PREVALENCE OF ANTIMICROBIAL RESISTANCE AND CHARACTERIZATION OF RESISTANCE GENES IN *ESCHERICHIA COLI* ISOLATES FROM CAPTIVE BABOONS IN KENYA

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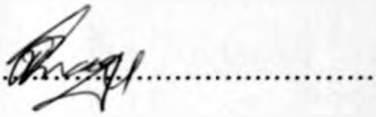
DECRALATION

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



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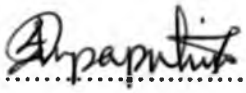

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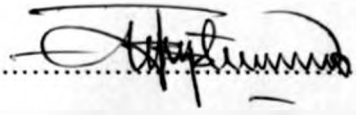

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DEDICATION

To my parents Charles and Florence for their support throughout my academic journey.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

The increasing prevalence of antimicrobial resistance is a major public health problem globally. In Kenya and the rest of developing countries the problem of antimicrobial resistance is much pressing especially due to high levels of infectious diseases and cost constraints which prevent the widespread use of newer and more expensive agents. Multiple studies drawn from point-prevalence assessment have recognized the role of commensal bacteria in the spread of antimicrobial resistance. In veterinary medicine, the alarming state of bacterial antimicrobial resistance is seen in examining the *Escherichia coli* isolates, where attention has been given to food-producing animals such as pigs, cattle and domestic fowl. However, few studies have looked at the antimicrobial resistance profiles in non-human primates. Therefore this study had the objective of assessing antimicrobial drug susceptibility and genetic characteristics of antimicrobial resistance in *Escherichia coli* from non-human primates.

A total of 100 faecal samples were collected using aseptic techniques from two groups of baboons at the Institute of Primate Research (IPR), Nairobi Kenya. Thirty six faecal samples were from group I baboons made up of 20 adult males and 16 females captured from Aberdare National park and transported to IPR one month earlier. Sixty four faecal samples were from group II baboons consisting of 64 adult male baboons that had lived at the IPR for a period of between one and 5 years. Ninety seven *Escherichia coli* were isolated by standard cultural, biochemical tests and final identification using the API 20E system (BioMe´rieux) test. Antimicrobial susceptibility was performed by the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI). A total of 16 antimicrobial agents commonly used were tested and these were: - ampicillin 10 µg, piperacillin 100 µg, amoxyclav 30 µg, ceftriaxone 30 µg, ceftazidime 30

µg, meropenem 10 µg, gentamicin 10 µg, amikacin 30 µg, kanamycin 30 µg, streptomycin 10 µg, tetracycline 30 µg, co-trimoxazole 25 µg, sulfamethoxazole 100 µg, ciprofloxacin 5 µg, ofloxacin 5 µg and chloramphenicol 30 µg. *E. coli* ATCC 25922 was used as a reference organism. DNA of *E.coli* isolates were extracted by boiling method and thereafter specific polymerase chain reaction (PCR) assays were used for the detection of genes for ampicillin resistance (bla_{TEM}, bla_{SHV} and bla_{CTX-M}), chloramphenicol resistance (*cmlA*), and streptomycin resistance (*aadA1* and *aadA2*) using specific primers. The PCR products were detected by electrophoresis in 1.5% agarose gels. To make sure that the amplification reaction had produced the desired gene segment TrackIt™ 1 Kb DNA ladder was used to estimate the PCR products size.

Most common resistance observed in *E.coli* isolated from both group of baboons was to ampicillin (36.1, 34.4%), sulphamethoxazole (33, 36.1%), amoxyclav (amoxicillin-clavulanic acid) (30.6, 26.2%), piperacillin (22.2, 23%), tetracycline (22.2, 19.7%), streptomycin (11.1, 21.3%), and co-trimoxazole (25, 9.8%) in group I and group II respectively. Resistance to chloramphenicol and ceftazidime was observed in 8 (8.2%) of the isolates. No *E.coli* isolates from group I baboons showed resistance to ceftriaxone, gentamicin, amikacin and ofloxacin. However, 1 (1.6%) isolate from group II baboons showed resistance against these four antimicrobial agents. No resistant isolates from both groups of baboons were detected for meropenem and ciprofloxacin. Nine isolates were resistant to ceftazidime and ceftriaxone, five of these isolates harbored the gene CTX-M. Resistance to co-trimoxazole was significantly higher ($p \leq 0.05$) in group I baboons as compared to group II baboons isolates. The proportion of strains showing multidrug resistance was 38.9 % and 41% of *E. coli* isolates from group I baboons and group II baboons, respectively. Although sulphamethoxazole resistance was the most frequently observed among *E. coli* isolates (4.9%) from

group II baboons, combined resistance to ampicillin-piperacillin-amoxyclav-streptomycin-tetracycline-co-trimoxazole-sulphamethoxazole was the most common among isolates (8.3%) from group I baboons. No significant difference was observed in the patterns of multidrug resistance between the isolates from group I and group II baboons. Twenty eight (75.7%) of the thirty seven ampicillin resistant *E.coli* isolates were positive for at least one of the three β-lactamase genes tested. The majority of the strains showed 21 (56.7%) positive amplification for *bla* $_{TEM}$. This was followed by *bla* $_{SHV}$ 19 (51.4%) and *bla* $_{CTX-M}$ in 5 (62.5%). Of the thirty seven ampicillin resistant *E.coli* isolates, one isolate showed resistance to ceftriaxone and eight to ceftazidime. Five of these *E.coli* isolates harboured the gene encoding CTX-M β-lactamase. The *cmlA* gene was detected in 5 of 8 chloramphenicol resistant *E.coli* isolate. The *aadA1* or *aadA2* gene was detected in 5 (25%) streptomycin resistant isolates.

Data from the present study shows that a moderate resistance to ampicillin, streptomycin, tetracycline, piperacillin, amoxyclav, co-trimoxazole, sulphamerthoxazole was prevalent in *E.coli* isolates from baboons. It also shows that resistance to ceftazidime and ceftiaxone, third generation cephalosporin is emerging in commensal bacteria and multidrug resistance *E.coli* harboring β-lactamase genes, *cmlA* and *aadA* resistance genes are common in IPR captive baboons. It may be concluded that captive baboons may be a potential reservoir for zoonotic transmission of multidrug resistant genes to humans and therefore minimal contact with these animals should be maintained to prevent possible horizontal transfer of resistant commensal bacteria to humans. I recommend epidemiological and molecular study on acquisition of resistance genes and their distribution among wild baboons and whether some of the genes are associated with known mobile elements.

CHAPTER ONE

1.0 INTRODUCTION

The increasing prevalence of antibiotic resistance is a major public health problem worldwide. The World Health Organization (WHO) and the European Commision (EC) have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control (Cryilmaz *et al.*, 2010). The increase in incidence of antibiotic-resistant bacteria has been attributed to the indiscriminate widespread use of antimicrobial agents in medicine and agriculture (Angulo *et al.*, 2004; Jeters *et al.*, 2009).

The role of commensal bacteria in the spread of antimicrobial resistance is being recognized as a vital component in understanding how to preserve the therapeutic usefulness of antibiotics. *Escherichia coli*, a common inhabitant of gastrointestinal tract of humans and the majority of animals is considered as practical "indicator bacteria" that could be used to track the evolution of antimicrobial resistance in different ecosystems. However, it has also emerged as an important cause of nosocomial and community acquired infections (Paterson and Bonomo, 2005; Barreto *et al.*, 2009).

In fact, antibiotic-resistant intestinal bacteria, at least in the minority populations of enterics and enterococci, have been found widely in environments where antibiotics are used. Antibiotic-resistant bacteria have also been found in settings where antibiotic exposure is expected to be rare or nonexistent. Surveys of antibiotic-resistant bacteria in wild animals have detected resistant bacteria in intestinal contents (Anderson *et al*, 2008; Costa *et al.*, 2008). These studies, however, have also been limited to the numerically minor bacterial populations (Jeters *et al.*, 2009).

The origin of antibiotic resistance in the environment is relevant to human health because of the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens (Allen *et al.* 2010). These antibiotic-resistant pathogens are profoundly important to human health, but the environmental reservoirs of resistance determinants are poorly understood.

Proximity to human activities influences the antibiotic resistance profiles of the gut bacteria of wild mammals, which live in densely populated microbial habitats in which antibiotics select for resistance (Allen *et al.*, 2010). About 13.3% of *E.coli* isolates from domestic and wild rats captured in peri-urban areas of Kenya were fully sensitive to all the eleven antimicrobials tested (Gakuya *et al.*, 2001). However, in Finland the faecal enterobacteria of wild elk, deer and voles showed almost no resistance (Osterblad *et al.*, 2001). Other studies have reported that African baboons and apes that are in contact with humans harbour more antibiotic-resistant enteric bacteria than those that dwell in areas that are remote from human activity (Rolland *et al.* 1985; Rwego *et al.*, 2008). Therefore, these reports might suggest that human activities influence antibiotic resistance profiles in bacterial communities in wild animals, although other factors that affect the frequency of antibiotic resistance cannot be eliminated (including differences in the testing methodologies used or variation in the intrinsic antibiotic resistance of the isolate populations).

Several factors are known to promote the importance of commensals in mediating the dissemination of antibiotic resistance genes, including the presence of antibiotic resistance gene reservoirs in commensal microbes in various environmental and host ecosystems (Gilliver *et al.*, 1999; Osterblad *et al.*, 2001; Lancaster *et al.*, 2003; Ready *et al.*, 2003; Nandi *et al.*, 2004; Salyers *et al.*, 2004; Smith *et al.*, 2004; Costa *et al.*, 2008). The second factor is the illustration of commensals as facilitators for antibiotic resistance gene dissemination (Luo *et al.*, 2005b), and finally the

correlation of antibiotic usage in animals with increased antibiotic resistance in human microbiota (Levy *et al.*, 1976; Smith *et al.*, 2002).

These resistance genes are commonly present on mobile genetic elements such as plasmids and integrons in clinical isolates of gram-negative microorganisms (Alekshun *et al.*, 2007). Furthermore, resistance genes selected for in non-pathogenic bacteria may later transfer the acquired resistance to pathogenic bacterial species (Phillips *et al.*, 2004; Wassenaar, 2005). Thus, normal bacterial flora can play a key role as an acceptor and donor of antimicrobial resistant determinant (Saenz *et al.*, 2004).

Attention has been given to some species of wild mammals regarding the occurrence of commensal resistant *E. coli* in various parts of the world (Rolland *et al.* 1985; Routman *et al.* 1985; Graves *et al.* 1988; Kinjo *et al.* 1992; Gilliver *et al.* 1999; Livermore *et al.* 2001; Swiecicka *et al.* 2003; Costa *et al.* 2006; Kozak *et al.* 2009; Schierack *et al.* 2009). An extensive study analysing antibiotic resistance in 449 *E. coli* isolates from 77 wild mammal species of 14 families was carried out in Australia (Sherley *et al.* 2000). The results from Australia demonstrated a low but widespread prevalence of antimicrobial resistance in wild isolates. Geographical location and host group significantly influenced the antibiotic resistance profile of isolates. However, in Kenya and especially in veterinary medicine, the alarming state of bacterial antibiotic resistance is seen in examining the *Escherichia coli* isolates, where attention has been given especially to food-producing animals such as pigs, cattle and domestic fowl (Kariuki *et al.*, 1997; Mapenay *et al.*, 2007; Kikuvi *et al.*, 2007a; Kikuvi *et al.*, 2010).

Whilst several studies in different animals including baboons have analysed *E. coli* for their susceptibility to antimicrobial agents and genetic determinants (Saenz *et al.*, 2004; Dunowska *et al.*,2006; Kadlec and Schwarz, 2008) zoonotic components of antimicrobial resistance varies between countries (De Jong *et al.*, 2009) and studies of *Enterobacteriaceae* of non human primates origin in Kenya are limited. Therefore, this study was undertaken to investigate the role that may be played by non human primates in the transmission of antimicrobial resistance in Kenya.

1.2 OBJECTIVES

1.2.1 Overall objective

The overall objective of this study was to determine the phenotypic and genetic characteristics of antimicrobiasl resistance in *Esherichia coli* isolates from healthy baboons in Kenya.

