutions of the antibiotic, and each succeeding dilution step represents a 50% decrease in concentration. In general, dilution tests are considered satisfactory if the MIC's vary no more than ± 1 dilution step around the mean on repeated tests. Conventional MIC testing

procedures are not able to differentiate satisfactorily between cultures that are suppressed overnight but residual turbidity remains, those unaffected by the antibiotic and those that are inhibited by antibiotic but regrowth occurs after antibiotic inactivation (Greenwood and Eley, 1982).

For most purposes, a concentration of 128 μg\ml is a satisfactory upper limit for routine testing with any antimicrobial agent. The lowest concentration selected will vary according to the antimicrobial agent. In general, however, this concentration should be below the upper limit of a high degree of susceptibility. The range of concentrations should include the end point for appropriate standard strains such as *E. coli* ATCC 25922 to permit adequate control (Washington, 1985). The agar dilution method has advantages of being able to test a number of strains simultaneously, detect microbial heterogeneity or contamination, and has a slightly better reproducibility than broth dilution method (Ericsson and Sherris, 1971).

2.7 Molecular epidemiology

2.7.1 Molecular epidemiology of bacterial infections

Bacteria have traditionally been defined by genus, species, biovar, serovar, pathovar, phagovar and morphovar. These phenotypic characterizations are based on specific assays for gene products. The identification of epidemic strains by specific markers offers the potential of bypassing a number of problems inherent in the classical methods of bacterial identification and characterization (Staley and Krieg, 1984).

2.7.2 Molecular epidemiology of *E. coli*

The carriage of antimicrobial resistance by normal gut flora in humans (Lester *et al.*, 1990) and in animals (Spika *et al.*, 1987) has been reported as the major reservoir for resistance genes which can be disseminated to pathogenic bacteria by various genetic processes. For example, in the Netherlands, Bonten *et al.*, (1990) observed that more than 50% resistance to sulphamethoxazole, ampicillin and tetracycline occurred among faecal flora from a group of students. In animals, these resistances in the normal gut flora have been observed in chicken and pigs in the United Kingdom and in turkeys in the USA (Chaslus-Dancla *et al.*, 1987).

Kariuki *et al.* (1999) observed that a plasmid of approximately 100-110 MDa was present in all *E. coli* isolates from environmental sources as well as from diarrhoeal cases in children. This plasmid was able to transfer 5 different resistance patterns to recipient *E. coli* K12 strain. The ability of multi-drug resistant *E. coli* to transfer resistance to *E. coli* K12 has been reported to range from 26% to 50% in human isolates, about 50% to 76% in pig isolates (Niljsten *et al.*, 1996b) and 24% in poultry isolates (O'Brien *et al.*, 1993).

Banatvala *et al.* (1996) was able to characterize an outbreak of *E coli* 0157:H7 infection associated with food contamination in retail supermarkets in Connecticut, USA, using PFGE (Pulsed Field Gel Electrophoresis). In their study, RFLP (Restriction Fragment Length Polymorphism) of PFGE was used to distinguish between outbreak-related strains and sporadic cases of *E. coli*

0157:H7. Kariuki (1996) reported that the similarities in compatibility groups of R-plasmids from *E. coli* isolated from children compared to isolates in chicken suggested some degree of relatedness in clusters of strains from various study sites.

2.7.3 Analytical techniques used in molecular epidemiology

Plasmid profile analysis, plasmid and chromosomal bacterial restriction endonuclease DNA analysis, and DNA hybridization are increasingly being used in clinical microbiology and epidemiology (Senerwa *et al.*, 1989). Plasmid profile analysis is the only one reviewed in the following subsection because it was the one used in this study.

2.7.3.1 Plasmid-DNA isolation

Plasmid DNA can be isolated from an overnight bacterial broth (Birmboim and Doly 1979; Kado and Liu, 1981). The principle of the method is based upon the disruption of the bacterial cell wall by treatment with lysozyme, lysis of the internal cell membranes with detergents and the denaturation of chromosomal DNA by acidic pH. Plasmid DNA is recovered by ethanol precipitation in the cold. Plasmid DNA is electrophoresed through vertical or horizontal agarose slab gel which separates it on the basis of molecular mass during migration towards the anode.

Most of bacterial plasmid DNA exists in a covalently closed circle (CCC) form (Hardy, 1981). If one of the two polynucleotide strands of CCC plasmid is

broken, an open circle (OC) is formed. When both polynucleotide strands are broken, a linear (L) molecule is formed if the breaks are either exactly opposite or so close together that the hydrogen bonds between intervening complementary bases are not strong enough to hold the two strands together. Application of shearing forces during plasmid DNA isolation results in formation of OC form(s). If shearing forces are excessive, L form(s) may result. Some of the large plasmids are difficult to keep in CCC form during isolation and purification. Different forms of plasmid DNA migrate through the agarose gel at different rates during electrophoresis. The rates are in the following decreasing order; CCC, OC and L (Hardy, 1981).

Different forms of the same plasmid can be identified by restriction endonuclease resistance. Plasmid may be maintained in the CCC form during isolation by applying minimum shearing forces. Some linear plasmids with covalently closed ends have been described. These plasmids encode for outer surface proteins in *Borrelia bungdorferi* and antibiotic biosynthesis in *Streptomyces* species (Mayer, 1988). During chromosomal DNA isolation, the cells are lysed and proteins are removed by sequential extraction with phenol and chloroform (Brenner *et al.*, 1969).

The DNA isolated by the above procedure is of sufficient quality for restriction endonuclease analysis. Restriction endonuclease are enzymes that recognize specific, Palindromic, base sequences at a defined position (Sambrook *et al.*, 1989). The number of fragments generated should be sufficient for specificity,

but not so many that DNA restriction fragments of the same size results (Platt *et al.*, 1986).

2.7.3.2 Plasmid DNA profiling and analysis

Plasmid profiling has been shown to be a good epidemiological tool in the investigation of epidemics or outbreaks of bacterial diseases (Mayer, 1988). In 20 different outbreaks of gastroenteritis studied by Centre for Disease Control (Holmberg *et al.*, 1984), plasmid profile analysis was used in identifying the clonal origin of *S. typhimurium* isolates from the cases involved in these outbreaks.

The use of plasmids as an epidemiological tool in the study of antimicrobial resistances has been referred to as "molecular fingerprinting". The principle of molecular fingerprinting is that plasmids of the same molecular weights, tend to have similar phenotypes and distinct "fingerprints". Plasmid fingerprintings of *Enterobacteriaceae* and *Pseudomonas* species have demonstrated that these methods are more accurate than other phenotyping methods including biotyping, antibiotic resistance patterns and serotyping (O'Brien *et al.*, 1993).

Results from various studies (Senerwa *et al.*, 1989; Olsvik *et al.*, 1985; O'Brien *et al.*, 1993), have shown plasmids to be useful markers for tracing the dissemination of antimicrobial resistance genes. The wide range of plasmid sizes from 5 to 150 kilobases (kb) allow individual plasmids to have characteristic band migration in agarose electrophoresis usually corresponding to their specific

phenotypic characteristics. In addition, Senerwa *et al.* (1989), in a study of diarrhoeal outbreak in preterm neonates in a hospital in Kenya, found that plasmid profiling was a useful epidemiological marker in the identification of enteropathogenic *E. coli* strains.

O'Brien *et al.* (1982) examined strains of three different *Salmonella* serotypes (*S. typhimurium var copenhagen*, *S.newport* and *S. dublin*) collected from reference laboratories in the United States. They found identical or nearly identical strains of *Salmonella* isolated from animals and humans in widely separated parts of the country. Restriction endonuclease enzyme analysis showed that plasmids from animal and human isolates were often identical or nearly so, substantiating the belief that human infection is usually acquired from animal sources (O'Brien *et al.*, 1982).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites

Rats were trapped in the densely populated area (slums) of Kibera and the less densely populated areas of Kabete and Kawangware in Nairobi, Kenya. Closed traps which enclosed life rats without killing them were used. The residents were encouraged to trap rats by giving a small fee for every rat trapped alive. The rats were transported to the Centre for Microbiology Research, Kenya Medical Research Institute (KEMRI) laboratories and classified as either domestic or wild according to the colour of their coats (Kingdom, 1974) and from the history of the residents as to where they trapped them (inside or outside the house).

3.2 Collection of biological specimens

The rats were sacrificed using chloroform (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England). Dissection of the abdomen was performed and faecal material was collected by opening the intestines of each rat. Intestinal scrapings were also collected by scrapping the intestinal mucosa using a sterile blunt scalpel blade. The samples were inoculated into peptone water (Oxoid) and incubated at 37°C for 18 hours for enrichment.

3.3 Processing of specimens

After 18 hours incubation at 37°C 1ml of each specimen in peptone water was subcultured into Rappaports Vassiliaidis soy peptone (RVS) broth (Difco, USA) and incubated overnight at 42°C. The RVS is important a selective media while

the 42°C temperature is important for suppressing growth of normal environmental microflora. After an overnight growth, the specimen was subcultured on MacConkey agar (MCA) plates (Oxoid) and Salmonella Shigella agar (SSA) plates (Oxoid). Lactose fermenting colonies (pink) were identified in MCA and non-hydrogen sulphide producers (pink) identified in SSA plates. These were sub-cultured again in new MCA plates in order to get pure colonies. The indole test was performed to differentiate *E. coli* from other lactose fermenting enterobactericeae.

Identification of *E. coli* was confirmed using analytical profile index (API) 20E strips (Bio Merieux, Marcy, France). The API 20E is a part of biochemical tests on a strip for the identification of bacteria in the family enterobactericeae. The API 20E strips test for H_2S and indole production, citrate utilization, lysine and ornithine decarboxylases, arginine dehydrolase, nitrate reduction, ß-galactosidase and fermentation of arabinose, rhamnose, mannitol and glucose (Mitruka, 1977). The results are fed into the computer programme which gives the identity of the bacteria.

A loopful of pure isolates of *E. coli* was inoculated into well- labelled sarstedt tubes each containing 1 ml tryptic soy broth + 5% glycerol. They were frozen at –80°C until required for analyses.

Other non-lactose fermenters in MCA plates and H_2S producers (dark colonies) in SSA plates were also followed and identified using API 20E system. These

were important in identification of other members of the family enterobactericeae present in the intestines of rats.