1.2.2 Specific objectives

1. To determine the prevalence of *E.coli* bacteria in healthy baboons in Kenya;
2. To determine the antimicrobial drug susceptibility patterns of the *E.coli* isolates
3. To investigate the presence and distribution of β-lactamase genes bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, streptomycin resistance genes *aadA1* and *aadA2*, chrolamphenicol resistance genes *cmlA* among the *E.coli* isolates

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 OLIVE BABOONS (*PAPIO ANUBIS*)

2.1.1 Morphology

Olive baboons have a greenish-grey coat covering their bodies. The individual hairs are green-grey with rings of black and yellowish-brown, giving the coat a multi-color appearance from up-close (Rowe, 1996; Groves, 2001). Males and females are sexually dimorphic, with the males being about twice as large as females. Wild male olive baboons weigh 24 kg on average and wild females weigh 14.7 kg on average. When their ecosystem is close to agricultural production they can raid crops, supplementing their natural diets with fruits, vegetables, and grains grown by local people, the average weights are slightly higher. Captive olive baboons weigh more than their wild counterparts, with the weight of captive males averaging 29 kg and females averaging 17 kg (Coelho, 1985).

2.1.2 Habitat

Olive baboons live in a variety of habitats across their broad range. Baboons are generally characterized as savanna species, inhabiting open grassland near wooded areas (Rowell, 1966). While olive baboons do inhabit grassland in much of their range, they are also found in moist, evergreen forests and near areas of human habitation and cultivation (Naughton-Treves *et al.* 1998). In Kenya, olive baboons are found in Gilgil which is open grassland with few trees, Laikipia plateau (dry woodland and grassland dotted with stands of trees and thick shrubbery), Aberdare forest, Masai-Mara National Reserve, Amboseli National Park (Barton *et al.* 1992).

2.1.3 Ecology

Baboons are omnivores and consume a huge variety of items including roots, tubers, corms, fruits, leaves, flowers, buds, seeds, bark, exudates, cacti, grasses, insects, birds, bird eggs, and vertebrates (including other primates) up to the size of a small antelope (Rowell 1966; Harding, 1976; Whiten *et al.*, 1991; Hassan, 2001). Olive baboons are generally opportunistic hunters, capturing prey as they come across it, but at Gilgil, Kenya, olive baboons exhibit simple and complex hunting patterns (Strum, 1981). In many areas of their range where human populations are increasing, olive baboons raid agricultural crops for food and feed on garbage and human refuse (Forthman-Quick 1986; Naughton-Treves *et al.*, 1998). Feeding close to human populations influences group behavior among olive baboons and may also influence social structure (Forthman-Quick, 1986). At Gilgil, the conflict between farmers and baboons became so intense that by 1984, more than 130 baboons were trapped and translocated in an attempt to appease farmers and save the baboons from persecution (Strum, 1987).

2.2 *ESHERICHIA COLI*

2.2.1 Introduction

Escherichia coli are one of the common microbial floras of gastrointestinal tract of animals and human beings (Akond *et al.*, 2009). However, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Moreover, even the most robust members of our species may be susceptible to infection by one of several highly adapted *E. coli* clones which together have evolved the ability to cause a broad spectrum of human diseases. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal diseases (Nataro and Kaper, 1998; Schroeder *et al.*, 2002).

2.2.2 Isolation and identification

E. coli can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions. *E. coli* in stool are most often recovered on MacConkey or eosin methylene-blue agar, which selectively grow members of the *Enterobacteriaceae* and permit differentiation of enteric organisms on the basis of morphology (Nataro and Kaper, 1998).

2.2.3 Serotyping

Serotyping of *E. coli* occupies a central place in the history of these pathogens. Prior to the identification of specific virulence factors in diarrheagenic *E. coli* strains, serotypic analysis was the predominant means by which pathogenic strains were differentiated. In 1933, Adam showed by

serologic typing those strains of "dyspepsiekoli" could be implicated in outbreaks of pediatric diarrhea. In 1944, Kauffman proposed a scheme for the serologic classification of *E. coli* which is still used in modified form today. According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles. A total of 170 different O antigens, each defining a serogroup, are recognized currently. The presence of K antigens was determined originally by means of bacterial agglutination tests: an *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture was heated was considered to have a K antigen. The discovery that several different molecular structures, including fimbriae, conferred the K phenotype led experts to suggest restructuring the K antigen designation to include only acidic polysaccharides. Proteinaceous fimbrial antigens have therefore been removed from the K series and have been given F designations. A specific combination of O and H antigens defines the "serotype" of an isolate. *E. coli* of specific serogroups can be associated reproducibly with certain clinical syndromes but it is not in general the serologic antigens themselves that confer virulence. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Nataro and Kaper, 1998).

2.2.4 Classification of enterovirulent *Esherichia coli*

2.2.4.1 Enterotoxigenic *E. coli* (ETEC)

ETEC is defined as containing the *E. coli* strains that elaborate at least one member of two defined groups of enterotoxins: heat stable toxin (ST) and heat labile toxin (LT) (Levine, 1987). ETEC strains were first recognized as causes of diarrheal disease in piglets, where the disease continues to cause lethal infection in newborn animals. Studies of ETEC in piglets first elucidated the

mechanisms of disease, including the existence of two plasmid encoded enterotoxins. The first descriptions of ETEC in humans reported that certain *E. coli* isolates from the stools of children with diarrhea elicited fluid secretion in ligated rabbit intestinal loops. DuPont *et al.* (1971) subsequently showed that ETEC strains were able to cause diarrhea in adult volunreers (Nataro and Kaper, 1998).

2.2.4.1.1 Pathogenesis

ETEC strains are generally considered to represent a pathogenic prototype: the organisms colonize the surface of the small bowel mucosa and elaborate their enterotoxins, giving rise to a net secretory state. Some investigators have reported that ETEC strains may exhibit limited invasiveness in cell cultures, but this has not been demonstrated in vivo. ETEC strains cause diarrhea through the action of the enterotoxins LT and ST. These strains may express an LT only, an ST only, or both an LT and an ST (Nataro and Kaper, 1998).

Heat-labile toxins. The LTs of *E. coli* are oligomeric toxins that are closely related in structure and function to the cholera enterotoxin (CT) expressed by *Vibrio cholerae*. LT and CT share many characteristics including holotoxin structure, protein sequence (ca. 80% identity), primary receptor identity, enzymatic activity, and activity in animal and cell culture assays; some differences are seen in toxin processing and secretion and in helper T-lymphocyte responses. There are two major serogroups of LT, LT-I and LT-II, which do not cross-react immunologically. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates, but in neither animals nor humans has it been associated with disease (Nataro and Kaper, 1998).

Heat-stable toxins. In contrast to the large, oligomeric LTs, the STs are small, monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins. There are two unrelated classes of STs that differ in structure and mechanism of action. Genes for both classes are found predominantly on plasmids, and some ST-encoding genes have been found on transposons. STa (also called ST-I) toxins are produced by ETEC and several other gram-negative bacteria including *Yersinia enterocolitica* and *V. cholerae* non-O1. STa has about 50% protein identity to the EAST1 ST of EAEC, which is described further below. It has recently been reported (Nataro and Kaper, 1998) that some strains of ETEC may also express EAST1 in addition to STa. STb has been found only in ETEC.

2.2.4.2 Enteropathogenic *E. coli* (EPEC)

EPEC is an important category of diarrheagenic *E. coli* which has been linked to infant diarrhea in the developing world. Once defined solely on the basis of O and H serotypes, EPEC is now defined on the basis of pathogenetic characteristics, as described below.

2.2.4.2.1 Pathogenesis

Attaching-and-effacing histopathology. The hallmark of infections due to EPEC is the attaching-and-effacing (A/E) histopathology, which can be observed in intestinal biopsy specimens from patients or infected animals and can be reproduced in cell culture. This striking phenotype is characterized by effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane. Marked cytoskeletal changes, including accumulation of polymerized actin, are seen directly beneath the adherent bacteria; the bacteria sometimes sit upon a pedestal-like

structure. These pedestal structures can extend up to 10 mm out from the epithelial cell in pseudopod- like structures. This lesion is quite different from the histopathology seen with ETEC strains and *V. cholerae*, in which the organisms adhere in a nonintimate fashion without causing microvillous effacement or actin polymerization.

Although earlier studies had also reported this histopathology, it was not until the report by Moon et al. (1983) that the phenotype became widely associated with EPEC and the term "attaching and effacing" was coined. The initial observation by Knutton *et al.* (1997) that the composition of the A/E lesion contained high concentrations of polymerized filamentous actin (F-actin) led to the development of the fluorescent-actin staining (FAS) test. In this test, fluorescein isothiocyanate (FITC)-labeled phalloidin binds specifically to filamentous actin in cultured epithelial cells directly beneath the adherent bacteria. Prior to the development of this test, the A/E histopathology could be detected only by the use of electron microscopy and intact animals or freshly isolated intestinal epithelial cells. Besides providing a diagnostic test for EPEC strains and other organisms capable of causing this histopathology, the FAS test enabled the screening of clones and mutants, leading to the identification of the bacterial genes involved in producing this pathognomonic lesion. In addition to F-actin, the composition of the A/E lesion includes other cytoskeletal components such as a-actinin, talin, ezrin, and myosin light chain. At the tip of the pedestals beneath the plasma membrane are located proteins that are phosphorylated on a tyrosine residue in response to EPEC infection.

The formation of the pedestal is a dynamic process, and video microscopy shows that these EPEC pedestals can bend and undulate, alternatively growing longer and shorter while remaining tethered

in place on the cell surface .Some of the attached EPEC organisms can actually move along the surface of the cultured epithelial cell, reaching speeds up to 0.07 mm/s in a process driven by polymerization of actin at the base of the pedestal. This motility resembles that seen with *Listeria* spp. (Tilney and Portnoy, 1989) inside eukaryotic cells, except that the motile EPEC organisms are located extracellularly. The significance of this motility observed in vitro to the pathogenesis of disease caused by EPEC is unknown. Similar A/E lesions are seen in animal and cell culture models of enterohemorhagic *E .coli* (EHEC) and *Hafnia alvei* isolated from children with diarrhea. However, only a small, highly conserved subset of *H. alvei* strains produce the A/E lesion (Ridell *et al.*, 1995), and detailed taxonomic studies suggest that the A/E-positive *H. alvei* strains should not be included in the same species as the A/E-negative *H. alvei* strains. The A/E lesion is also produced by strains of *Citrobacter rodentium* (formerly *Citrobacter freundii* biotype 4280) that cause murine colonic hyperplasia (although diarrhea is not seen in infection due to this species) (Schauer and Falkow, 1993). In addition to EPEC and EHEC, a variety of *E. coli* strains capable of A/E have been isolated from rabbits, calves, pigs, and dogs. Thus, EPEC strains are the prototype of an entire family of enteric pathogens that produce A/E lesions on epithelial cells (Ridell *et al.*, 1995).

2.2.4.3 Enterohemorrhagic *E. coli* (EHEC)

Most of the work on pathogenic factors of *E. coli* O157:H7 has focused on the *Stx*, which are encoded on a bacteriophage inserted into the chromosome. Additional potential virulence factors are encoded in the chromosome and on a ca. 60-MDa plasmid found in all EHEC strains of serotype O157:H7 (Elliot *et al.*, 1994).

2.2.4.3.1 Shiga toxins

The major virulence factor, and a defining characteristic of EHEC, is *Stx*. This potent cytotoxin is the factor that leads to death and many other symptoms in patients infected with EHEC.

Structure and genetics. The *Stx* family contains two major, immunologically non-cross-reactive groups called *Stx1* and *Stx2*. A single EHEC strain may express *Stx1* only, *Stx2* only, or both toxins or even multiple forms of *Stx2*, *Stx1* from EHEC is identical to Shiga toxin from *S. dysenteriae Stx1* from some strains may differ from *Stx* in one residue, while *Stx1* from other strains shows no sequence variation. The prototypical *Stx1* and *Stx2* toxins have 55 and 57% sequence identity in the A and B subunits, respectively. While *Stx1* is highly conserved, sequence variation exists within *Stx2*. The different variants are designated *Stx2c*, *Stx2v*, *Stx2vhb*, *Stx2e*, etc., and the various subtypes are wholly interchangeable between the *Stx* and VT nomenclatures (Calderwood *et al.*, 1996).

2.2.4.4 Enteroaggregative *E. coli* (EAEC)

The pathogenesis of EAEC infection is not well understood; however, a characteristic histopathologic lesion and several candidate virulence factors have been described.