3.4 Antimicrobial susceptibility testing

Sixty *E. coli* isolates were randomly sampled from the 131 *E. coli* isolated from rats using multistage sampling procedure. The primary sampling units were the areas where the rats were trapped (Kibera or Kabete) while the secondary sampling units were the type of rats (wild or domestic).

3.4.1 Disc susceptibility test

Disc Susceptibility Test was done using modified controlled disc diffusion technique (Stokes and Waterhouse, 1973) on Isosensitest^{R} agar plates (Oxoid, Unipath LTD, Hampshire, England). Commonly used antibiotics in treatment of human diseases were tested. These antibiotics and their disc strengths were as follows: ampicillin (10 μg), co-amoxyclav (20:10 μg), tetracycline (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), trimethoprim (5 μg), sulphamethoxazole (10 μg), cefuroxime (30 μg), ceftazidime (30 μg), nalidixic acid (30 μg) and streptomycin (10 μg).

The micro-organisms stored in sarstedt tubes at -80 °C were allowed to thaw for 15 minutes. They were sub-cultured on blood agar (BA) plates and incubated at 37°C overnight. After overnight growth they were again sub-cultured on fresh BA plates and incubated at 37°C so as to get actively growing bacterial cells. Three to five discrete colonies on the BA plate were inoculated into a universal

bottle containing sterile distilled water. These were compared with the Barium chloride 0.5 McFarland turbidity standard. The turbidity of the test suspension was equated to the standard by adding more bacteria or more sterile distilled water. Isosensitest^{R} agar plates (Oxoid) were inoculated by dipping a sterile swab into the inoculum (test material suspended in sterile water) and pressing and rotating the swab firmly against the side of the bottle above the level of the liquid to remove excess inoculum. These were followed by streaking the swab all over the surface of the media 3 times and rotating the plate through an angle of 60^{o} after each application. Finally, the swab was passed round the edge of the agar surface and the inoculum left to dry at room temperature for a few minutes with the lid closed. Antibiotic discs were placed on the inoculated plates using a pair of sterile forceps. Gentle pressing was required to ensure even contact with the medium.

Plates were incubated at 37°C overnight and diameter of each zone (including the diameter of the disc) was measured in millimetres using a metre rule. Results were interpreted according to critical diameters set by National Committee for Laboratory Standards (1997) (NCCLS), USA (Appendix 1). A separate plate was inoculated with *E. coli* ATCC 25922 which have known zone diameters and was used as a control.

3.4.2 Minimum inhibitory concentration (MIC)

A loopful of discrete colonies of *E. coli* which had been incubated on blood agar plate at 37°C for 18 hours was picked and inoculated into 4 ml of sterile distilled

water. The inoculum was adjusted to a concentration of about 10^4 cfu\ml using a barium chloride 0.5 McFarland density standard solution.

Double dilutions of each antimicrobial agent were prepared in sterile distilled water from commercial tablets of known potency and concentration (Adatabs, Mast Pharmaceuticals, Merseyside, U.K.). The potency of the antibiotic tablets were as follows: ampicillin (3.2 mg), co-amoxyclav (3.2 mg), streptomycin (1.6 mg), co-trimoxazole (6.4 mg), tetracycline (1.6 mg), cefuroxime (3.2 mg), ceftazidime (3.2 mg), ciprofloxacin (0.8 mg), nalidixic acid (1.6 mg) and gentamicin (0.8mg). Two mililitres (ml) of the antibiotic dilutions were dispensed into 20 ml Isosensistest agar (Oxoid) at 55°C (to keep media liquid and to prevent denaturing the drugs). The media were allowed to set overnight.

The dilutions were as follows: ampicillin (128, 64, 32, 16, and 8 μg\ml), co-amoxyclav (32, 16, 8, 4, and 2 μg\ml), streptomycin (128, 64, 32, 16, and 8 μg\ml), co-trimoxazole (64, 32, 16, 8, and 4 μg\ml), tetracycline (64, 32, 16, 8, and 4 μg\ml), Cefuroxime (32, 16, 8, 4, and 2 μg\ml), ceftazidime (32, 16, 8, 4, and 2 μg\ml), nalidixic acid (32, 16, 8, 4, and 2 μg\ml), gentamicin (32, 16, 8, 4, and 2 μg\ml) and ciprofloxacin (16, 8, 4, 2, and 1 μg\ml).

The inoculum was inoculated on the Isosensitest/antibiotic dilution media plates using a multiple inoculator. Control plates (without antibiotics) were inoculated last to ensure that viable bacteria were present throughout the experiment. The inoculated agar plates were allowed to stand undisturbed until the inoculum

spots were completely dry. Incubation was done at 37°C for 18 hours.

MIC was interpreted according to criterion set by NCCLS (Appendix 2). MIC is the minimum concentration of antibiotic that inhibits the visible growth of test micro-organisms. *E. coli* strain ATTC 25922 with known MIC values of each antimicrobial agent was included in each test as a control (Appendix 3).

3.4.3 ß-lactamase production

For isolates resistant to ß-lactam antibiotics (ampicillin and co-amoxyclav), ß-lactamase production test was performed in order to verify if resistance was through production of ß-lactamase enzyme.

A loopful of fresh culture of bacteria on blood agar was suspended in 5ml tryptic soy broth and incubated at 37°C overnight. ß-lactamase production was tested using a strip coated with chromogenic cephalosporin (nitrocephin). Change of colour from white to pink was confirmatory. A ß-lactam antibiotic susceptible *E. coli* isolate was used as a control.

3.5 Plasmid isolation

Plasmid DNA was isolated using a commercial plasmid preparation kit (Hybaid Limited, Middlesex, London). The micro-organisms stored in sarstedt tubes at -80°C were allowed to thaw for 15 minutes, sub-cultured on Blood agar (BA) plates and then incubated at 37°C overnight. After overnight growth the micro-organisms were again sub-cultured on fresh BA plates and incubated at 37°C so

as to get actively growing bacterial cells.

The bacterial cells were suspended in a eppendorf tube containing 50µl pre-lysis buffer (150mM sodium chloride in 10:1 mM Tris-EDTA buffer) by pipetting up and down. A 100 microlitres (µl) of alkaline lysis solution (sodium dodecyl sulphate (SDS)) were added directly into the cell suspension and mixed by pipetting up and down until it was clear and viscous. Seventy five microlitres of neutralising solution (3M sodium acetate) were added to the it and mixed by vortexing. It was centrifuged for 2 minutes at 13000 revolutions per minute (rev\min) in a microcentrifuge and the supernatant transferred into a spin filter while avoiding the white precipitate.

After shaking to resuspend gel matrix 250µl of binding buffer (Silica gel) was added to it. The binding buffer and the centrifuged mixture were mixed by pippeting up and down. The mixture was centrifuged for 1 minute at 13000 rev\min and the solution at the bottom of the vial was discarded. Three hundred and fifty microlitres of wash solution (ethanol) were added into the spin filter and centrifuged for 1 minute at 13000 rev\min. The liquid in the collection vial was discarded and further centrifugation performed for 1 minute at 13000 rev\min to dry the pellet. The spin filter was transferred into a new eppendorf tube. Fifty microlitres of sterile distilled water was added into the spin filter. Vortexing was briefly done in order to resuspend binding matrix/DNA. The suspension was centrifuged for 30 seconds at 13000 rev\min and DNA was collected at the bottom of the catch tube. Twenty microlitres of loading buffer was added and

the DNA was ready for use. The procedure took place at room temperature.

3.6 Preparation of agarose gel and staining

Plasmids were analyzed by electrophoresis on 1% agarose gel on horizontal tanks containing 0.5X TBE (0.1M Tris, 0.1M boric acid, 0.2M EDTA) buffer. In preparing the gel, 1gm of agarose (Sigma, St. Louis, USA) was dissolved in a 100ml of TBE. For staining, 0.05 mg % ethidium bromide solution (Sigma) was added into the agarose solution. The mixture was heated to dissolve the agarose gel completely. This was cooled to 55°C and poured on horizontal gel tanks. A comb for making wells was placed on the gel solution and the gel was allowed to set for 30 minutes. It was then was placed on an electrophoretic machine filled with TBE buffer. The plasmid extracts of the test organisms were loaded onto the wells. *E. coli* strains 39R861 and V517 containing plasmids of known molecular weights were included in each of the plasmid gels used in electrophoresis. Electrophoresis was performed at 90 volts for 3 hours.

3.7 Photography and determination of the molecular sizes of plasmid DNA

The gel was placed on an ultraviolet transilluminator (VVP INC., San Gabriel, California, USA) and pictures taken using a Polaroid MP-3 Camera (Polaroid, Cambridge, Ma., USA). Molecular weights for test plasmid DNA were calculated from a standard curve (Appendix 4) obtained from the natural log of the molecular weights (MDa) against the migration distance in millimetres of the molecular weight standards.

3.8 Studies of transferable resistance plasmids

In vitro conjugation tests on transferable antimicrobial resistance was performed according to the method of Walia *et al.* (1987) with modifications. Single discrete colonies of each donor bacteria (*E. coli* isolate from rat) and recipient *E. coli* K12 F- (nalidixic acid resistant and carrying no plasmids) were subcultured into 5ml tryptic soy broth and incubated at 37□C overnight. The donor and the recipient bacterial broth cultures were then diluted 1:10 in fresh tryptic soy broth and allowed to multiply to the logarithmic phase by keeping them at room temperature for 4 hours. The recipient and donor bacterial broths were mixed at a ratio of 1:2 respectively (to allow as many donors as possible to conjugate with the recipients without leaving excess donors) and incubated at 37°C for conjugation to take place overnight.

To select transconjugants (progeny of donors and recipients), 3μl-samples were drawn from the overnight culture and plated on isosensitest agar plates containing 16 μg\ml nalidixic acid + 8 μg\ml tetracycline, 16 μg\ml nalidixic acid + 16 μg\ml streptomycin and 16 μg\ml nalidixic acid + 16 μg\ml co-trimoxazole using a multipoint inoculator. For ß-lactam antibiotics where resistance was shown (ampicillin and amoxyclav) the overnight broth was centrifuged at 15000 rev\min for 5 minutes and the supernatant discarded. This was to remove ß-lactamase enzyme from the solution which could interfere with the process. The precipitate was resuspended on fresh tryptic soy broth and 3μl samples were plated on isosensitest agar plates containing 16 μg\ml nalidixic acid + 16 μg\ml ampicillin and 16 μg\ml nalidixic acid + 8 μg\ml co-amoxyclav. The plates were

incubated at 37°C overnight and growth on agar plates confirmed presence of transconjugants.

Plasmid isolation and identification for the transconjugants was performed as described in section 3.5.

3.9 Statistical analysis

Chi-square tests with Yate's correction in STATISTIX computer package were used to compare proportions of *E. coli* isolates obtained from rats trapped in different areas of (Kibera, Kabete and Kawangware) and also the proportions of *E. coli* isolates obtained from rats categorized as wild or domestic. The proportions of antimicrobial resistant *E. coli* isolates were also compared depending on the areas of trapping and types of rats, respectively. *E. coli* isolates and their resistance were taken as the "diseases" while the areas of trapping the rats and type of rats were taken as the "risk factors".

CHAPTER FOUR

4.0 RESULTS

4.1 Isolates

A total of 215 rats were trapped, dissected and their intestinal contents and scrapings obtained. From these, a total of 131 *E. coli* isolates and 71 other bacterial isolates of the family *Enterobacteriaceae* were obtained as shown in Tables 2, 3 and Appendix 5. These included, 56 *Proteus mirabilis*, 3 *Proteus vulgaris*, 4 *Enterobacter cloacae*, 1 *Enterobacter sakazakii*, 3 *Citrobacter freundii*, 2 *Morganella morganii*, 1 *Klebsiella pneumoniae* and 1 *Salmonella* spp.

The proportions of *E. coli* isolated from rats did not significantly (P=0.5658) differ between those trapped in Kibera and Kabete areas. Kawangware area was not included in the analysis as no *E. coli* was isolated from rats trapped there. The proportions of *E. coli* isolates did not significantly (P=0.8696) differ between wild and domestic rats.

Table 2: *Enterobacteriaceae* isolates from rats trapped from Kabete, Kibera and Kawangware areas in Kenya, 1998

Area	Number of rats trapped	micro-organisms isolated	Number of isolates
Kabete	97	*E. coli*	58
		P. mirabilis	28
		P. vulgaris	2
		E. cloacae	2
		E. sakazakii	1
		C. freundii	1
		M. morganii	2
Kibera	113	*E. coli*	73
		P. mirabilis	26
		P. vulgaris	1
		K. pneumonia	1
		C. freundii	1
		Salmonella spp.	1
Kawangware	5	*P. mirabilis*	2
		E. cloacae	2
		C. freundii	1

Table 3: Proportions of various types of *Enterobacteriaceae* isolated from rats trapped from Kabete, Kibera and Kawangware areas in Kenya, 1998

Isolates	Proportion (%)
E. coli	60.93
Proteus spp.	27.44
Enterobacter spp.	2.32
Citrobacter freundii	1.40
Morganella morganii	0.93
Klebsiella pneumoniae	0.47
Salmonella spp.	0.47

4.2 Susceptibility testing of *E. coli* isolates.

4.2.1 Disc susceptibility test

Eight (13.3%) of the 60 *E. coli* isolates were fully sensitive to the 11 antimicrobials tested. Thirty two (53.3%) were either sensitive to one or more but not all antimicrobials. Some had intermediate susceptibility. All isolates were susceptible to ciprofloxacin, ceftazidime, cefuroxime, nalidixic acid and gentamicin. Table 4 and Appendix 6 shows antimicrobial susceptibility profiles of these isolates.

Twenty (33.3%) were resistant to one or more of the following antimicrobials: ampicillin, streptomycin, sulphamethoxazole, co-amoxyclav, trimethoprim and tetracycline (Table 5). Twelve (20%) of the isolates were resistant to a single antimicrobial mostly ampicillin, streptomycin and sulphamethoxazole. Eight (13.3%) of the isolates were multidrug resistant (i.e. resistant to two or more antimicrobials). One (12.5%) out of the 8 *E. coli* isolates showing multi-drug resistance was isolated from wild rats. Figure 1 shows the multiple antimicrobial resistance patterns. Visually, *E. coli* isolates from domestic rats showed high percent resistance.

Table 4: Antimicrobial susceptibility profile of *E. coli* isolates from rats trapped from Kabete and Kibera areas in Kenya, 1998

Antimicrobial agent	No. (%) *E. coli* n=60		
	Resistant	Intermediate	Susceptible
Amp	12 (20.0)	32 (53.3)	16 (26.7)
Amc	1 (1.7)	5 (8.3)	54 (90.0)
Strept	8 (13.3)	40 (66.7)	12 (20.0)
Sulpha	15 (25.0)	22 (36.7)	23 (38.3)
Tet	2 (3.3)	23 (38.3)	35 (58.3)
Trim	3 (5.0)	7 (11.7)	50 (83.3)
Cefu	0 (0.0)	9 (15.0)	51 (85.0)
Ceft	0 (0.0)	8 (13.3)	52 (86.7)
Nal	0 (0.0)	7 (11.7)	53 (88.3)
Gent	0 (0.0)	8 (13.3)	52 (86.7)
Cip	0 (0.0)	2 (3.3)	58 (96.7)

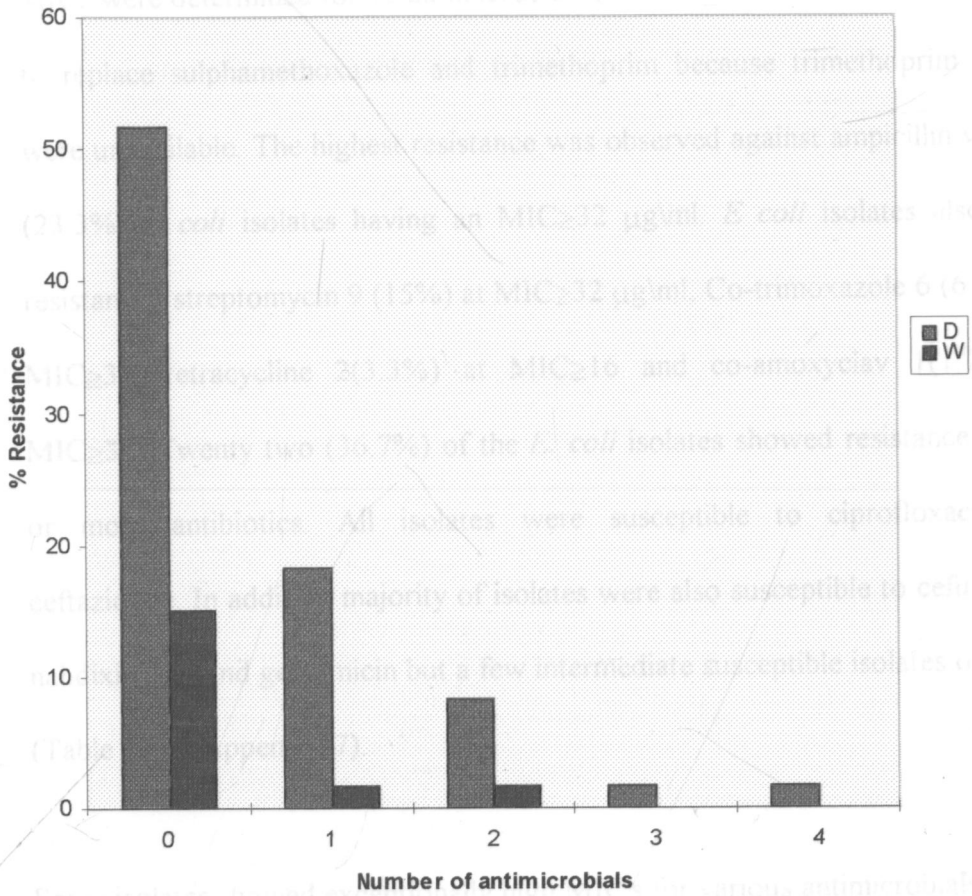
Key: Amp=Ampicillin, Amc=Co-amoxyclav, Strept=Streptomycin, Sulpha=Sulphamethoxazole, Tet=tetracycline, Trim=Trimethoprim, Cefu=Cefuroxime, Ceft=ceftazidime, Nal=Nalidixic acid, Gent=Gentamicin, Cip=Ciprofloxacin.

Table 5: Multi-drug resistant *E. coli* isolates from rats trapped from Kabete and Kibera areas in Kenya, 1998

Type of rat	No. of resistant *E. coli* (isolates)	No. of Antimicrobials	Antimicrobial combination
Domestic	1	4	Amp, Sulpha, Tet, Trim.
Domestic	1	3	Strept, Sulpha, Trim.
Domestic	1	2	Amp, Strept.
Domestic	1	2	Amp, Amp.
Domestic	1	2	Strep, Sulpha.
Wild	1	2	Amp, Tet.
Domestic	1	2	Amp, Sulpha.
Domestic	1	2	Sulpha, Trim.

Key: Amp=Ampicillin, Strept=Streptomycin, Sulpha=Sulphamethoxazole, Tet=tetracycline, Trim=Trimethoprim,

Figure 1: Multiple antimicrobial resistance (%) from E. Coli isolated in rats.



Key: D = Domestic rats
W = Wild rats

4.2.2 Minimum inhibitory concentrations (MICs)

MICs were determined for 10 antimicrobials (Table 6). Co-trimoxazole was used to replace sulphamethoxazole and trimethoprim because trimethoprim tablets were unavailable. The highest resistance was observed against ampicillin with 14 (23.3%) *E coli* isolates having an MIC≥32 μg\ml. *E coli* isolates also were resistant to streptomycin 9 (15%) at MIC≥32 μg\ml, Co-trimoxazole 6 (6.6%) at MIC≥32, tetracycline 2(3.3%) at MIC≥16 and co-amoxyclav 1(1.7%) at MIC≥32. Twenty two (36.7%) of the *E. coli* isolates showed resistance to one or more antibiotics. All isolates were susceptible to ciprofloxacin and ceftazidime. In addition majority of isolates were also susceptible to cefuroxime, nalidixic acid and gentamicin but a few intermediate susceptible isolates occurred (Table 7 and Appendix 7).

Some isolates showed exceptionally high MIC's for various antimicrobials. These included the following: ampicillin with 5 (8.3%) isolates and 1 (1.7%) isolate showing MIC≥64 and MIC≥128 μg\ml respectively, streptomycin with 2 (3.3%) and 1(1.7%) isolate showing MIC≥64 and MIC≥128 μg\ml respectively and co-trimoxazole with 1 (3.3%) isolate showing MIC≥64 μg\ml. The mode MIC, MIC_{50} and MIC_{90} were generally low for all antimicrobials. This indicated that the isolates were highly susceptible to all the antimicrobials.