Histopathology. Important clues to EAEC pathogenesis may be found by histopathologic examination of infected patients and animal models. EAEC strains characteristically enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm. Tzipori *et al.* (1989) fed a series of EAEC strains to gnotobiotic piglets; although some of these animals did not experience diarrhea, all animals tested developed an unusual mucoid gel closely adherent to the small intestinal epithelium. High-power examination of this gel revealed the presence of large numbers of densely packed, aggregating bacteria. In addition, the intestinal epithelium displayed

pitting of goblet cells, suggesting stimulation of mucus hypersecretion. Ligated rabbit ileal loops injected with EAEC also displayed pitting of goblet cells and embedding of aggregating bacteria within a periodic acid-Schiff (PAS)-staining blanket. Hicks *et al.*, (1996) reported that EAEC strains adhere to sections of pediatric small bowel mucosa in an *in vitro* organ culture model. In this series of experiments, as above, EAEC strains were observed to be embedded within a mucuscontaining biofilm. The ability of EAEC to bind mucus has been demonstrated *in vitro*, and volunteers fed EAEC develop diarrhea which is predominantly mucoid. The role of excess mucus production in EAEC pathogenesis is unclear; however, the formation of a heavy biofilm may be related to the diarrheagenicity of the organism and, perhaps, to its ability to cause persistent colonization and diarrhea. In addition to the formation of the characteristic mucus biofilm, experimental evidence suggests that EAEC infection is accompanied by cytotoxic effects on the intestinal mucosa (Hicks *et al.*, 1996). Vial *et al.* (1990) were the first to show that infection with EAEC strains in rabbit and rat ileal loop models resulted in a destructive lesion demonstrable on light microscopy (Nataro and Kaper, 1998).

2.2.4.5 Enteroinvasive *E. coli* (EIEC)

EIEC strains were first shown to be capable of causing diarrhea in volunteer studies conducted by DuPont *et al.*, (1971). EIEC strains are biochemically, genetically, and pathogenetically closely related to *Shigella* spp; like *Shigella* spp., EIEC strains are generally lysine decarboxylase negative, nonmotile, and lactose negative.

2.2.4.5.1 Pathogenesis

The precise pathogenetic scheme of EIEC has yet to be elucidated; however, pathogenesis studies of EIEC suggest that its pathogenetic features are virtually identical to those of *Shigella species*. Both

organisms have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci. In addition, both EIEC and *Shigella spp.* elaborate one or more secretory enterotoxins that may play roles in diarrheal pathogenesis.

Invasiveness. The current model of *Shigella* and EIEC pathogenesis comprises (i) epithelial cell penetration, (ii) lysis of the endocytic vacuole, (iii) intracellular multiplication, (iv) directional movement through the cytoplasm, and (v) extension into adjacent epithelial cells. When the infection is severe, this sequence of events elicits a strong inflammatory reaction which is manifested grossly as ulceration at the site of *Shigella* and EIEC infection in the colonic mucosa (Nataro and Kaper, 1998).

2.3 BETA-LACTAM ANTIBIOTICS

2.3.1 Introduction

β-Lactam antibiotics are a broad class of antibiotics that includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems, that is, any antibiotic agent that contains a β-lactam nucleus in its molecular structure. They are the most widely-used group of antibiotics. While not true antibiotics, the β-lactamase inhibitors are often included in this group (Basseti *et al.*, 2008).

The history of the brilliant research that led to the discovery and development of penicillins is well chronicled. In 1928, while studying staphylococcus variant in the laboratory at St Mary's Hospital in London, Alexander Fleming observed that mold contaminating one of his cultures caused the bacteria in the vicinity to undergo lysis. Because the mold belonged to the genus *Penicillium*, Fleming named the antibacterial substance penicillin. A decade later, penicillin was developed as a systemic therapeutic. First therapeutic trials were conducted in 1941. A vast research program was soon initiated in the United States. During 1942, 122 million units of penicillin were made available and the first clinical trials were conducted at Yale University and Mayo clinic, with dramatic results (Hardman *et al.*, 2001)

2.3.2 Mechanism of action

The β-lactam antibiotics kill susceptible bacteria inhibiting the last step in the synthesis of peptidoglycan: a transpeptidation reaction occurring outside the cell membrane. The transpeptidase is membrane bound and probably is acylated by penicillin. Although inhibition of the transpeptidase is important for the mechanism of action of penicillins and cephalosporins, they have other important targets termed *penicillin-binding proteins* (PBPs) (Hardman *et al.*, 2001).

There are several PBPs; for example, *Staphylococcus aureus* has four PBPs. The PBPs have different affinities for different β-lactam antibiotics. The higher molecular weight PBPs of *Escherichia coli* (PBP 1a and 1b) include the transpeptidases involved in the synthesis of peptidoglycan. Other PBPs maintain the rodlike shape, or are involved in the septum formation at division. The inhibition of some PBPs results in spheroplasts and rapid lysis, while the inhibition of other PBPs may cause delayed lysis (PBP 2) or the production of long filamentous forms of the bacterium (PBP 3) (Bayles, 2000).

The lysis subsequent to β-lactam antibiotics ultimately involves the activity of cell-wall lytic enzymes (autolysins or murein hydrolases). The relationship between the inhibition of PBPs and the activation of autolysins is unclear: an abnormal peptidoglycan formation may result in cell lysis, or β-lactam antibiotics may cause the loss of an autolysin inhibitor. Saturation of at least two of the three essential PBPs leads to a fast killing rate (Hardman *et al.*, 2001).

2.3.4 Mechanism of resistance

2.3.4.1 History

Only a few years after the introduction of penicillin into clinical practice, *S. aureus* developed resistance caused by a β-lactamase coded for it by a plasmid gene. Although this problem was solved by the introduction of methicillin and similar compounds that resisted the enzymatic hydrolysis, another enzyme, TEM β-lactamase, was reported in gram-negative bacteria in strains containing multiple-drug-resistant R plasmids that date from 1962 (Bradford, 2001). This enzyme became widespread throughout the world, making penicillins with gram-negative activity, such as

ampicillin, almost useless (Methicillin and its relatives are inactive against gram-negative bacteria because they are pumped out efficiently by the multidrug efflux pump). β-Lactamases are classified into several phylogenetic families. Class A includes both the *S. aureus* and TEM enzymes, whereas Class C represents chromosomally coded enzymes (e.g., AmpC) that are present in many gram-negative bacteria. These two classes are both similar to serine proteases in their mechanism, whereas Class B enzymes are metalloenzymes that hydrolyze carbapenems efficiently (Nikaido, 2009)

In response to the spread of β-lactam resistance, various β-lactams were developed. Although the first-generation cephalosporins, such as cephaloridine and cefazolin, were rapidly hydrolyzed by both TEM and AmpC, both cephamycins (such as cefoxitin) and the third-generation cephalosporins containing an oxyimino side chain (such as cefotaxime) were initially reported to resist both types of enzymes. However, the former was inactive against some gram-negative bacteria such as *Enterobacter* and *Serratia* (Vu and Nikaido 1985). Although the latter was capable of killing these organisms, their introduction into the clinics was followed by the emergence of resistant strains that overproduced the chromosomal AmpC enzyme. In fact, the AmpC enzymes have very low *KM* values for these compounds, and the values of Vmax/*KM* were quite high (Nikaido, 2009). The AmpC enzyme, however, needs to be induced, and the third-generation cephalosporins were effective against these bacteria simply because they were ineffective inducers of this enzyme. Thus, the third-generation cephalosporins selected for constitutive mutants of *ampC*. Furthermore, strong expression of plasmid-coded AmpC has been found recently in species that do not express the chromosomally coded *ampC* (PAGE, M.G.P., 1993)

Subsequently, fourth-generation cephalosporins (cefepime, cefpirome) that are more resistant to hydrolysis by the AmpC enzyme have been developed. However, continued selective pressure resulted in the selection of plasmids that produced mutants of common enzymes, such as TEM or its relative SHV, which can now hydrolyze third and sometimes even fourth-generation cephalosporins. These enzymes are called ESBL (extended spectrum β-lactamases) (Jacoby and Medeiros, 1991).

Especially troublesome among the ESBL enzymes are those called CTX-M (Bonnet, 2004). The genes coding for these enzymes appear to have originated from the chromosome of an infrequently encountered gram-negative bacterium *Kluyvera* and have transferred to R plasmids. This transfer or mobilization unusually appears to have occurred many times, and consequently the enzyme rapidly became widespread among R-plasmid-containing pathogenic bacteria (Barlow et al., 2008).

β-Lactams with a new nucleus, such as carbapenems (e.g., imipenem), still remain quite effective, but their use may eventually result in the increased prevalence of enzymes capable of hydrolyzing these compounds (Queenan and Bush, 2007). Table 2.1 below indicate key dates showing the emergence of β-lactamases.

Table 2.1: Key dates showing emergence of ß-lactamases

Year	Enzyme	Organism	Place
1944	Penicillinase	*S.aureus*	-
1963	TEM-1	*E. coli*	Athens
1974	SHV-1	*E. coli*	Switzerland
1978	OXA-10	*P. aeruginosa* -	-
1982	SME-1	*S. marcescens*	London
1984	IMI-1	*E. cloacae*	California
1988	Metallo ß-lactamase	*P. aeruginosa*	Japan
1989	Inhibitor-resistant penicillinase	*E. coli, K. pneumoniae*	France, Spain, Greece
1990	NMc A	*E. cloacae*	Paris
1991	OXA-11 OXA-14	*P. aeruginosa*	Turkey
1991	PER-1	*P. aeruginosa, S. typhimurium*	Turkey
1992	MEN-1	*E. coli, K. pneumoniae*	France
1994	TOHO-1	*E. coli*	Japan
1996	PER-2	*E. coli, K. pneumoniae, S. typhimurium, P. mirabilis*	Germany
1997	VEB-1	*E. coli*	Germany

Adapted from Chaudhary and Aggawal, (2004)

2.3.4.2 Types of Extended-Spectrum β-Lactamases (ESBLs)

Most ESBLs are derivatives of TEM or SHV enzymes. There are now >90 TEM-type β-lactamases and >25 SHV-type enzymes. With both of these groups of enzymes, a few point mutations at selected loci within the gene give rise to the extended-spectrum phenotype. TEM- and SHV-type ESBLs are most often found in *E. coli* and *K. pneumoniae*; however, they have also been found in *Proteus* spp., *Providencia* spp., and other genera of *Enterobacteriaceae*. (Bradford, 2001; Chaudhary and Aggarwal, 2004).

2.3.4.2.1 TEM

TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Chaudhary and Aggarwal, 2004). This enzyme is also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original β-lactamase (Chaudhary and Aggarwal, 2004). This caused a shift in the isoelectric point from a pI of 5.4 to 5.6, but it did not change the substrate profile. TEM-3, originally reported in 1989, was the first TEM-type β-lactamase that displayed the ESBL phenotype. In the years since that first report, over 90 additional TEM derivatives have been described (for amino acid sequences for TEM, SHV, and OXA extended-spectrum and inhibitor-resistant β-lactamases, see http://www.lahey.org/studies/webt.htm). Some of these β-lactamases are inhibitor resistant enzymes, but the majority of the new derivatives are ESBLs. (Bradford, 2001).

2.3.4.2.2 SHV

The SHV-1 β-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Bradford, 2001). In many strains of *K. pneumoniae*, bla_{SHV-1} or a related gene is integrated into the bacterial chromosome (Livermore, 1995). Although it has been hypothesized that the gene encoding SHV-1 may exist as part of a transposable element, it has never been proven (Bradford, 2001). Unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1. Furthermore, the changes that have been observed in bla_{SHV} to give rise to the SHV variants occur in fewer positions within the structural gene. The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitution of lysine for glutamate at position 240. It is interesting that both the Gly238Ser and Glu240Lys amino acid substitutions mirror those seen in TEM-type ESBLs. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Ho *et al.*, 2000).

To date, the majority of SHV-type derivatives possess the ESBL phenotype. However, one variant, SHV-10, is reported to have an inhibitor-resistant phenotype. This enzyme appears to be derived from SHV-5 and contains one additional amino acid substitution of glycine for serine at position 130. It is interesting that the inhibitor-resistant phenotype conferred by the Ser140Gly mutation seems to override the strong ESBL phenotype usually seen in enzymes containing the Gly238Ser and the Glu240Lys mutations seen in other SHV-5-type enzymes. The majority of SHV-type ESBLs are found in strains of *K. pneumoniae*. However, these enzymes have also been found in *Citrobacter diversus*, *E. coli*, and *P. aeruginosa* (Bradford, 2001).