There was statistical difference (P=0.0431) between resistant isolates from rats trapped in Kibera and Kabete. In general, no statistical differences (P=0.2884) were found in resistance between isolates from wild and domestic rats.

Table 6: Minimum inhibitory concentrations (MICs) of 10 antimicrobials tested against 60 *E. coli* isolates from rats trapped from Kabete and Kibera areas in Kenya, 1998.

Antimicrobial	MIC range (μg\ml)	Mode	MIC_{50}	MIC_{90}	* No. of resistant isolates	% resistant
Ampicillin	8-128	<8	16	64	14	23.3
Streptomycin	8-128	<8	8	32	9	15.0
Co-trimixazole	4-64	<4	<4	16	4	6.6
Tetracycline	4-64	<4	<4	4	2	3.3
Co-amoxyclav	4-64	<2	<2	4	1	1.7
Gentamicin	2-32	<2	<2	8	0	0.0
Ceftazidime	2-32	<2	<2	2	0	0.0
Cefuroxime	2-32	<2	<2	16	0	0.0
Nalidixic acid	2-32	<2	<2	4	0	0.0
Ciprofloxacin	1-16	<1	<1	1	0	0.0

Key: * The number of resistant *E. coli* was determined using NCCLS (1997) cut-off MICs (μg\ml)

Ampicillin=8, Streptomycin=8, Co-trimoxazole=16, Tetracycline=4, Co-amoxyclav=8, Gentamicin=4, Ceftazidime=8, Cefuroxime=8, Nalidixic acid=16, Ciprofloxacin=1.

Table 7: Minimum inhibitory concentrations (MICs) and susceptibility patterns of 60 *E. coli* isolated from rats trapped from Kabete and Kibera areas in Kenya, 1998.

Antimicrobial	Resistant	Intermediate	Susceptible
Amp	14 (23.3)	18 (30.0)	28 (46.7)
Amx	1 (1.7)	2 (3.3)	57 (95.0)
Strept	9 (15.0)	8 (13.3)	43 (71.7)
Tet	2 (3.3)	4 (6.7)	54 (90.0)
Trim	4 (6.7)	5 (8.3)	51 (85.0)
Cefu	0 (0.0)	8 (13.3)	52 (86.7)
Nal	0 (0.0)	2 (3.3)	58 (96.7)
Gent	0 (0.0)	8 (13.3)	52 (86.7)
Ceft	0 (0.0)	0 (0.0)	60 (100)
Cip	0 (0.0)	0 (0.0)	60 (100.0)

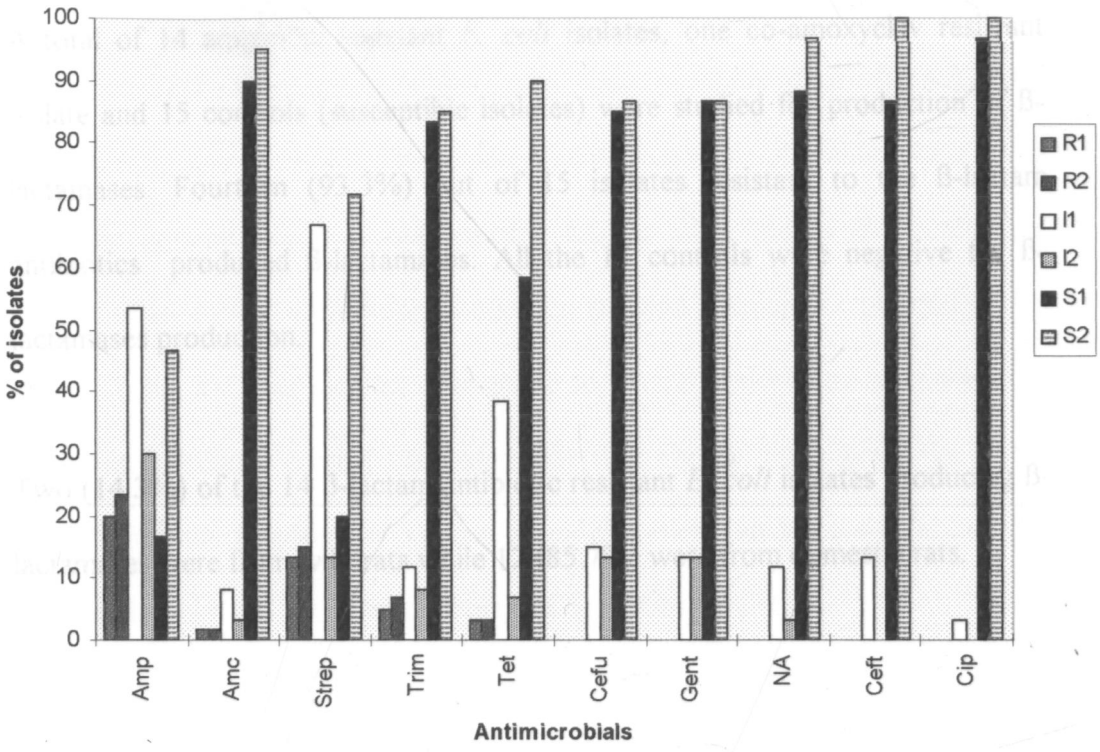
Key: Amp=Ampicillin, Amx=Co-amoxyclav, Trim=Co-trimoxazole, Tet=tetracycline, Cefu=Cefuroxime, Ceft=ceftazidime, Nal=Nalidixic acid, Gent=Gentamicin, Cip=Ciprofloxacin.

4.2 .3 Comparison between MICs and Disc susceptibility test results

Three *E. coli* isolates which showed intermediate susceptibility in disc susceptibility test showed full resistance in MIC tests. Two were resistant to ampicillin and 1 to streptomycin. Changes also occurred among intermediate and susceptible isolates. Some which had intermediate susceptibility in disc susceptibility test showed full susceptibility in MIC (Figure 2).

Statistically, there were no significant (P=0.2627) differences between resistant *E. coli* isolates in MIC and disc susceptibility tests . Further, no significant (P=0.3369) differences were found between susceptible isolates. However, significant (P=0.0001) differences occurred in intermediate susceptible isolates between the two tests. There was no antimicrobial agent which showed significant (P>0.05) differences in susceptibility patterns between the two tests.

Figure 2: Comparison between MIC and Disc susceptibility tests.



Key: R1 = Resistance in disc susceptibility test.
R2 = Resistance in MIC test.
I1 = Intermediate susceptibility in disc susceptibility test
I2 = Intermediate susceptibility in MIC test.
S1 = Susceptible in disc susceptibility test
S2 = Susceptible in MIC test.

Amp = Ampicillin,	Amc = Co-amoxyclav,	Trim = Co-trimoxazole,
Tet = Tetracycline,	Cefu = Cefuroxime,	Ceft = Ceftazidime,
NA = Nalidixic acid,	Gent - Gentamicin,	Cip = Ciprofloxacin.

(Co-trimoxazole in MIC was compared with Trimethoprim in disc susceptibility test.)

4.2.4 ß-lactamases production from *E. coli* isolates from rats

A total of 14 ampicillin resistant *E. coli* isolates, one co-amoxyclav resistant isolate and 15 controls (susceptible isolates) were studied for production of ß-lactamases. Fourteen (93.3%) out of 15 isolates resistant to the ß-lactam antibiotics produced ß-lactamases. All the 15 controls were negative for ß-lactamases production.

Two (14.3%) of the 14 ß-lactam antibiotic resistant *E. coli* isolates producing ß-lactamases were from wild rats while 12 (85.7%) were from domestic rats.

4.3 Plasmid profile analysis of the *E. coli* isolates from rats.

Sixteen (72.7%) of the 22 *E. coli* isolates resistant to one or more antimicrobials carried plasmids. The molecular weights of these plasmids ranged from 2 to 98 megadaltons (Mda). Six (27.3%) isolates did not carry any plasmids. The isolates were categorized into 5 plasmid profile groups according to the number of plasmids they carried with those without any plasmid forming one plasmid profile group (Table 8). Four isolates susceptible to all antibiotics were also analysed. Of these, 2 isolates carried plasmids while the other 2 did not. Of the 2 that carried plasmids 1 isolate had 3 plasmids and the other had 1 plasmid. Figure 3 is a reference gel showing plasmids from the *E. coli* isolates.

A plasmid of approximately 90-100 Mda was carried by 9 (40.9%) of the resistant *E. coli* isolates (Appendix 8). Others of approximately 55-65 Mda and 40-50 Mda were carried by 8 (36.4%) and 5 (22.7%) of the resistant isolates respectively. Others carried by the isolates included 2 Mda by 3 (13.6%) isolates, 4 Mda by 4 (18.2%) isolates and 18 and 17 Mda by 1 (4.5%) isolate each.

Two (25%) of the 8 multidrug resistant isolates carried both the plasmids of 90-100 and 55-65 Mda while four (50%) carried either the 90-100 or 55-65 Mda plasmids. Two (25%) did not carry any plasmids.

The two *E. coli* isolates from wild rats which showed resistance carried plasmids. One had 2 plasmids of molecular weights 95 and 42 Mda while the other had one plasmid of molecular weight 95 Mda.

Table 8: Plasmid profile groups of antimicrobial resistant *E. coli* isolates from rats trapped from Kabete and Kibera areas in Kenya, 1998

{PRIVATE } Plasmid profile group	No. of plasmids carried	Molecular weight sizes of plasmids (Mda)	No. of isolates
1	0	-	6
2	1	95, 90, 60, 2	6
3	2	98, 96, 95, 60, 56, 59, 42, 2	5
4	3	96, 62, 56, 50, 42, 40, 18, 8, 4, 2	4
5	4	95, 56, 40, 4	1

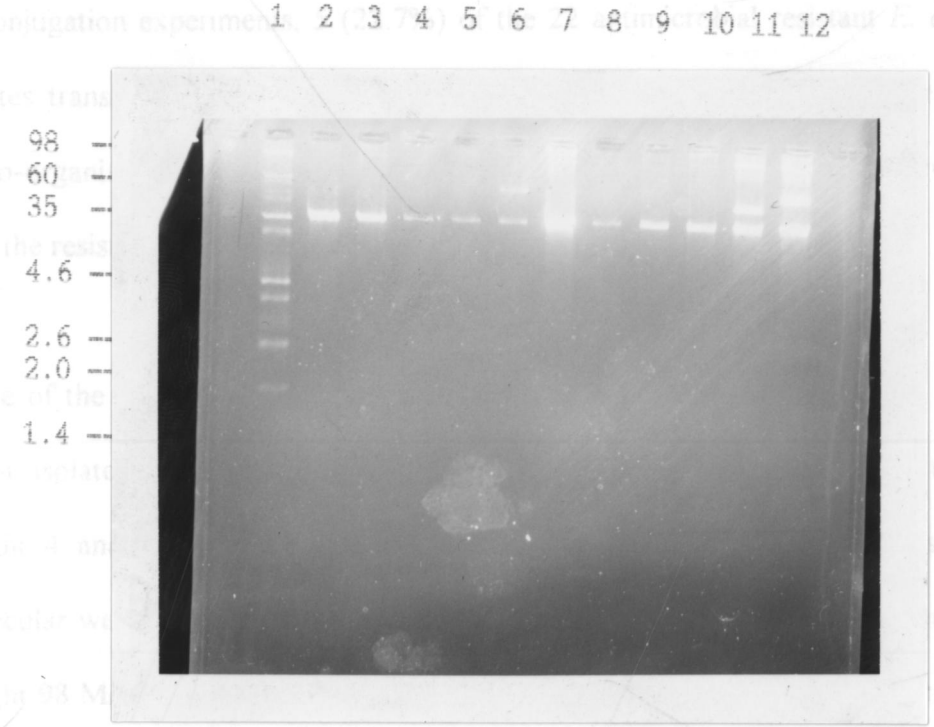


Figure 3: Molecular weights in megadaltons of plasmids of reference drug-resistant *E. coli*. Plasmids of *E. coli* V517 and 39R861 are in lane 1 and 12 respectively and those of plasmids of *E. coli* isolates from rats in lane 2 to 11.

4.4 Transfer of antimicrobial resistance of *E. coli* isolates from rats to E. coli K12.

In conjugation experiments, 5 (22.7%) of the 22 antimicrobial resistant *E. coli* isolates transferred ampicillin resistance to *E. coli* K12 (susceptible reference micro-organisms). All the 5 isolates were obtained from domestic rats. It was only the resistance to ampicillin which was transferable.

Three of the transconjugants carried plasmids which were transferred from the donor isolates (Figure 4). One transconjugant had 2 plasmids of molecular weight 4 and 60 Mda transferred from its donor which had 3 plasmids of molecular weights 4, 60 and 98. This shows that only the plasmid of molecular weight 98 Mda was not transferred from the donor to the recipient. The second transconjugant had a plasmid of molecular weight 95 Mda transferred from a donor with a single plasmid of the same molecular weight. The third transconjugant had a plasmid of molecular weight 56 Mda transferred from a donor with 2 plasmids of molecular weights 95 and 56 Mda showing that only the plasmid of molecular weight 56 Mda was transferred. For the other 2 trans-conjugants which had no plasmids, one of the donors had a plasmid of molecular weight 60 Mda which was not transferred while the other donor did not carry any plasmid.

Large plasmids of approximately 90-100 Mda and 55-60 Mda were transferred by all the 3 isolates while a small plasmid of 4 Mda was transferred by only 1 isolate.

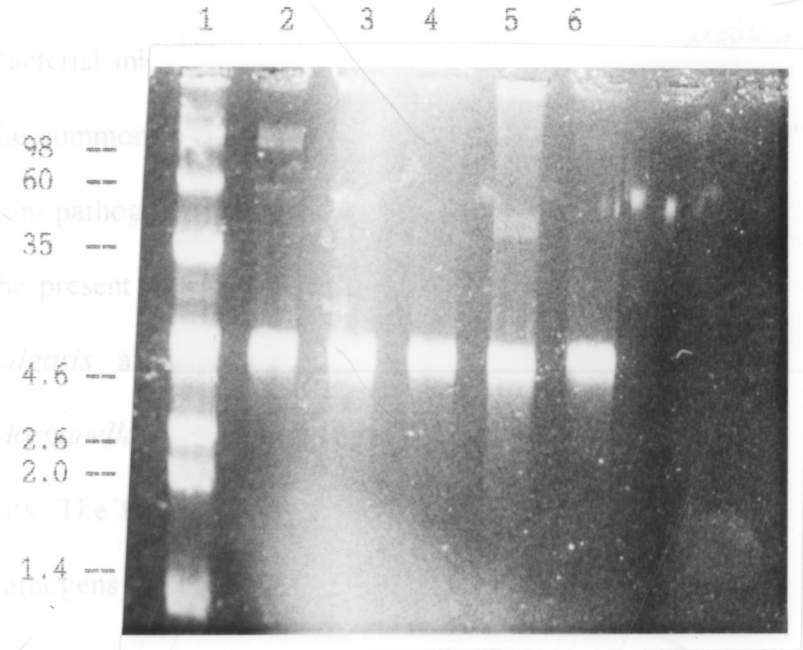


Figure 4: Molecular weights in megadaltons of plasmids of the *E. coli* transconjugants (lane 2 to 6) and those of the marker *E. coli* V517 (lane 1).

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

Bacterial micro-organisms which belong to the family *enterobacteriaceae* are the commonest microflora of the gut of humans and animals. They are usually non- pathogenic. However, pathogenic and opportunistic pathogens do occur. In the present study *E. coli*, *Salmonella* species, *Klebsiella pneumoniae*, *Proteus vulgaris* and *P. mirabilis*, *Enterobacter cloacae* and *E. sakazakii* and *Morganella morganii* were isolated from the intestines of domestic and sylvatic rats. The first two can be pathogenic while the rest are mostly opportunistic pathogens (Holts *et al.*, 1994).

Salmonella species are the most investigated among the genera in the family *enterobacteriaceae* in rats. This is probably due to their pathogenicity and ease of transmission among various hosts. In this study, a low proportion of 0.47% was found as compared to 7.3% observed in a study by Kayihura (1982), 19.71% by Singh *et al.* (1980) and 6.3% by Abdi El-Ghan (1977). The proportion however, was similar to 0.57% reported in a study by Steele (1969).

The proportions of *Klebsiella pneumoniae*, *Enterobacter* species, *Citrobacter* species and *Morganella* species were low (0.47%, 2.32%, 1.4%, 0.93% respectively) This agrees with the study by Holts *et al.* (1994) that these organisms occur in low numbers in intestines of human and animals. On the other hand, the proportion of *Proteus* species was higher (27.44%) and this may have

resulted from rats picking and ingesting high loads of these micro-organisms from the environment because *Proteus* species are the predominant environmental contaminants occurring in manure dumps, soil and polluted water (Atlas, 1984; Holts *et al.*, 1994). The presence of these bacteria in the intestines of rats is important in that, being opportunistic pathogens, rats may act as reservoirs transmitting them to immunosupressed individuals hence complicating infections in these people.

E. coli was the most predominant *enterobacteriaceae* in the gut of domestic and wild rats with a proportion of 60.93%. A paucity of literature on the isolation of *E. coli* from the guts of rats exists except for Moine *et al.* (1987) who reported presence of *E. coli* in the guts of rats and mice living in various pig herds. Isolations in other hosts have been carried out in poultry, pigs and cattle (Kariuki, 1996; Niljesten *et al.*, 1996a; Orden *et al.*, 1999). Since there exists no literature on *E. coli* studies in rats in Kenya, the present study was conceived with the objectives of isolating *E. coli* from rats, determining the antimicrobial resistance of these isolates, determining antimicrobial resistance profiles and minimum inhibitory concentrations (MICs) of these isolates and investigating if this resistance was encoded in plasmids.

The *E. coli* isolates from rats showed resistance to commonly used antimicrobials in human medicine notably, ampicillin, sulphamethoxazole, trimethoprim, co-trimoxazole, streptomycin and tetracycline. No resistance was observed for gentamicin, nalidixic acid, ceftazidime and ciprofloxacin. These

results agree with those of Kariuki (1996) in a study of antimicrobial resistant *E. coli* from normal intestinal microflora of chicken. However, full susceptibility was observed for ceftazidime and ciprofloxacin. Further, the results compare with those reported by Niljesten *et al.* (1996a) in a study of *E. coli* in pigs except that full susceptibility was only found for ciprofloxacin. In this study the differences, in the ranges of susceptiblities of *E. coli* isolates to various antimicrobials could have arisen from the fact that rats get into contact with these antimicrobials via general sources, for example food, water, faeces etc. These antimicrobial sources are from different carriers exposed at various times as well as development of resistance.

Eight (13.3%) *E. coli* isolates from rats were fully susceptible to all antimicrobials tested while 8 (13.3%) showed multidrug resistance mainly to ampicillin, sulphamethoxazole and streptomycin. These results agree with those reported by Niljesten *et al.* (1996a) in pigs and Al-Ghamdi (1999) in humans but differed from those of Kariuki *et al.* (1999) in a study of *E. coli* in chickens and Ombui *et al.* (1995) in a study of *E. coli* in cow milk. The highest resistance was observed for sulphamethoxazole followed by that of ampicillin and streptomycin. Similar observations have been reported by other authors (Kariuki *et al.*, 1999; Niljesten *et al.*, 1996a; Ombui *et al.*, 1995). These authors reported resistance frequencies of 10% to 100% for sulphamethoxazole, 5% to 98% for ampicillin and 10 to 50% for streptomycin. In contrast, these investigators reported high level of tetracycline resistance which was overtly absent in the present study. Probably this could have been due to the fact that these authors carried out their

studies in different areas where tetracyclines are intensively used and hence easy development of resistance to it by *E. coli*; in this study the drug could have been lowly used, thus minimum exposure to sources of *E. coli* to rats. It is worthy noting that ampicillin, streptomycin and sulphamethoxazole (mostly in combination with trimethoprim as co-trimoxazole) and tetracycline are used as "first" line agents for treatment of bacterial infections in humans hence have very high residue effect in faeces and other wastes of humans. These can be passed to the rats through eating human feces or fecal contaminated foods, leading to acquisition of resistance. These drugs are also used in veterinary medicine and therefore their residues can pass through faeces or fecally-contaminated water or feeds from treated animals or animals fed on antibiotic supplemented feeds.