2.3.4.2.3 CTX-M

In recent years a new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyze cefotaxime has arisen. They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae*. They include the CTX-M-type enzymes CTX-M-1 (formerly called MEN-1), CTX-M-2 through to CTX-M-10. These enzymes are not very closely related to TEM or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases (Tzouvelekis *et al.*, 2000). Previously, the most closely related enzymes outside this family were thought to be the chromosomally encoded class A cephalosporinases found in *K. oxytoca, C. diversus, Proteus vulgaris*, and *Serratia fonticola* (73 to 77% homology) (Bonnet et al 1999). However, it was recently reported by Humeniuk *et al.* (2000) that there is a high degree of homology between the chromosomal AmpC enzyme of *Kluyvera ascorbata* (designated Klu-1 and Klu-2) and the CTX-M-type enzymes, suggesting that the latter probably originated from this species.

2.3.4.2.4 OXA

The OXA-type enzymes are another growing family of ESBLs. These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bradford, 2001). The OXA β-lactamase family was originally created as a phenotypic rather than a genotypic group for a few β-lactamases that had a specific hydrolysis profile. Therefore, there is as little as 20% sequence homology among some of the members of this

family. However, recent additions to this family show some degree of homology to one or more of the existing members of the OXA β-lactamase family.

While most ESBLs have been found in *E. coli*, *K. pneumoniae*, and other *Enterobacteriaceae*, the OXA-type ESBLs have been found mainly in *P. aeruginosa*. Several of the OXA-type ESBLs have been derived from OXA-10 (OXA-11, -14, -16, and -17). OXA-14 differs from OXA-10 by only one amino acid residue, OXA-11 and OXA-16 differ by two, and OXA-13 and OXA-19 differ by nine. Among the enzymes related to OXA-10, the ESBL variants have one of two amino acid substitutions: an asparagine for serine at position 73, or an aspartate for glycine at position 157. In particular, the Gly157 Asp substitution may be necessary for high-level resistance to ceftazidime (Bradford, 2001).

2.3.4.2.5 Other ESBLs

While the majority of ESBLs are derived from TEM or SHV β-lactamases and others can be categorized with one of the newer families of ESBLs, a few ESBLs have been reported that are not closely related to any of the established families of β-lactamases (Bradford, 2001).

The PER-1 β-lactamase was first discovered in strains of *P. aeruginosa* isolated from patients in Turkey (Bradford, 2001). Later, it was also found among isolates of *S. enterica* serovar *Typhimurium* and *A. baumanii* (Vahaboglu *et al.*, 1998). The PER-1 β-lactamase is widespread across Turkey and is found in up to 60% of ceftazidime-resistant strains of *A. baumanii*, which represent 46% of total isolates. A common plasmid encoding PER-1 was found in multiple nosocomial isolates of *S. enterica* serovar *Typhimurium*, suggesting that the strains acquired the resistance plasmids in the hospital setting (Bradford, 2001).

A related enzyme, PER-2, which has 86% amino acid homology with PER-1, was found among *S. enterica* serovar *Typhimurium* strains in Argentina. It is interesting that PER-1 is found almost exclusively in Turkey, while PER-2 has been found almost exclusively in South America.

Another enzyme that is somewhat related to PER-1 is the VEB-1 β-lactamase. VEB-1 was first found in a single isolate of *E. coli* in a patient from Vietnam, but was subsequently also found in a *P. aeruginosa* isolate from a patient from Thailand (Bradford, 2001).

A third related enzyme is CME-1, which was isolated from *Chryseobacterium meningosepticum* (Rossolini *et al.*, 1999). A fourth enzyme in this group is TLA-1, which was identified in an *E. coli* isolate from a patient in Mexico (Silva *et al.*, 2000). The PER-1, PER-2, VEB-1, CME-1, and TLA-1 β-lactamases are related but show only 40 to 50% homology. These enzymes all confer resistance to oxyimino-cephalosporins, especially ceftazidime, and aztreonam. They also show some homology to the chromosomal cephalosporinases in *Bacteroides* spp. and may have originated from this genus (Rossolini *et al.*, 1999).

2.4 AMINOGLYCOSIDES

2.4.1 Sources of aminoglycosides

The aminoglycosides are a group of antibiotics derived from either *Streptomyces* species (those with names ending in "mycin") or *Micromonospora* species (those with names ending in "micin") (Gilbert, 1995). Streptomycin, introduced in 1944, was the first aminoglycoside in clinical use. Subsequently, it has been followed by several, related compounds including gentamicin, tobramycin, netilmicin, kanamycin, and amikacin (a semisynthetic derivative of kanamycin). In the 1970s, the semisynthetic aminoglycosides dibekacin, amikacin, and netilmicin demonstrated the possibility of obtaining compounds which were active against strains that had developed resistance mechanisms towards earlier aminoglycosides as well as displaying distinct toxicological profiles. Since then, however, the pace of development of new aminoglycosides has markedly slowed down (Mingeot-Leclercq *et al.*, 1999).

2.4.2 Mechanism of action and spectrum of activity

The aminoglycosides are generally bactericidal agents. They penetrate the bacterial cell wall and membrane, and block protein synthesis by binding to components of the 30s ribosomal subunit, a process that requires energy derived from aerobic metabolism. This binding leads to the bacterial misreading of mRNA, with production of nonfunctional proteins, and may also cause detachment of ribosomes from mRNA with subsequent cell death. Aminoglycosides, like quinolones, exhibit killing that is concentration dependent, i.e., they kill more rapidly and effectively when present at higher concentrations at the site of infection. This distinguishes the aminoglycosides from vancomycin and from the β-lactam drugs, which kill in a time-dependent fashion (Joseph and McGowan, 1998).

Aminoglycosides, particularly amikacin, have broad activity against Gram-negative aerobes such as *Klebsiella, Enterobacter, Serrutia,* and most *Acinetobacter* species. They are also active against *Pseudomonas aeroginosa* (tobramycin > gentamicin), but less so for non *aeruginosa* strains of *Pseudomonas* (Joseph and Mcgowan, 1998). Some organisms resistant to both gentamicin and tobramycin may be susceptible to amikacin. Aminoglycosides are not active against anaerobes because their uptake across bacterial cell membranes depends on energy derived from aerobic metabolism. Their dependence on aerobic metabolism is the cause of markedly reduced activity of these agents in areas with low pH and oxygen tension such as abscesses and other infected tissues. Among Gram-positive organisms, the aminoglycosides are active against *Staphylococcus aureus* and coagulase- negative staphylococci (amikacin and gentamicin are most effective). Other Gram-positive organisms, such as *Streptococcus pyogenes, Streptococcus pneumoniae,* and enterococci are relatively resistant (Joseph and Mcgowan, 1998).

Aminoglycosides should not be used as single agents in infections caused by these organisms. Synergy has been seen between penicillin and gentamicin toward sensitive strains of *Enrerococcus faecalis* and *E. faecium,* Group A and B streptococci, and *Streptococcus viridians.* Synergy has been described between aminoglycosides and antimicrobials that inhibit cell-wall synthesis (β-lactam drugs, vancomycin, monobactams, and carbapenems). *Enterococci* with high-level resistance to gentamicin may remain susceptible to streptomycin, and both of these drugs should therefore be tested in infections caused by such organisms (Joseph and McGowan, 1998).

2.4.3 Resistance Mechanisms

The emergence of resistant strains has somewhat reduced the potential of aminoglycosides in empiric therapies. The main mechanisms which may affect all aminoglycosides are (i) a decreased

uptake and/or accumulation of the drug in bacteria and (ii) the bacterial expression of enzymes which modify the antibiotic and thereby inactivate it (Mingeot-Leclercq *et al.*, 1999).

2.4.3.1 Decrease in drug uptake and accumulation.

Reduced drug uptake, mostly seen in *Pseudomonas* spp. and other non-fermenting Gram-negative bacilli, is likely to be due to membrane impermeabilization, but the underlying molecular mechanisms are largely unknown. It is highly significant in the clinic since it affects all aminoglycosides, is a stable characteristic, and results in a moderate level of resistance (intermediate susceptibility). Aerobic Gram-negative bacilli in general also show a phenomenon of adaptive resistance (transiently reduced antimicrobial killing in originally susceptible bacteria). Membrane protein changes and alteration in the regulation of genes of the anaerobic respiratory pathway in bacteria exposed to aminoglycosides are probably responsible for this phenomenon which gives a pharmacodynamic rationale for high dosages associated with long intervals between successive administrations. Active efflux has been evidenced for neomycin, kanamycin, and hygromycin A in *Escherichia coli* (protein Mdfa, a member of the family of multidrug resistance proteins), but its clinical significance is still uncertain compared to that of other antibiotics (Mingeot-Leclercq et al., 1999).

2.4.3.2 Aminoglycoside-modifying enzymes.

Aminoglycoside-modifying enzymes catalyze the covalent modification of specific amino or hydroxyl functions, leading to a chemically modified drug which binds poorly to ribosomes and for which the EDP-II of accelerated drug uptake also fails to occur, thereby resulting most often in high-level resistance. The enzymes modifying aminoglycosides are *N*-acetyltransferases (AAC), which use acetyl-coenzyme A as donor and affect amino functions, and *O*- nucleotidyltransferases

(ANT) and *O*-phosphotransferases (APH), which both use ATP as donor and affect hydroxyl functions (Mingeot-Leclercq *et al.*, 1999).

2.4.3.2.1 Aminoglycoside acetyltransferase (*AAC*)

Acetylation of aminoglycosides can occur at the 1, 3, 6', and 2' amino groups and involves virtually all medically useful compounds (e.g. gentamicin, tobramycin, netilmicin, and amikacin). Enzymes that modify the 3 position (3-*N*-aminoglycoside acetyltransferases [AAC(3)]) and the 6' position (6'-*N*-aminoglycoside acetyltransferases [AAC(6')]) were discovered early in *P. aeruginosa* and remain the most common acetyltransferases and, with ANT(2'), the most common enzymes providing for aminoglycoside resistance in this organism. The AAC (3)-I family, of which three variants (Ia, Ib and Ic) have been described in *P. aeruginosa*, is a common determinant of gentamicin resistance in this organism. AAC (3)-II and AAC(3)-III are less commonly described AAC(3) enzymes that determine gentamicin resistance as well as tobramycin and netilmicin resistance [AAC(3)-II] or tobramycin and kanamycin resistance [AAC(3)-III] in *P. aeruginosa* (Mingeot-Leclercq et al., 1999).

The AAC (6') family of enzymes mediate resistance to tobramycin, netilmicin, kanamycin, and either amikacin (I subfamily) or gentamicin (II subfamily). AAC (6')-II is not only the most common AAC (6') but also the most common AAC in *P. aeruginosa* and is thus a significant determinant of gentamicin and tobramycin resistance in this organism. While AAC(6')-I [also referred to as AAC(6')- Ia] is less common, it is significant for amikacin resistance in *P. aeruginosa*, although a variant of this enzyme that fails to provide for amikacin resistance, AAC(6')- Ib, has been reported in clinical isolates resistant to tobramycin. A variant of the latter enzyme, AAC (6')-Ib, that differs from AAC (6')-Ib by a single amino acid and that has the same

activity as AAC (6')-II has been described in a few CF patient isolates resistant to tobramycin. A fused *aac(3)-Iaac(6')-Ib* gene encoding an enzyme active against gentamicin, tobramycin, and kanamycin has also been described . Novel AAC(6') enzymes similar in sequence to AAC(6')-I but significantly shorter and dubbed AAC(6')-29a and AAC(6')- 29b that provide resistance to all typical AAC(6')-I substrates except netilmicin have been described. AAC (6')-29b displays weak acetyltransferase activity, and aminoglycoside resistance appears to result from very tight binding (i.e., sequestering) of aminoglycosides by this enzyme (Vakulenko and Mobashery, 2003). Table 2.2 summarizes the substrate profile of the aminoglycoside acetyltranferase

Table 2.2: Substrate profiles of aminoglycoside acetyltransferases

Acetyltransferase	Substrate(s)
AAC(6')	
I (at least 24 different enzymes)	Tobramycin, amikacin, netilmicin, dibekacin, sisomicin, kanamycin, isepamicin
II	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, kanamycin
AAC(3)	
Ia, Ib	Gentamicin, sisomicin, fortimicin
IIa, IIb, IIc	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin
IIIa, IIIb, IIIc	Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin, neomycin, paromomycin, lividomycin
IV	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, apramycin
VII	Gentamicin
AAC(1)	Paromomycin, lividomycin, ribostamycin, apramycin
AAC(2')-Ia	Tobramycin, gentamicin, netilmicin, dibekacin, neomycin

Adapted from Vakulenko and Mobashery (2003)

2.4.3.2.2 Aminoglycoside Phosphoryltransferase [APH]

Inactivation of aminoglycosides such as kanamycin, neomycin, and streptomycin by resistant strains of *P. aeruginosa* as a result of phosphorylation has been known for over 30 years. Inactivation is carried out by phosphotransferases [APH (3')] that modify the 3'-OH of these antimicrobials, and these phosphotransferases are commonly encountered in *P. aeruginosa*. Several APH (3') enzymes have been described in *P. aeruginosa*, with APH (3')-I and -II being predominant in clinical isolates resistant to kanamycin (and neomycin). Indeed, a chromosomal *aphA*-encoded APH (3')-II-type enzyme, APH (3')-IIb, is likely responsible for the general insensitivity of *P. aeruginosa* to, e.g., kanamycin that was noted in the 1960s, when this drug was first being used clinically (Vakulenko and Mobashery, 2003). Interestingly, a gene, *hpaA*, encoding an AraCtype positive regulator of *aph(3')-IIb* and genes involved in the metabolism of 4-hydroxyphenylacetic acid (4-HPA) occur immediately upstream of the *aph(3')-IIb* gene and form an operon with the *aph(3')-IIb* gene. HpaA activation of these genes is stimulated by 4-HPA, suggesting that the phosphotransferase may, in fact, play an intended role in metabolism and only fortuitously provides resistance to aminoglycosides. APH (3') enzymes that provide resistance to other aminoglycosides have also been described in *P. aeruginosa* and include APH(3')-VI (amikacin and isepamicin) and APH(2') (gentamicin and tobramycin) (Vakulenko and Mobashery, 2003). Table 2.3 summarizes the substrate profile of the aminoglycoside phosphotranferase.

Table 2.3: Substrate profiles of aminoglycoside phosphotransferases

Phosphotransferase	Substrate (s)
APH (3')	
I	Kanamycin, neomycin, lividomycin, paromomycin, ribostamycin
II	Kanamycin, neomycin, butirosin, paromomycin, ribostamycin
III	Kanamycin, neomycin, lividomycin, paromomycin, ribostamycin, butirosin, amikacin, isepamicin
IV	Kanamycin, neomycin, butirosin, paromomycin, ribostamycin
V	Neomycin, paromomycin, ribostamycin
VI	Kanamycin, neomycin, paromomycin, ribostamycin, butirosin amikacin, isepamicin
VII	Kanamycin, neomycin
APH(2)	
Ia (bifunctional enzyme)	Kanamycin, gentamicin, tobramycin, sisomicin, dibekacin
Ib, Id	Kanamycin, gentamicin, tobramycin, netilmicin, dibekacin
Ic	Kanamycin, gentamicin, tobramycin
APH(3')-Ia, -Ib	Streptomycin
APH(7')-Ia	Hygromycin
APH(4)-Ia, -Ib	Hygromycin
APH(6)-Ia, -Ib, -Ic, -Id	Streptomycin
APH(9)-Ia, -Ib	Spectinomycin

Adapted from Vakulenko and Mobashery (2003)

2.4.3.2.3 Aminoglycoside nucleotidyltransferase [ANT]

The adenylation of aminoglycosides such as streptomycin and gentamicin by resistant strains of *P. aeruginosa* has been known for over 20 years. The most prevalent nucleotidyltransferase is the ANT (2')-I enzyme, which, with AAC (6') [and, to some extent, AAC(3)], represents the most common determinant of enzyme-dependent aminoglycoside resistance in *P. aeruginosa* (Vakulenko and Mobashery, 2003). The ANT (2')-I enzyme inactivates gentamicin and tobramycin but not netilmicin or amikacin and is thus found in gentamicinresistant (Vakulenko and Mobashery, 2003) and tobramycin-resistant clinical isolates. Other adenyltransferases associated with aminoglycoside resistance in *P. aeruginosa* include ANT (3') (streptomycin resistance) and ANT (4')-II (amikacin, tobramycin, and isepamicin resistance). Two variants of ANT(4')-II, ANT(4')-IIa and ANT(4')-IIb, have been reported and are encoded by genes present in the chromosome and/or on plasmids of amikacin-resistant clinical isolates (Vakulenko and Mobashery, 2003). Table 2.4 summarizes the substrate profiles of aminoglycoside nucleotidyltransferases.

Table 2.4: Substrate profiles of aminoglycoside nucleotidyltransferases

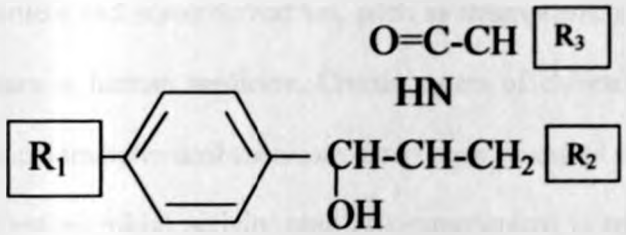
Nucleotidyltransferase	Substrate(s)
ANT(2')-I	Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin
ANT(3')-I	Streptomycin, spectinomycin
ANT(4')-Ia	Tobramycin, amikacin, dibekacin, kanamycin, isepamicin
ANT(4')-IIa	Tobramycin, amikacin, kanamycin, isepamicin
ANT(6')-I	Streptomycin
ANT(9)-I	Spectinomycin

Adapted from Vakulenko and Mobashery, (2003).

2.5 CHLORAMPHENICOL

2.5.1 Chemical structures and properties of chloramphenicol

Chloramphenicol, originally referred to as chloromycetin, was isolated from *Streptomyces venezuelae* in 1947 (Ehrlich *et al.*, 1947; Shwarz *et al.*, 2004) and shown to be a broad spectrum antibiotic with a novel structure (Fig. 2.1), remarkable both for a p-nitrophenyl group (at C-1) and an N-dichloroacetyl substituent (at C-2) attached to a 1,3-propanediol with two chiral centers (C-1 and C-2).Chloramphenicol was the first naturally occurring substance described to contain a nitro group. The relative simplicity of cloramphenicol made it the first antibiotic to be marketed as the product of chemical synthesis and chloramphenicol has been produced exclusively this way since 1950. Only one (D-threo) of the four possible diastereoisomers possesses antibiotic activity. The C-3 primary hydroxyl group, initially thought to be essential for inhibition of protein synthesis through its affinity for the peptidyltransferase of 50S ribosomes, can be replaced with fluorine. Besides the fluoro substitution at C-3 (in florfenicol), very few other substitutions are tolerated without adverse effects on antimicrobial activity. Among them, the substitution of the nitro group (–NO2), which was considered to be responsible for the dose-unrelated aplastic anemia, by a sulfomethyl group (–SO2CH3) at the para position of the 1-phenyl moiety became effective in thiamphenicol, and florfenicol (Fig.2.1) (Schwarz *et al.*, 2004).



	R_1	R_2	R_3
Chloramphenicol	$-NO_2$	$-OH$	$=Cl_2$
Azidamfenicol	$-NO_2$	$-OH$	H, N=N=N
Thiamphenicol	$-SO_2CH_3$	$-OH$	$=Cl_2$
Florfenicol	$-SO_2CH_3$	$-F$	$=Cl_2$

Fig. 2.1: Structure of chloramphenicol and related substances.

Source: Schwarz *et al.*, 2004

2.5.2 Use in human and veterinary medicine

Chloramphenicol and some derivatives, such as thiamphenicol and azidamfenicol, have been used over the years in human medicine. Certain esters of chloramphenicol, such as chloramphenicol palmitate or chloramphenicol succinate, have been produced for therapeutic applications. They do not exhibit antimicrobial activity until chloramphenicol is released after hydrolysis by esterases (Simon and Stille, 2000). Chloramphenicol succinate shows a good solubility in water and therefore is used for parenteral applications. The water soluble azidamphenicol is only used in eye drops (Simon and Stille, 2000). In the early years after its introduction into clinical use, chloramphenicol was considered as a promising broad spectrum antibiotic. However, a number of adverse effects have been observed since the mid-1960s in connection with the application of chloramphenicol (Schwarz *et al.*, 2004). These side-effects include a dose-unrelated irreversible aplastic anemia which occurs at frequencies of 1:10,000–1:40,000 (Simon and Stille, 2000). or 1:20,000–1:600,000 (Schwarz *et al.*,2004), a dose-related reversible bone marrow suppression, or the Gray syndrome in neonates and infants. Occasionally, hypersensitivity to chloramphenicol ranging from skin rashes to anaphylaxis has been observed, too. Based on these adverse effects and on the availability of less toxic antimicrobial agents with a similar spectrum of activity, the use of chloramphenicol in humans is nowadays limited to the therapy of a small number of life threatening infections. Since chloramphenicol readily crosses the blood–brain barrier, it remains an alternative therapeutic agent for the treatment of meningitis caused by susceptible strains of *Haemophilus influenzae, Neisseria meningitidis or Streptococcus pneumoniae* when no other antimicrobial agents can be used, e.g. in penicillin allergic patients (Mascaretti, 2003).

The use of chloramphenicol in veterinary medicine in the European Union (EU) is currently limited to pets and nonfood- producing animals. It was banned in 1994 from use in any food-producing animals in the EU. The main reason for this ban was protection of the consumer from potential adverse effects arising from chloramphenicol residues in carcasses of food animals. Because of the dose-independent irreversible aplastic anemia in humans, the "non-observed effect level" (NOEL) could not be determined for chloramphenicol. In toxicological studies, NOEL represents the dose at and below which adverse effects do not occur (Schwarz *et al.*, 2001). The calculation of the "maximum residue level" (MRL), which represents the maximum level of antibiotic residues acceptable in carcasses at slaughter without any adverse effect on public health, is based on the NOEL and, therefore, could not be determined. As a consequence, EU legislation banned chloramphenicol along with several antibiotics, including nitroimidazoles and furazolidinones, from use in food-producing animals (Schwarz *et al.*, 2001).

Since adverse side-effects, in particular the dose-independent irreversible aplastic anemia, have not been observed in animals, the fluorinated chloramphenicol derivative forfenicol has been licensed for the control of bacterial respiratory tract infections in cattle and pigs. Forfenicol was approved in the EU for the use in cattle and in pigs in 1995 and in 2000, respectively. Moreover, forfenicol is also approved for the treatment of infectious pododermatitis (interdigital phlegmon) in cattle due to *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* in the USA. In commercial salmon farming, a forfenicol premix is used for the treatment of furunculosis in salmons caused by *Aeromonas salmonicida*. Fluorinated chloramphenicol derivatives are currently not used in human medicine (Schwarz, *et al.*, 2001).

2.5.3 Mode of action and spectrum of activity

In procaryotes, chloramphenicol is a highly specific and potent inhibitor of protein biosynthesis. Chloramphenicol dependent inhibition of bacterial protein biosynthesis is mainly due to the prevention of peptide chain elongation. Its bacteriostatic activity is based on a reversible binding to the peptidyltransferase centre at the 50S ribosomal subunit of 70S ribosomes (Schlunzen *et al.*, 2001). 80S ribosomes of eucaryotic cells are not targets of chloramphenicol and its derivatives. However, it has been assumed that chloramphenicol may interact with mitochondrial ribosomes which are similar in their structure to 70S ribosomes rather than to 80S ribosomes. As a possible consequence, the mitochondrial function of stem cells in bone marrow may be impaired resulting in a suppression of the bone marrow function (Schwarz *et al.*, 2004). The substrate spectrum of chloramphenicol includes Gram-positive and Gram-negative, aerobic and anaerobic bacteria, but also *chlamydiae*, *mycoplasmas*, and *rickettsiae* (Yao and Moellering, 1999). Chloramphenicol analogs including the fluorinated derivative florfenicol have a similar spectrum of antimicrobial activity as chloramphenicol (Yao and Moellering, 1999). Intrinsic resistance to chloramphenicol and florfenicol has not been observed although members of different bacterial species and genera may differ in their basic levels of susceptibility to both drugs as confirmed by the determination of minimum inhibitory concentrations (MICs) (Priebe and Schwarz, 2003).