Statistical difference was recorded between the proportions of antimicrobial resistant *E. coli* isolated in rats trapped in Kibera and Kabete areas. Kibera is a densely populated area (slum) with poor drainage and sanitation conditions, hence human faeces and other wastes easily get into the drainage systems and waste dumping sites where rats frequent to feed. This probably could lead to ingestion of the resistant *E. coli* micro-organisms with subsequent transfer of resistance to normal gut microflora. Kabete is less densely populated with better sewer systems and relatively well managed dumping sites hence contacts of rats with human waste was minimal. This led to the low frequency of antimicrobial resistant *E. coli* isolated from rats trapped in Kabete area.

Disc susceptibility and minimum inhibitory concentration (MIC) tests were used

in combination because the former is qualitative while the latter is quantitative. The results from each did not differ statistically from the other hence their similarities in sensitivities. Disc susceptibility test is important for getting a susceptibility profile while MIC is important for determining at what concentration of a drug an isolate is inhibited hence giving light to the level of exposure to the specific antimicrobial. The similarities in the sensitivities of these 2 tests was also reported by Kariuki (1996) and Niljesten *et al.* (1996a). These authors found no statistical difference between the two tests in their studies on *E. coli* in chickens and pigs, respectively.

Fourteen (93.3%) of the 15 *E. coli* isolates resistant to ß-lactam antibiotics in this study (ampicillin and Co-amoxyclav) produced ß-lactamases. This agrees with the results by Sanders (1992) and Ling et al. (1994) who reported that 90% of resistance to ß-lactams is through production of ß-lactamases. Resistance to ampicillin presents a major problem since it is one of the most widely available orally administered antibiotic. It is used in most hospitals for the treatments of enterobacterial infections and pneumonia in children. Therefore, its resistance in *E. coli* isolated from rats via production of ß-lactamases with probable transmission of the resistant characteristics to humans is of a major concern. From the results of this study, it seems ß-lactamases produced by *E. coli* isolated from rats were susceptible to inhibition by clavulanate since only one isolate was resistant to co-amoxyclav.

The transfer of antimicrobial resistant *E. coli* isolates and their genes from

humans to rats and vice versa is still debatable. Considering that in Kenya, *E. coli* is an important cause of bacteraemia in nosocomial infection (Gilks et al. 1990) and a significant public health problem, and that antibiotics are widely used in clinical practice, the need to avert the spread of resistance is important. It seems rats can act as reservoirs of genetic pools of antimicrobial resistance genes which could be transferred to humans. On the other hand, humans may act as the reservoirs of genetic pools of antimicrobial resistance genes which could be transferred to rats. These rats can thus act as the foci for multiplication of these genes with subsequent transmissions to humans and other animals. This may lead to a cyclic phenomenon of antimicrobial resistance gene transfers from humans to rats and from rats to other animals and then to rats. They eventually reach humans. These cycles may lead to the emergence of highly antimicrobial resistant *E. coli* due to selective pressure. This could pose a major threat in treatment of diseases in humans and other animals. These cycles can form important epidemiological chains of transfer of antimicrobial resistant *E. coli* and their genes. The hypothesis, therefore warrants studies to verify if antimicrobial resistant foci occur, with an aim of coming up with strategies of averting their spread as well as further development.

The number of plasmids per *E. coli* isolate ranged from 0 to 4. These results compare well with those of Ombui *et al.* (1995) and Bebora *et al.* (1994) who reported similar ranges in the number of plasmids. However, resistance to various antimicrobial agents was not closely associated with the presence of plasmids since resistance was found in isolates with no plasmids as well as those

that had plasmids. Nevertheless, large plasmids of 90-100 Mda and 55-65 Mda were more associated with the resistance as they occurred in 75% of the antimicrobial resistant *E. coli* isolates. A similar observation was reported by Kariuki *et al.* (1999). They reported that a plasmid of 90-100 Mda was associated with antimicrobial resistant *E. coli* isolates from chicken and children who lived in close proximities. Bebora *et al.* (1994) also reported an association of 60 Mda plasmid with drug resistance of *E. coli* from chickens.

Resistance was only transferred in 5 (22.7%) of ampicillin resistant *E. coli* isolates to *E. coli* K12. The ability of multidrug resistant *E. coli* isolates to transfer resistance to *E. coli* K12 has been reported to range from 26% to 50% in isolates from human, about 50% to 76% in isolates from pigs (Niljesten *et al.*, 1996b) and 24% in isolates from poultly (O'Brein *et al.*, 1993). Although the results of this study agree with the findings in Niljesten *et al.* (1996b) for human *E. coli* isolates, they do not agree with ranges obtained in pig isolates and those in O'Brein *et al.* (1993). Furthermore, resistance was transferrable for only one antimicrobial namely penicillin, as opposed to their findings where resistance was transferrable to one, two or more antimicrobials. Large plasmids of 90-100 MDa and 55-65 MDa were transferred in 3 (60%) of the 5 isolates hence it can be concluded that these plasmids were responsible for transfer of ampicillin resistance.

In this study, there was no specific molecular weight plasmid that could be incriminated as the one responsible for the resistance of *E. coli* isolates from rats

to antimicrobial agents. This is because resistance occurred in isolates with various molecular weight plasmids as well as those that had no plasmids. It is possible that some resistance genes were on the chromosomes. Resistance genes may be located on plasmids, or on chromosomes, or on transposons (Clowes, 1972). It is therefore possible that resistance to various antimicrobial agents of *E. coli* isolates in this study was mediated by genes located on chromosomes, plasmids and\or on transposons. This could be further explained by the observation that some trans-conjugants did not have any plasmids though the donors had plasmids showing that transposons responsible for resistance could have been transferred (Davies, 1994). The lack of relationship between plasmid profile groups and antimicrobial resistance patterns was not unexpected because the same antimicrobial resistance patterns can be encoded by unrelated plasmids, transposons, phages and chromosomal genes. Even identical enzyme mediated antibiotic modification has been shown to be encoded by two different genes (Mayer, 1988). Antimicrobial resistance may occur due to spontaneous mutation or may be acquired from other bacteria. The resistance factor (R-factor) can transfer resistance among bacterial species and even among genera of bacteria (O'Brien *et al.*, 1982). Thus, the control of rats is very important because rats are capable of transferring antimicrobial resistance to *E. coli* and other pathogenic micro-organisms such as *Salmonella* and *Shigella* in humans.

There are adverse implications for the failure of treatment of infections due to resistant micro-organisms. Therapeutic failure leads to increased period of hospitalization and in some cases to increases in morbidity and mortality. The

use of newer and more expensive antimicrobials as a result of resistance to the commonly used ones may result in high costs which may be unaffordable (Kariuki, 1996). These problems are more compounded in the developing world where the range of antimicrobials used in treatment of human diseases is limited and the sale of antibiotics over the counter is not controlled (Niljesten *et al.*, 1996a). In the rats, since resistance transfer was only found for ampicillin there could be a possibility of lack of co-selection for resistance to other related or non-related antimicrobials which could be an advantage in that the rate of spread of resistance through rats to the newer antimicrobials may be limited. In addition, it would be easier to control this type of resistance in *E. coli* from rats by improving environmental sanitation.

Results of this study showed that rats do carry antimicrobial resistant *E. coli* and their plasmids. A study with an aim of determining if there is a relationship between resistant *E. coli* plasmids in rats and in humans is required so as to determine if transmission of resistant *E. coli* micro-organisms and their plasmids occurs from rats to humans and vice versa. A wider epidemiological study of drug resistance in bacteria from rats is therefore important in areas where large populations of rats share the same ecosystem with humans and other animals.

The study also show that resistance of *E. coli* to antimicrobial agents is present even in wild rats which, to our knowledge, could not have been exposed to antimicrobials. This undermines the presumption that resistance will decline in absence of, or controlled, antimicrobial treatment. The origin of this resistance

and the selection mechanisms for it is unknown. It is important that these problems are addressed because similar mechanisms may occur in domestic animals and humans.

In conclusion the present study observed that: -

1. Wild and domestic rats harbour micro-organisms of the family *Enterobacteriaceae* in their gastro-intestinal tract with *E. coli* being the most prevalent in most study areas.
2. Antimicrobial resistance occurs in *E. coli* isolates from intestinal tract of wild and domestic rats.
3. Higher levels of antimicrobial resistant *E. coli* isolates from rats occurred in the more crowded areas with poor drainage systems and poor hygienic conditions.
4. Antimicrobial resistant *E. coli* isolates from rats do have plasmids which could be used as epidemiological tools for determining if transfer of resistance occurred between humans and rats.
5. Ampicillin resistance in *E. coli* isolates from rats is transferable and there is a possibility of this resistance being transferred to pathogenic bacteria in rats, humans and other animals.
6. Control of rat population by the improvement of sewer systems, dumping sites personal hygiene and storage of foodstuffs is highly recommended so as to reduce any acquisition of resistance characteristics of *E. coli* in rats.

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APPENDICES

Appendix 1: A table for interpretation of disc susceptibility tests.

Antimicrobial agent	Disc content (μg)	Disc diameter (mm)		
		Resistance	Intermediate	Susceptible
Ampicillin	10	≤13	14-16	≥17
Co-amoxyclav (Amoxycillin\ Clavulanic acid)	20\10	≤13	14-17	≥18
Cefuroxime	30	≤14	15-17	≥18
Ceftazidime	30	≤14	15-17	≥18
Ciprofloxacin	5	≤15	16-20	≥21
Gentamicin	10	≤12	13-14	≥17
Nalidixic acid	30	≤13	14-18	≥19
Trimethoprim	5	≤10	11-15	≥16
Sulphamethaxazole	100	≤12	13-16	≥17
Tetracycline	10	≤14	15-18	≥19
Streptomycin	10	≤11	12-14	≥15

Source: National Clinical Control of Laboratory Stardards (NCCLS), USA. (1987).