2.5.4 Bacterial resistance to chloramphenicol

Over the years, bacteria have developed a number of mechanisms which enable them to circumvent the inhibitory effects of chloramphenicol. The first and still most frequently encountered mechanism of bacterial resistance to chloramphenicol is enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases (CATs) (Murray and Shaw, 1997). However, there are also reports on other mechanisms of chloramphenicol resistance, such as

efflux systems, inactivation by phosphotransferases, mutations of the target site and permeability barriers (Schwarz *et al.*, 2004). As the number of genes associated with resistance to chloramphenicol and related drugs increases, inconsistencies of their nomenclature were observed. These included the assignment of identical designations for different chloramphenicol resistance genes and that of different designations for virtually the same resistance gene (Tables 2.5-2.7).

2.5.4.1 Chloramphenicol acetyltransferases

Chloramphenicol acetyltransferases (CATs) are able to inactivate chloramphenicol as well as thiamphenicol and azidamfenicol. Due to the replacement of the hydroxyl group at C-3 by a fluor residue, the acceptor site for acetyl groups was structurally altered in florfenicol. This modification rendered florfenicol resistant to inactivation by CAT enzymes, and consequently, chloramphenicol-resistant strains, in which resistance is exclusively based on the activity of CAT, are susceptible to florfenicol (Schwarz *et al.*, 2004). There are two defined types of CATs which distinctly differ in their structure: the classical CATs, referred to in this review as type A CATs and the novel CATs, also known as xenobiotic CATs (Murray and Shaw, (1997), but referred to in this review as type B CATs. In addition, annotations of cat genes were found in the whole genome sequences of *Rhodobacter capsulatus, Mesorhizobium loti, S. agalactiae* strain 2306 (Tettelin *et al.*, 2002), *Bacillus cereus* (Ivanova *et al.*, 2003), and *Brucella melitensis* (DelVecchio *et al.*, 2002). The potential CAT variants encoded by these five presumed cat genes do not exhibit structural features that allow their assignment to either type A or type B. As long as functional activity has not been confirmed, assignment of these CAT-like proteins to further novel subtypes has to be postponed.

2.5.4.1.1 Type A chloramphenicol acetyltransferases

Type A CATs have been detected in a wide variety of bacteria. Despite the differences in their amino acid sequences, the type A CATs share some common properties. The native CAT is usually composed of three identical polypeptides each ranging in size between 207 and 238 amino acids (aa). In cells in which two different, but related, CATs are present, functionally active heterotrimers may also occur. The cat gene codes for the CAT monomer. In all currently known type A CATs, some amino acids, which are involved in substrate binding, catalytic activities, folding of the monomers, or assembly of the monomers to a trimer, appear to be conserved. Some of the type A CATs have specific properties, such as the capability to mediate resistance also to fusidic acid or sensitivity to inhibition by thiol-reactive reagents (Schwarz *et al.*, 2004). One type A CAT enzyme, CAT III from *Shigella flexneri*, has been studied by X-ray crystallography and the data derived have been the basis for the understanding of the catalytic activities and the assembly of the CAT monomers. There are at least 16 distinct groups, A-1–A-16, of cat A genes. The corresponding type A CAT proteins assigned to the same group exhibit amino acid sequence identities of more than 80% (Schwarz *et al.*, 2004). The different groups and their representatives are listed in Table 2.5

Table 2.5: Type A chloramphenicol acetyltranferase

Group	Gene designation (s)	Bacteria sources	Plasmid/transposon/ chromosome	% identity		Database Accession no
				DNA	AA	
A-1	*cat1*	*Eshericia coli*	Tn9,R429	98.3-100	97.3-100	V00622
	cat1	*Acinetobacter baumannii*	Chromosome (Tn2670)			M62822
	cat	*Acinetobacter calcoaceticus*	Tn2670-like			M37690
	pp-cat	*Photobacterium damselae subsp. piscicida*	pSP9351			D16171
	-	*Pseudomonas putida*	Unknown			E02706
	cat	*Serratia marcescens*	R478			NC_005211, BX664015
A-2	*cat*	*Shigella flexneri*	Chromosome			AF326777
	catIII	*Shigella flexneri*	R387	99.8–100	99.5–100	X07848
	catA3	*Mannheimia taxon* 10	pMHSCS1			AJ249249
	catA3	*Mannheimia varigena*	pMVSCS1			AJ319822
	cat	*Uncultured eubacterium*	pIE1130			NC_004973, AJ271879
A-4	*cat*	*Proteus mirabilis*	Chromosome			M11587
A-5	*cat*	*Streptomyces acrimycini*	Chromosome			P20074
A-5	*cat*	*Streptomyces acrimycini*	Chromosome			P20074
A-6	*cat86*	*Bacillus pumilus*	Chromosome			K00544
A-7	*cat(pC221),*	*Staphylococcus aureus*	pC221	96.6–100	95.3–100	X02529
	cat	*Staphylococcus aureus*	pKH7			U38429
	cat	*Staphylococcus aureus*	pUB112			X02872
	cat	*Staphylococcus intermedius*	pSCS1			M64281
	cat	*Staphylococcus aureus*	pSCS6			X60827

Group	Gene designation (s)	Bacteria sources	Plasmid/Transposon/ Chromosome	% identity		Database accession no.
				DNA	AA	
	cat	*Bacillus subtilis*	pTZ12			M16192
	cat	*Streptococcus agalactiae*	pGB354			U83488
	cat	*Streptococcus agalactiae*	pIP501			X65462
A-8	*cat*(*pC223*).	*Staphylococcus aureus*	pC223 89	2–100 85	2–100	NC_005243, AY355285
	cat	*Staphylococcus aureus*	pSCS7			M58516
A-9	*cat*(*pC194*)	*Staphylococcus aureus*	pC194	93.9–99.8	87.0–94.9	V01277
	cat-TC	*Lactobacillus reuteri*	pTC82			U75299
A-10	*cat*	*Bacillus clausii*	Chromosome			AY238971
A-11	*catP*	*Clostridium perfringens*	pIP401 : Tn4451	100	100	U15027
A-12	*catS*	Streptococcus pyogenes	Chromosome			X74948
A-13	*cat*	*Campylobacter coli*	pNR9589			M35190
A-14	*cat*	*Listonella anguillarum*	pJA7324			S48276
A-15	*catB*	*Clostridum butyricum*	Chromosome			M93113
A-16	*catQ*	*Clostridium perfringens*	Chromosome			M55620

Source: S. Schwarz *et al.*, 2004

2.5.4.1.2 Type B chloramphenicol acetyltransferases

Type B CATs, occasionally referred to as xenobiotic acetyltransferases, also inactivate chloramphenicol by acetylation. Type B CATs share some common properties with the type A CATs: native type B CATs are also homotrimers composed of monomers which are in the range of 209– 212 aa. However, on the basis of their amino acid sequences, type B CATs differ distinctly in their structure from type A CATs and appear to be related to other acetylating enzymes of staphylococci and enterococci involved in resistance to A compounds of the streptogramins, such as Vat (D) (formerly known as SatA), Vat (E) (formerly known as SatG; Vat (A) (formerly known as Vat), or Vat(B) (Schwarz *et al.*, 2004). The different groups and their representatives are listed in Table 2.6

Table 2.6: Type B chloramphenicol acetyltranferases

Group	Gene designations	Bactria sources	Plasmid/transosons/ Chromosomes	% identity		Database Accession no.
				DNA	AA	
B-1	*cat, catB1*	*Agrobacterium tumefaciens*	Chromosome	100	100	M58472
B-2	*catB2*	*Escherichia* coli	pNR79:Tn2424	99.5–99.8	99.0–100	F047479A
B-3	*catB3*	*Salmonella Typhimurium*	pWBH301	84.3–100	84.8–100	AJ009818
B-4	*catB7*	*Pseudomonas aeruginosa*	Chromosome	100	100	AF036933
B-5	*catB9*	*Vibrio cholerae*	Chromosome	100	100	AF462019
	catB9	*Vibrio cholerae*	Chromosome			NC_002506

Source: Schwarz *et al.*, 2004

2.5.4.2 Chloramphenicol exporters

The export of chloramphenicol from the bacterial cell can be mediated by either specific transporters and/or multidrug transporters. Specific transporters have a substrate spectrum which is commonly limited to a small number of structurally closely related compounds whereas that of the multidrug transporters often includes a wide range of unrelated substances. Specific transporters commonly mediate distinctly higher levels of resistance as compared to those of multidrug transporters. While specific transporters involved in the export of chloramphenicol have no known function in the physiological cell metabolism, multidrug transporters play an important role in the excretion of toxic compounds, occasionally also including specific antimicrobial agents such as chloramphenicol and florfenicol, from the bacterial cell (Schwarz *et al.*, 2004).

2.5.4.2.1 Specific exporters

At least eight different groups of specific exporters, E-1–E-8, are currently known (Table 2.7). Resistance to chloramphenicol not due to enzymatic inactivation was first detected in 1979 in *P. aeruginosa* and later on shown to be based on the presence of the transposon Tn1696. Sequence analysis of the chloramphenicol resistance gene of Tn1696, *cmlA*, revealed that the corresponding protein of 419 aa had 12 transmembrane domains and thus resembled closely other transmembrane transport proteins of the major facilitator superfamily (George and Hall, 2002). The *cmlA* gene proved to be part of a gene cassette. However, in contrast to other cassette-borne resistance genes, the *cmlA* gene had its own promoter and regulation of *cmlA* expression was inducibly regulated via translational attenuation. An attenuator-like structure – similar to that of inducibly expressed catA genes was detected upstream of the cmlA gene. During the last decade, a number of genes closely related to or indistinguishable from *cmlA* and all assigned to group E-1 have been identified in a

wide variety of gram-negative bacteria, including *E. coli*, *S. Typhimurium*, *Klebsiella pneumoniae*, and *P. aeruginosa*, but also from an uncultured eubacterium (Schwarz *et al.*,2004). *E. coli* strains carrying the gene cmlA were reported to exhibit MICs of chloramphenicol P32 to >256 mg l^{-1} and MICs of Ff of 68 to 64 mgl/1 (Bischoff, *et al.*, 2002). As compared to the other members of group E-1, the gene cmlA2 (also referred to as cmlB) from Enterobacter aerogenes showed only 84% nucleotide sequence identity and 85% identity in the amino acid sequence. Database search also identified a chloramphenicol resistance gene, designated cml, which was located on plasmid R26 . The Cml protein, which does not mediate resistance to fluorinated chloramphenicol analogs, consists of 302 amino acids and exhibits only five transmembrane segments. It is in part similar to the distinctly larger *CmlA* protein of Tn1696 and represents group E-2. A number of genes referred to in the published literature as pp-flo, cmlA-like, *floSt*, *flo*, or *floR*, mediate combined resistance to chloramphenicol and florfenicol and were grouped together in group E-3 (Bischoff, *et al.*, 2002).

Table 2.7: Specific exporters mediating resistance to chloramphenicol

Group	Gene designation	Bacteria sources	Plasmid/transposon/ chromosome	%identity		Database accession no.
				DNA	AA	
E-1,	*cmlB cmlA2*	*Enterobacter aerogenes*	pIP833	83.1–100	83.9–100	AF034958
E-2	*cml*	*Escherichia coli*	R26			M22614
E-3	*cmlA*-like	*Salmonella Typhimurium*	Chromosome DT104	95.6–100	87.7–100	
	floR	*Escherichia coli*	Plasmid			AF231986
E-4	*fexA*	*Staphylococcus lentus*	pSCFS2			AJ549214
E-5	*cml*	*Streptomyces* lividans	Chromosome			X59968
E-6	*cmlv*	*Streptomyces venezuelae*	Chromosome			U09991
E-7	*cmrA*	*Rhodococcus rhodochrous*	Tn5561	77.5	86.2	AF015087
	cmr	*Rhodococcus fascians*	pRF2			Z12001
E-8	*cmr*	*Corynebacterium glutamicum*	pXZ10145	99.9	99.7	U85507