Appendix 2: Minimum inhibitory concentrations (MICs) interpretive standard (mg\ml) for *Enterobacteriaceae*

Antimicrobial agent	Tablet potency (mg)	MIC (μg\ml)		
		Sensitive	Intermediate	Resistant
Ampicillin	3.2	≤8	16	≥32
Co-amoxyclav	3.2	≤8	16	≥32
Cefuroxime	3.2	≤8	16	≥32
Ceftazidime	3.2	≤8	16	≥32
Ciprofloxacin	1.6	≤1	2	≥4
Gentamicin	0.8	≤4	8	≥16
Tetracycline	1.6	≤4	8	≥16
Nalidixic acid	1.6	≤16	-	≥32
Co-trimoxazole	6.4	≤16	-	≥32
Streptomycin	1.6	≤8	16	≥32

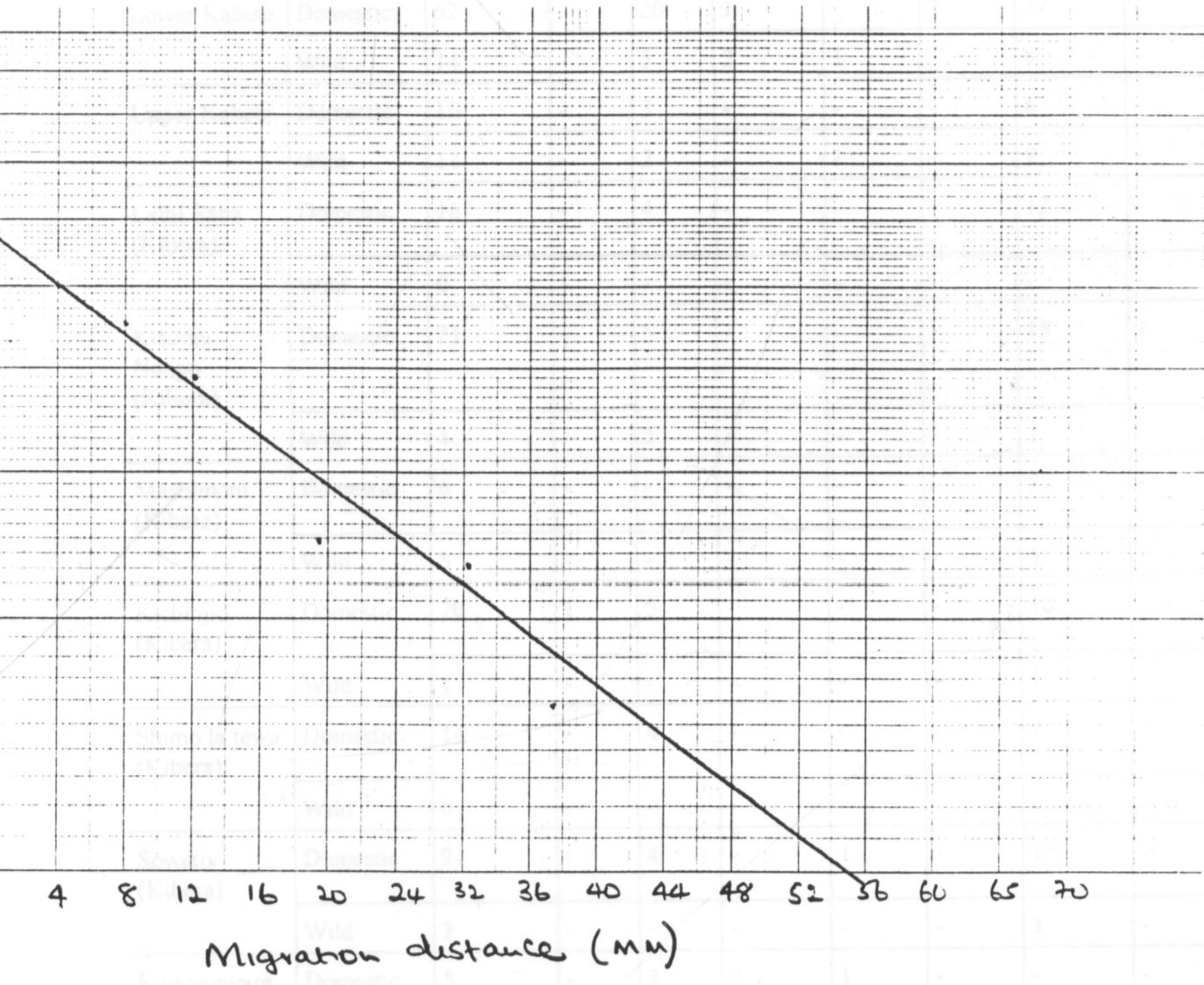
Source: National Clinical Control of Laboratory Stardards (NCCLS), USA. (1987).

Appendix 3: Disc susceptibility test: Disc diameter sizes (mm) of the standard *E. coli* ATCC 25922

Antimicrobial agent	Disc potency (μg)	Disc diameter (mm)
Ampicillin	10	16-22
Co-amoxyclav	20\10	19-25
Tetracycline	10	18-25
Trimethoprim	5	24-32
Sulphamethoxazole	100	15-23
Gentamicin	10	19-26
Streptomycin	10	12-20
Cefuroxime	30	20-26
Nalidixic acid	30	22-28
Ceftazidime	30	25-32
Ciprofloxacin	5	30-40

Source: National Clinical Control of Laboratory Stardards (NCCLS), USA. (1987).

Appendix 4: An example of a standard graph of the molecular weight standards (*E. coli* strains 39R861 and V517) used for estimating the molecular weights of plasmids of *E. coli* isolates from rats.



Appendix 5: Bacterial isolates from rats

Location	Type of rats	No. trapped	Bacterial isolations						
			Kleb	Prot	Entero	Citro	Morga	E.coli	Salmo
Lower Kabete	Domestic	62	-	20	1	_	2	37	-
	Wild	14	-	5	2	1	_	6	-
Upper Kabete	Domestic	10	-	3	-	-	-	6	-
	Wild	11	-	2	-	-	-	9	-
Laini Saba (Kibera)	Domestic	16	-	4	-	-	-	12	-
	Wild	0	-	-	-	-	-	-	-
Kisumu Ndogo (Kibera)	Domestic	21	-	5	-	-	-	15	1
	Wild	4	-	3	-	-	-	1	-
Mashimoni (Kibera)	Domestic	6	-	-	-	-	-	3	-
	Wild	5	-	-	-	-	-	5	-
Kichinjio (Kibera)	Domestic	20	1	5	-	-	-	10	-
	Wild	1	-	-	-	-	-	1	-
Shimo la tewa (Kibera)	Domestic	14	-	4	-	-	-	7	-
	Wild	0	-	-	-	-	-	-	-
Soweto (Kibera)	Domestic	21	-	4	-	1	-	15	-
	Wild	3	-	-	-	-	-	3	-
Kawangware	Domestic	5	-	2	2	1	-	-	-
	Wild	0	-	-	-	-	-	-	-

Kleb=Klebsiella spp, Prot=proteus spp, Entero=Enterobacter spp,

Citro=Citrobacterspp, Morg=Morganella spp, E.coli=Escherichia coli,

Salmo=Salmonella spp

Appendix 6: Disc susceptibility test zone sizes for *E. coli* isolates from rats

Isolates	Amp	Amx	Cefu	Ceft	Cip	Gent	Tet	NA	Trim	Sulf	Stre
6121	14 I	24 S	24 S	32 S	28 S	20 S	22 S	24 S	28 S	16 I	14 I
6104	18 S	27 S	28 S	31 S	30 S	16 S	26 S	22 S	26 S	15 I	12 I
6111	8 R	30 S	16 I	30 S	42 S	28 S	22 S	30 S	30 S	28 S	14 I
6101	10 R	30 S	32 S	32 S	30 S	14 I	20 S	22 S	28 S	14 I	8 R
5084	14 I	18 S	16 I	28 S	30 S	20 S	18 I	20 S	14 I	14 I	14 I
6011	6 R	24 S	20 S	26 S	28 S	19 S	26 S	28 S	26 S	8 R	10 R
6084	22 S	24 S	28 S	30 S	40 S	20 S	20 S	30 S	30 S	30 S	13 I
6090	14 I	30 S	20 S	28 S	28 S	14 I	20 S	20 S	28 S	16 I	12 I
6030	6 R	18 S	20 S	30 S	30 S	14 I	16 I	24 S	20 S	16 I	14 I
6095	20 S	18 S	20 S	30 S	30 S	18 S	18 I	22 S	20 S	18 S	16 S
6009	10 R	20 S	20 S	30 S	28 S	18 S	18 I	22 S	26 S	10 R	14 I
6094	10 R	30 S	20 S	26 S	22 S	16 S	20 S	20 S	22 S	10 R	14 I
6123	14 I	20 S	20 S	30 S	20 S	16 S	16 I	18 I	18 S	12 R	14 I
6128	18 S	22 S	20 S	30 S	24 S	14 I	24 S	20 S	10 R	6 R	10 R
6029	13 I	24 S	22 S	32 S	30 S	18 S	20 S	22 S	22 S	18 S	16 S
6138	20 S	28 S	20 S	30 S	30 S	16 I	24 S	22 S	10 R	6 R	12 I
6062	14 I	28 S	22 S	24 S	28 S	18 S	18 I	20 S	22 S	16 I	14 I
6139	16 I	26 S	24 S	32 S	38 S	18 S	18 I	24 S	24 S	18 S	12 I
5087	18 S	20 S	18 S	24 S	24 S	18 S	18 I	20 S	20 S	20 S	16 S
6116	16 I	24 S	18 S	30 S	24 S	18 S	24 S	28 S	22 S	20 S	14 I
6093	8 R	22 S	18 S	28 S	24 S	17 S	26 S	18 I	14 I	6 R	14 I
6081	14 I	22 S	15 I	16 I	28 S	13 I	16 I	22 S	15 I	16 I	16 S
6092	19 S	22 S	20 S	20 S	40 S	18 S	17 I	20 S	20 S	16 I	20 S
6067	16 I	21 S	19 S	25 S	30 S	15 S	16 I	21 S	21 S	18 S	13 I
6119	10 R	22 S	28 S	30 S	20 S	25 S	8 R	16 I	24 S	18 S	14 I
6122	15 I	23 S	19 S	24 S	37 S	14 I	17 I	22 S	25 S	18 S	16 S
6117	16 I	22 S	26 S	30 S	20 S	17 S	19 S	26 S	15 I	19 S	11 R
5098	14 I	17 I	20 S	30 S	35 S	20 S	23 S	25 S	26 S	25 S	17 S