2.5.4.2.2 Multidrug transporters.

In addition to specific exporters, a number of multidrug transporter systems have been identified whose substrate spectrum includes chloramphenicol and/or florfenicol. In general, the levels of chloramphenicol and florfenicol resistance mediated by multidrug transporters are lower than those mediated by specific exporters ((Baucheron *et al.*, 2002). The AcrAB-TolC multidrug efflux system is able to export chloramphenicol and florfenicol at low levels (MICs of chloramphenicol and florfenicol of 4 mgl/1). Overproduction of this system, due to mutations at regulator loci, however, leads to clinical levels of resistance to chloramphenicol (MIC 16–32 mg l/1), Ff (MIC 32 mg l/1) and other antimicrobials by active efflux (Baucheron *et al.*, 2002). Another multidrug transporter, MdfA, which also exports chloramphenicol has been identified in *E. coli*. It shows 96% amino acid identity to the *E. coli* protein Cmr, a 12 TMS protein of 411 aminoacid which specifies a chloramphenicol efflux pump. Multidrug transporters whose substrate spectrums include chloramphenicol have also been described in *P. aeruginosa*. Similar to the AcrAB–TolC system in *E. coli*, these multidrug transporters are also composed of three components, a protein of the resistance/nodulation cell division family (MexB, MexD or MexF), a membrane fusion protein (MexA, MexC or MexE) and an outer membrane protein (OmpM, OmpJ or OprN), which interact cooperatively to enable export of the drugs (Poole, 2002). Multidrug transporters of a similar structure which can also export chloramphenicol have been identified in *Burkholderia cepacia* (CeoAB–OpcM) and *P. putida* (ArpAB–ArpC; TtgAB–TtgC). Overexpression of most of these multidrug transporter systems led to a distinct increase of the MIC of chloramphenicol whereas functional deletion resulted in a distinctly more susceptible phenotype (Sulavik *et al.*, 2001). It should be noted that several types of multidrug transporters may be present in the same bacterial strain and that specific transporters may occur side-by-side with multidrug transporters. Lee *et al.*,

(2000) investigated the effects of simultaneous expression of several efflux pumps, including specific exporters such as CmlA and multidrug transporters such as MdfA, AcrAB–TolC or MexAB–OprM, and observed additive as well as multiplicative effects on chloramphenicol resistance of *E. coli* and *P. aeruginosa* (Lee *et al.*, 2000). Some multidrug transporters from Gram-positive bacteria, such as NorA from *S. aureus* or Blt from *B. subtilis* were reported to be able to export chloramphenicol. However, studies on strains expressing the gene norA at elevated levels showed that their MICs of chloramphenicol and florfenicol were in the same low range as those of strains not carrying the gene *norA* . This observation suggested that carriage of the gene *norA* is most probably not a relevant factor in chloramphenicol resistance in staphylococci (Lee *et al.*, 2000).

2.5.4.3 Other resistance mechanisms

Besides inactivation by acetylation, there are other ways to inactivate chloramphenicol some of which, such as dehalogination, glucuronidation, and reduction of the nitro group, are usually seen during biotransformation in hepatocytes of humans and animals, but have not been identified in bacteria. Other mechanisms such as ophosphorylation and hydrolytic degradation of chloramphenicol to p-nitrophenylserinol are seen in the chloramphenical producer *S. venezuelae* ISP5230. These latter mechanisms seem to have a self-defense function in the antibiotic producer. Recently, the 3-O-phosphotransferase was crystallized and its X-ray structure was determined (Izard and Ellis, 2000).

Non-enzymatic chloramphenicol resistance mechanisms based on permeability barriers have been described in various bacteria. The loss of an outer membrane protein was considered to play a role in chloramphenicol resistance of *H. influenzae* strains which did not exhibit CAT activity.

Chloramphenicol resistance due to decreased permeability of the outer membrane was also observed in *B. cepacia*. The absence of a 50 kDa porin in *Tn1696*-carrying strains may also enhance chloramphenicol resistance. In *S. Typhi*, the lack of the OmpF protein, which is required for the entry of chloramphenicol into the bacterial cell, was found to result in high level resistance to chloramphenicol (Quintiliani *et al.*, 1999).

The mar locus which is present in bacteria of many enterobacterial genera has also been reported to contribute to chloramphenicol resistance of *E. coli*. The transcriptional activator MarA is able to activate the gene *micF* which produces an antisense RNA that effectively inhibits ompF translation (Quintiliani *et al.*, 1999). Mutations in the major ribosomal protein gene cluster of *E. coli* and *B. subtilis* as well as in the 23S rRNA gene of *E. coli* are known to confer resistance to chloramphenicol. However, in contrast to resistance to other protein biosynthesis inhibitors, e.g. macrolide, lincosamide–streptogramin antibiotics (Vester and Douthwaite, 2001), chloramphenicol resistance as a consequence of target site mutation/modification is rarely seen. One plausible suggestion for this observation is that structural changes at the peptidyl transferase center that might prevent chloramphenicol binding are incompatible with satisfactory ribosome function (Murray, 2000). Finally, a novel gene, *cfr*, which mediates resistance to chloramphenicol and florfenicol by a yet unidentified mechanism has recently been detected on plasmid pSCFS1 from *S. sciuri* (Schwarz, *et al.*, 2001). The corresponding gene product shows no homology to any of the so far known chloramphenicol resistance proteins, does not inactivate chloramphenicol, and also does not display transmembrane topology. Structural comparisons revealed a certain degree of similarity with Fe-S binding oxidoreductases of the MoaA/NifB/PqqE family. Two domains were detectable: the N-terminal domain represented a putative Cys-rich Fe-S binding sequence (CISSQCGCNFGCKFC),

whereas the C-terminal domain might contain a NAD-binding Rossman fold. However, the target site of *Cfr* as well as the *Cfr* mediated mechanism of resistance remains to be determined. The MICs of chloramphenicol of the *S. sciuri* strain carrying pSCFS1 were 32 and 64 mg/l and could be increased after induction to 64 and 512 mg/l, respectively. A potential regulatory region which resembled a translational attenuator was detected immediately upstream of the cfr reading frame (Schwarz *et al.*, 2004).

2.6 MULTIDRUG RESISTANCE

Treatment of infections is compromised worldwide by the emergence of bacteria that are resistant to multiple antibiotics. Although classically attributed to chromosomal mutations, resistance is most commonly associated with extrachromosomal elements acquired from other bacteria in the environment. These include different types of mobile DNA segments, such as plasmids, transposons, and integrons. However, intrinsic mechanisms not commonly specified by mobile elements such as efflux pumps that expel multiple kinds of antibiotics are now recognized as major contributors to multidrug resistance in bacteria. Once established, multidrug-resistant organisms persist and spread worldwide, causing clinical failures in the treatment of infections and public health crises (Alekshun and Levy, 2007).

"There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fitness' [resistance]." Efforts aimed at identifying new antibiotics were once a top research and development priority among pharmaceutical companies. The potent broad spectrum drugs that emerged from these endeavors provided extraordinary clinical efficacy. Success, however, has been compromised. We are now faced with a long list of microbes that have found ways to circumvent different structural classes of drugs and are no longer susceptible to most, if not all, therapeutic regimens (D'Costa et al., 2006).

The means that microbes use to evade antibiotics certainly predate and outnumber the therapeutic interventions themselves. In a recent collection of soil-dwelling *Streptomyces* (the producers of many clinical therapeutic agents), every organism was multidrug resistant. Most were resistant to at least seven different antibiotics, and the phenotype of some included resistance to 15–21 different

drugs (D'Costa *et al.*, 2006). Moreover, many isolates were resistant to daptomycin, quinupristin-dalfopristin, and telithromycin, all drugs approved by the United States Food and Drug Administration (FDA) within the last decade, as well as purely synthetic agents such as ciprofloxacin. These data not only suggest that our surroundings can act as a reservoir for new (and old) resistance mechanisms, but that the drugs we use to treat infectious diseases have long-lasting effects outside of the hospital. Many antimicrobial molecules exist for millennia stably within the environment (Cook *et al.*, 1989), where they select and promote growth of resistant strains. Resistance to single antibiotics became prominent in organisms that encountered the first commercially produced antibiotics. The most notable example is resistance to penicillin among staphylococci, specified by an enzyme (penicillinase) that degraded the antibiotic (Barber, 1947). Over the years, continued selective pressure by different drugs has resulted in organisms bearing additional kinds of resistance mechanisms that led to multidrug resistance (MDR) novel penicillin-binding proteins (PBPs), enzymatic mechanisms of drug modification, mutated drug targets, enhanced efflux pump expression, and altered membrane permeability (D'Costa *et al.*, 2006).

Some of the most problematic MDR organisms that are encountered currently include *Pseudomonas aeruginosa* (another microbe of soil origin), *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* bearing extended-spectrum β-lactamases (ESBL), vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (Table 2.8). Some like methicillin-resistant *S. aureus* couple MDR with exceptional virulence capabilities (Miller *et al.*, 2005). Others, including some strains of *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, manage to evade every drug within the physician's arsenal (Levin *et al.*, 1999).

Table 2.8: General Characteristics of Multidrug-Resistant Organisms

Organism	Common Infections	Key Antibiotic Resistances	Drugs Considered for Treatment of MDR[a]
P. aeruginosa	Lung, wound	β-lactams, fluoroquinolones, aminoglycosides	Colistin
Acinetobacter spp.	Lung, wound, bone, blood	β-lactams, fluoroquinolones, aminoglycosides	Colistin, tigecycline
E. coli and *K. pneumoniae* bearing extended-spectrum β-lactamases	Urinary, biliary, gastrointestinal tracts,lung, blood	β-lactams, fluoroquinolones, aminoglycosides	Colistin (for *K. pneumoniae*), tigecycline
Vancomycin-resistant enterococci	Blood, heart, intra-abdominal	Vancomycin	Quinupristin-dalfopristin, linezolid, daptomycin
Methicillin-resistant *S. aureus*	Skin and soft tissue, respiratory tract, blood	β-lactams, fluoroquinolones, macrolides	Quinupristin-dalfopristin, daptomycin, linezolid, tigecycline, vancomycin
Multidrug-resistant *S. pneumoniae*	Ear, lung, blood, cerebrospinal fluid	β-lactams, macrolides, tetracyclines, co-trimoxazole	Fluoroquinolones, tigecycline
Extensively drug-resistant *M. tuberculosis*	Lung	Rifampin, isoniazid, and three of the following: aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, or para-aminosalicylic acid	3rd line agents, drug combinations

[a] Agents either have been approved for use by a regulatory agency (e.g., FDA), have shown usefulness in treating infection, or exhibit promising in vitro activity and await a determination of clinical efficacy.

Adapted from Alekshun and Levy, 2007

2.6.1 Genetics of Multidrug Resistance

Bacterial antibiotic resistance can be attained through intrinsic or acquired mechanisms. Intrinsic mechanisms are those specified by naturally occurring genes found on the host's chromosome, such as, AmpC β-lactamase of Gram-negative bacteria and many MDR efflux systems. Acquired mechanisms involve mutations in genes targeted by the antibiotic and the transfer of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic material. In general, this exchange is accomplished through the processes of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosome of chromosomal DNA, plasmids, and other DNAs from dying organisms) (Levy and Marshall, 2004). Although gene transfer among organisms within the same genus is common, this process has also been observed between very different genera, including transfer between such evolutionarily distant organisms as Gram-positive and Gram-negative bacteria (Courvalin, 1994).

Plasmids contain genes for resistance and many other traits; they replicate independently of the host chromosome and can be distinguished by their origins of replication. Multiple plasmids can exist within a single bacterium, where their genes add to the total genetics of the organism. Transposons are mobile genetic elements that can exist on plasmids or integrate into other transposons or the host's chromosome. In general, these pieces of DNA contain terminal regions that participate in recombination and specify a protein(s) (e.g., transposase or recombinase) that facilitates incorporation into and from specific genomic regions. Conjugative transposons are unique in having qualities of plasmids and can facilitate the transfer of endogenous plasmids from one organism to another. Integrons contain collections of genes (gene cassettes) that are generally classified according to the sequence of the protein (integrase) that imparts the recombination function (Mazel,

2006). They have the ability to integrate stably into regions of other DNAs where they deliver, in a single exchange, multiple new genes, particularly for drug resistance. The super-integron, one which contains hundreds of gene cassettes (representing about ~3% of the host's genome), is distinct from other integrons; it was first identified in *Vibrio cholerae* (Mazel *et al.*, 1998).

Bacteria can become antibiotic resistant by mutation of the target gene in the chromosome. They can acquire foreign genetic material by incorporating free DNA segments into their chromosome (transformation). Genes are also transferred following infection by bacteriophage (transduction) and through plasmids and conjugative transposons (Alekshun and Levy, 2007).

2.6.1.2 Multidrug Resistance Efflux Systems

Historically, the Gram-negative cell envelope was thought to affect antibiotic susceptibility by greatly restricting drug penetration (Li and Nikaido, 2004). Contemporary studies, however, have shown that most antibacterial agents effectively penetrate Gram-negative organisms (Li and Nikaido, 2004) but fail to reach intracellular targets because of efflux (Levy, 1992).