Appendix 6 continued,

5040	14 I	22 S	24 S	30 S	33 S	22 S	18 S	27 S	15 I	16 I	12 I
6141	22 S	22 S	23 S	32 S	37 S	18 S	20 S	24 S	28 S	22 S	14 I
6140	16 I	15 I	24 S	20 S	25 S	19 S	26 S	21 S	26 S	20 S	16 S
6012	15 I	25 S	20 S	27 S	38 S	17 S	22 S	23 S	23 S	15 I	13 I
6013	14 I	25 S	28 S	31 S	40 S	20 S	22 S	25 S	28 S	10 R	10 R
6135	11 R	23 S	32 S	33 S	30 S	20 S	18 I	23 S	30 S	10 R	14 I
6000	14 I	14 I	21 S	28 S	26 S	19 S	22 S	20 S	25 S	23 S	14 I
6063	16 I	23 S	24 S	30 S	30 S	18 S	21 S	25 S	30 S	15 I	14 I
5093	16 I	18 S	16 I	28 S	19 S	18 S	16 I	22 S	22 S	20 S	12 I
6088	16 I	22 S	17 S	15 I	22 S	22 S	17 I	22 S	23 S	10 R	10 R
6110	15 I	18 S	18 S	28 S	26 S	15 S	15 I	18 I	20 S	16 I	14 I
6120	16 I	20 S	18 S	15 I	23 S	20 S	17 I	20 S	23 S	16 I	20 S
6067	16 I	21 S	18 S	17 I	35 S	18 S	18 I	23 S	23 S	14 I	12 I
6004	15 I	18 S	16 I	15 I	23 S	18 S	17 I	18 I	18 S	15 I	18 S
6114	10 R	22 S	18 I	15 I	18 I	18 S	6 R	22 S	6 R	6 R	10 R
6001	16 I	22 S	17 I	15 I	17 I	18 S	17 I	18 I	22 S	15 I	12 I
5099	14 I	13 R	15 I	15 I	22 S	20 S	15 I	17 I	16 S	6 R	13 I
6137	10 R	24 S	33 S	30 S	40 S	26 S	22 S	28 S	30 S	6 R	12 I
5096	20 S	20 S	22 S	30 S	30 S	22 S	24 S	22 S	30 S	26 S	12 I
6003	14 I	26 S	20 S	28 S	26 S	20 S	26 S	20 S	26 S	13 I	14 I
6087	22 S	28 S	26 S	30 S	40 S	20 S	22 S	28 S	36 S	20 S	14 I
6086	15 I	18 S	30 S	30 S	40 S	20 S	22 S	28 S	30 S	22 S	14 I
5090	22 S	28 S	24 S	30 S	42 S	22 S	22 S	30 S	26 S	30 S	14 I
5041	20 S	22 S	22 S	28 S	30 S	18 S	20 S	22 S	26 S	24 S	14 I
6129	16 I	14 I	28 S	24 S	30 S	14 I	22 S	20 S	30 S	14 I	13 I
6127	14 I	16 S	28 S	28 S	32 S	22 S	18 I	22 S	14 I	16 I	14 I
6066	16 S	14 I	26 S	32 S	40 S	22 S	18 I	22 S	14 I	6 R	14 I
6130	22 S	28 S	24 S	28 S	40 S	24 S	20 S	24 S	30 S	20 S	12 S
6148	14 I	20 S	22 S	26 S	30 S	20 S	18 S	24 S	28 S	13 I	14 I

Appendix 6 continued,

5094	20 S	18 S	28 S	30 S	30 S	20 S	24 S	22 S	28 S	8 R	10 R
6083	10 R	27 S	16 I	30 S	35 S	20 S	22 S	25 S	28 S	16 I	14 I
6010	18 S	18 S	24 S	28 S	32 S	18 S	20 S	22 S	22 S	22 S	20 S
ATCC	20 S	22 S	24 S	28 S	32 S	20 S	20 S	22 S	26 S	19 S	16 S

Appendix 7: Minimum inhibitory concentration (MICs) results for E. coli isolates from rats

Isolates	Amp	Amx	Cefu	Ceft	Cip	Gent	Tet	NA	Cotri	Strep
6121	16 I	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6104	8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6111	32 R	<2 S	16 I	<2 S	<1 S	2 S	<4 S	<2 S	<4 S	8 S
6101	32 R	<2 S	2 S	<2 S	1 S	8 I	<4 S	<2 S	<4 S	128 R
5084	8 S	4 S	16 I	2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6011	32 R	2 S	2 S	<2 S	<1 S	<2 S	4 S	2 S	4 S	32 R
6084	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6090	16 I	<2 S	<2 S	<2 S	<1 S	8 I	4 S	2 S	<4 S	8 S
6030	64 R	<2 S	<2 S	<2 S	<1 S	<2 S	8 I	4 S	4 S	16 I
6095	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	<8 S
6009	64 R	<2 S	4 S	4 S	<1 S	4 S	<4 S	2 S	16 I	8 S
6094	64 R	<2 S	2 S	2 S	1 S	4 S	4 S	8 S	16 I	16 I
6123	16 I	<2 S	<2 S	<2 S	<1 S	2 S	4 S	<2 S	<4 S	64 R
6128	<8 S	4 S	<2 S	<2 S	<1 S	8 I	8 I	<2 S	32 R	64 R
6029	64 R	<2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	8 S
6138	<8 S	<2 S	<2 S	<2 S	<1 S	8 I	<4 S	<2 S	32 R	16 I
6062	8 S	2 S	2 S	<2 S	<1 S	<2 S	4 S	<2 S	8 S	8 S
6139	16 I	<2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	<8 S
5087	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	<8 S
6116	16 I	8 S	2 S	2 S	<1 S	8 I	<4 S	4 S	4 S	8 S
6093	32 R	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	16 I	32 R	8 S
6081	16 I	<2 S	2 S	<2 S	<1 S	8 I	<4 S	2 S	8 S	8 S
6092	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	<8 S
6067	16 I	<2 S	<2 S	2 S	<1 S	<2 S	4 S	<2 S	4 S	8 S
6119	64 R	8 S	4 S	<2 S	<1 S	<2 S	64 R	8 S	<4 S	8 S
6122	8 S	<2 S	<2 S	<2 S	<1 S	8 I	<4 S	<2 S	<4 S	<8 S
6117	16 I	<2 S	4 S	2 S	1 S	<2 S	4 S	4 S	16 I	32 R
5098	16 I	8 S	4 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	8 S

Appendix 7 continued,

5040	16 I	2 S	<2 S	<2 S	<1 S	2 S	4 S	<2 S	<4 S	<8 S
6141	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	8 S
6140	16 I	2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6012	16 I	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6013	8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	32 R
6135	64 R	<2 S	8 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	8 S
6000	16 I	2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	8 S
6063	<8 S	<2 S	2 S	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	16 I
5093	16 I	32 R	16 I	8 S	1 S	4 S	8 I	16 I	16 I	16 I
6088	16 I	<2 S	4 S	8 S	<1 S	4 S	4 S	<2 S	<4 S	32 R
6110	8 S	<2 S	<2 S	<2 S	1 S	<2 S	<4 S	8 S	4 S	<8 S
6120	8 S	<2 S	<2 S	2 S	<1 S	<2 S	8 I	<2 S	<4 S	<8 S
6067	<8 S	<2 S	<2 S	2 S	<1 S	<2 S	<4 S	<2 S	4 S	<8 S
6004	16 I	<2 S	16 I	<2 S	1 S	4 S	<4 S	4 S	4 S	8 S
6114	32 R	<2 S	16 I	8 S	<1 S	<2 S	16 R	2 S	64 R	32 R
6001	<8 S	2 S	16 I	2 S	<1 S	<2 S	4 S	<2 S	<4 S	<8 S
5099	32 R	16 I	16 I	2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6137	32 R	<2 S	<2 S	<2 S	1 S	<2 S	4 S	8 S	8 S	<8 S
5096	<8 S	<2 S	<2 S	2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6003	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6087	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	16 I
6086	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
5090	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	4 S	16 I
5041	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6129	<8 S	16 I	<2 S	<2 S	<1 S	8 I	<4 S	<2 S	<4 S	<8 S
6127	16 I	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	8 S	8 S
6066	8 S	2 S	<2 S	<2 S	<1 S	<2 S	4 S	<2 S	16 I	8 S
6130	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S
6148	16 I	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	<4 S	<8 S

Appendix 7 continued,

5094	<8 S	<2 S	<2 S	<2 S	<1 S	<2 S	<4 S	<2 S	4 S	32 R
6010	8 S	2 S	2 S	2 S	1 S	4 S	4 S	2 S	4 S	8 S
6083	128 R	<2 S	16 I	<2 S	<1 S	<2 S	4 S	<2 S	<4 S	16 I

Appendix 8: Plasmid profile of 22 antimicrobial resistant *E. coli* and 4 non-resistant *E. coli* isolates (control) from rats

Lab Reference Number	M.W. of Plasmids in MDa	Antimicrobial resistance (i.e Resistance to:)
5094	-	Strept, Sulfa
5099	62, 4, 2	Amp, Amx, Co-trim, Sulfa
6009	17, 2	Amp, Sulfa
6011	95, 56	Amp, Sulfa, Srept
6013	95, 56, 40, 4	Strep, Sulfa
6029	90	Amp
6030	60	Amp
6083	50, 8, 4	Amp
6088	95, 42	Strept, Sulfa
6093	-	Amp, Sulfa
6094	-	Amp, Sulfa
6101	96, 62, 40	Amp, Strept
6111	-	Amp
6114	98, 60	Amp, strept, Sulfa, Tet, Trime, Co-trim
6117	95	Strept

Appendix 8 continued,

Lab Reference Number	M.W. of plasmids in MDA	Antimicrobial resistance (i.e. Resistance to:)
6119	-	Amp, Tet
6123	42, 18, 4	Strept, Sulfa
6128	90	Strept, Sulfa, Trime, Co-trim
6135	2	Amp, Sulfa
6138	60	Sulfa, Co-trim
6137	96, 59	Amp, Sulfa
6066	-	Sulfa, Trime, Co-trim
*6086	-	–
*6122	-	-
*6127	60	-
*6087	98, 70, 35	-

Key : M.W.= Molecular weights in Megadaltons (MDa)

Amp=Ampicillin, Amx=Co-amoxyclav, Sulfa=Sulphamethaxozole, Trime=Trimethoprim, Co-trim=Co-trimoxazole, Strept=Streptomycin, Tet=Tetracycline.

* Controls

Appendix 9: Schematic flow diagram of this epidemiological study