In Gram-negative bacteria, including *E. coli* and nonfermenting organisms such as *P. aeruginosa*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Burkholderia cepacia*, "intrinsic" resistance is attributed to the expression of the RND efflux system(s). This mechanism is an effective means for dealing with different antibiotic classes using a single resistance determinant. The natural function of the *E. coli* AcrAB efflux system is thought to have evolved to protect the cell from the inhibitory activity of toxic substances such as bile salts (Li and Nikaido, 2004). Other related systems function similarly in *Neisseria gonorrhoeae* (Hagman *et al.*, 1995) and export molecules involved in quorum sensing in *P. aeruginosa* (Pesci *et al.*, 1999). Tigecycline, which gained FDA

approval in 2005, has poor activity against *P. aeruginosa*, *Proteus mirabilis*, *Morganella morganii*, and *Klebsiella pneumonia*; this is attributed to RND efflux systems (references in Stein and Craig, 2006). Elimination of an AcrA ortholog in *M. morganii*, MexXY-OprM in *P. aeruginosa*, and an AcrB ortholog in *P. mirabilis* increased susceptibility to tigecycline 16- to 133-fold (references in Stein and Craig, 2006), whereas deletion in *E. coli* of AcrAB and AcrEF had a more modest (4-fold) effect (Hirata *et al.*, 2004). *Bacillus subtilis* Bmr (Bacillus multidrug resistance) (Neyfakh *et al.*, 1991) and *S. aureus* Qac (quaternary ammonium compound) (Tennent *et al.*, 1989) are two MDR efflux proteins (members of the MF superfamily) that were first characterized in Gram-positive cells. Like many members of the RND family in Gram-negative bacteria, Bmr is constitutively expressed and therefore engenders intrinsic resistance to chloramphenicol and fluoroquinolones. Another MDR efflux pump from *B. subtilis*, Blt, includes spermidine among its list of substrates. It is now thought that the natural function of Blt is to facilitate the removal of polyamines from the cell (Woolridge *et al.*, 1997). The staphylococcal Qac systems provide resistance to antiseptics and disinfectants (e.g., quaternary ammonium compounds, chlorhexidine, and diamidines). Unlike most other MDR efflux proteins, these are specified on plasmids, a feature that facilitates their dissemination (Hirata *et al.*, 2004).

2.6.1.2 Single Determinants of Multidrug Resistance

Except for the case of MDR efflux pumps, a single resistance mechanism commonly affords protection against one antibiotic or, at the most, drugs within the same general class, e.g., PBP2 of MRSA. Still, resistance determinants that specify erythromycin (Erm) methyltransferases in a variety of pathogens are single proteins that give rise to macrolide, lincosamide, and type B

streptogramin resistance: structurally unique agents that share a common target and mechanism of action (Roberts, 2004).

A mutant aminoglycoside acetyltransferase (specified by *aac(6')-Ib-cr*) that gave rise to aminoglycoside (amikacin, kanamycin, and tobramycin) and fluoroquinolone (ciprofloxacin and norfloxacin) resistance was identified recently (Robicsek *et al.*, 2006). Although the level of resistance conferred was low, *aac(6')-Ib-cr* was located on a plasmid bearing another unusual mechanism (the *qnr* determinant of fluoroquinolone resistance, and the two determinants together functioned in an additive manner to yield clinical levels of fluoroquinolone resistance.

Reduced susceptibility to the macrolides, chloramphenicol, and linezolid in clinical *S. pneumoniae* isolates has been attributed to mutations in large ribosomal protein (L4) (Wolter *et al.*, 2005). Although mutations in the genes that specify 23S rRNA, L4, or L22 commonly lead to macrolide resistance, susceptibility to chloramphenicol frequently occurs following the acquisition of a modifying enzyme. For linezolid, previous resistance in the enterococci and *S. aureus* was attributed solely to mutations in the locus that encodes 23S rRNA (Wolter *et al.*, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Animals

Faecal samples were taken from two groups of olive baboons (*Papio anubis*) housed in the Institute of Primate Research (Nairobi, Kenya). Faecal sample collections was performed following approval from Institutional Review Committee (IRC) reference number IRC/06/09.

3.1.1 Group I

This group consisted 20 adult male baboons and 16 female baboons which were captured from Aberdare National park (Located approximately 180 km north of Nairobi) and transported to Institute of Primate Research. This group of baboons had been in captivity for a period of less than one month. For the first two weeks they were housed in group cages and later in individual cages. This group had very minimal contact with people unless during feeding and general cleaning which was done in the morning. The concrete floors of the cage area were regularly cleaned with hoses. Access to these animals was restricted to cage caretaker and veterinarian in charge. No animal received antibiotic treatment prior to our sampling. All primates received comperable diet and they were fed a diet of Purina monkey chow (no less than 5% protein), fruit, and water.

3.1.2 Group II

This group consisted 64 adult male baboons weighing 15- 30kg that had lived at the Institute of Primate Research for a period of between one year and 5 years. These animals had been rested from any research work for a period of six months. All primates received comperable diet and they were housed in individual cages. The concrete floors of the cage area were regularly cleaned with hoses.

All captive primates were fed a diet of Purina monkey chow (no less than 5% protein), fruit, and water. No animal received antibiotic treatment during the several months before our sampling.

3.2 Sample collection and processing

A total of 100 faecal samples (36 of group I and 64 of group II) collected from healthy captive baboons were tested in this study. Faecal samples were collected immediately after passage as baboons move away from their own feces by climbing onto the cages. Faecal samples used in this study had generally been in contact with the ground for less than 5 minutes before collection. In all cases, the entire sample was carefully removed into a sterile plastic stool container and stored on ice for 1 to 2 hours until returned to the laboratory The fresh faecal bolus was then sampled from its center by using a sterile swab which was immediately placed into Stuart's transport medium (Oxoid, Basingstoke, United Kingdom), and processed on the same day.

3.3 Isolation and identification of *Escherichia coli*

Faecal swab samples were pre-enriched in buffered peptone water (Oxoid, Basingstoke, England) and incubated for 3-6 hours at 37°C. A loopful aliquot of the pre-enrichment broth was streaked on MacConkey agar (Oxoid Ltd, Basingstoke Hampshire, England) and incubated for 24 hours at 37°C. Three colonies per sample, with typical *E. coli* morphology, were selected and identified by classical biochemical methods (gram stain, indole, methyl red-voges proskauer (MRVP), and citrate), and by the API 20E system (BioMe´rieux) test

3.4 Storage of stock culture

Each *E.coli* isolate was purified and inoculated in cryovials containing tryptone soya broth and 50% glycerol and stored at -20°C in duplicate until analysed.

3.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed by the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2008). A total of 16 antimicrobial agents all from Himedia Laboratories Ltd, Mumbai, India were tested. These were: - ampicillin (10µg), piperacillin (100µg), amoxyclav (30µg), ceftriaxone (30µg), ceftazidime (30µg), meropenem (10µg), gentamicin (10µg), amikacin (30µg), Kanamycin (30µg), streptomycin (10µg), tetracycline (30µg), co-trimoxazole (25µg), sulfamethoxazole (25µg), ciprofloxacin (5µg), ofloxacin (5µg) and chloramphenicol (30µg). *E. coli* ATCC 25922 was used as a reference organism for growth of bacteria and potency of antibiotics. Table 3.1 shows zone diameter interpretive standards breakpoints for *Enterobacteriaciae* used in this study.

Table 3.1: Zone diameter interpretive standards breakpoints for Enterobactericiae

Antimicrobial agents	Zone diameter nearest whole mm			
	Disk content(µg)	Resistance	intermediate	susceptible
Ampicillin	10	≤13	14-16	≥17
Piperacillin	100	≤17	18-20	≥21
Amoxyclav	30	≤13	14-17	≥18
Ceftriaxone	30	≤13	14-20	≥21
Ceftazidime	30	≤14	15-17	≥18
Meropenem	10	≤13	14-15	≥16
Gentamicin	10	≤12	13-14	≥15
Amikacin	30	≤14	15-16	≥17
Kanamycin	30	≤13	14-17	≥18
Streptomycin	10	≤11	12-14	≥15
Tetracycline	30	≤11	12-14	≥15
Ciprofloxacin	5	≤15	16-20	≥21
Ofloxacin	5	≤12	13-15	≥16
Co-trimoxazole	25	≤10	11-15	≥16
Sulphamethoxazole	100	≤12	13-16	≥17
Chloramphenicol	30	≤12	13-15	≥16

Source: Clinical Laboratory Stardand Institute (CLSI), 2008.

3.5.1 Inoculum preparation

The *E.coli* bacterial strains including *E.coli* ATCC 25922 were subcultured onto Tryptone Soya (TSA) agar (Oxoid Ltd., Basingstoke, Hampshire, England.) and then incubated at 37°C for 18-24 hours. After incubation 4 or 5 colonies were suspended into 5ml of sterile saline. The suspension was adjusted to a turbidity of 0.5 McFarland Standard (1 to 2 x 10^8 CFU/ml).

3.5.2 Inoculation of test plates

Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was to remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60^0 each time, to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left partly open for three to five minutes, but not more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

3.5.3 Application of disks to inoculated agar plates

The antimicrobial disks were placed using a sterile forceps ensuring disks are not closer than 24 mm from center to center. Each disk was pressed down to ensure complete contact with the agar surface. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator set to 37^0 C

erpreting zone of inhibition

to 18 hours of incubation, each plate was examined. If the plate was well streaked and the
l size was correct, the resulting zone of inhibition was uniformly circular with confluent
growth. If individual colonies were apparent, the inoculum was too light and test was
The zones of complete inhibition (as judged by unaided eye), including the diameter of the
measured. Zones were measured to the nearest whole millimeter using a sliding caliper.
as held on the back of the inverted petri plate. The petri plate was held a few inches above a
on reflecting background illuminated with a reflected light. The zone diameters of all the
ept the sulphamethoxazole disk were interpreted according to the CLSI document M31-A2
2008). The breakpoints for sulphamethoxazole were those recommended by NCCLS
its M31-A2 (NCCLS, 2004). Multidrug resistance was defined as simultaneous resistance
t two of the antimicrobials tested.

otypic characterization of antimicrobial resistance genes using PCR

icillin resistant *E.coli* isolates were screened for β-lactamase encoding genes (*bla*$_{TEM}$, *bla*
bla$_{CTX-M}$). Streptomycin resistant *E.coli* isolates were screened for the presence of *aadA1*
A2 genes as shown below. In addition, chloramphenicol resistant *E.coli* isolates were
for the presence of *cmlA* genes. Positive and negative controls from the bacterial collection
niversity of Nairobi, Department of Public Health, Pharmacology and Toxicology, were
ll assays.

3.6.1 Oligonucleotide primers

The oligonucleotide PCR primers specific for the five genes in this study, annealing temperature and PCR product length listed in table 3.2 were used in the PCR analysis.

Table 3.2: Nucleotide sequence and annealing temperature of the primers used in the PCR reactions carried out in this study for detection of antimicrobial resistance genes.

Primers	Oligonucleotide sequence (5-3)	Annealing temp. °C	Amplicon Size (bp)	Reference
TEM-F	TCCGCTCATGAGACAATAACC		931	Kiratisin *et*
TEM-R	TTGGTCTGACAGTTACCAATGC	50		*al.*,2008
SHV-F	TGGTTATGCGTTATATTCGCC		868	Kiratisin *et*
SHV-R	GGTTAGCGTTGCCAGTGCT	55		*al.*,2008
CTX-M-F	TCTTCCAGAATAAGGAATCCC		909	Kiratisin *et*
CTX-M-R	CCGTTTCCGCTATTACAAAC	55		*al.*, 2008
AadA-F	GCAGCGCAATGACATTCTTG		282	Costa *et*
AadA-R	ATCCTTCGGCGCGATTTTG	60		*al.*,2008
cmlA-F	CCGCCACGGTGTTGTTGTTATC		698	Kikuvi *et*
cmlA-R	CACCTTGCCTGCCCATCATTAG	45		*al.*,2007

3.6.2 Bacterial DNA extraction

Using a bacteriological loop, five to six pure colonies of isolated strains of *E.coli* were each transferred to eppendorf tube containing 300µl of double distilled water. The colonies were resuspended in the distilled water by vortexing. DNA was extracted from bacterial samples by boiling bacterial suspensions in the tubes in a water bath at 100^0C for 30 minutes. The eppendorf tubes were then centrifuged at 12,000 rpm for 15 min and the supernatant containing DNAs was transferred to new tubes and stored at -20^0C until used for PCR amplification. The bacterial DNA concentration was determined by spectrophotometer at 260 wavelengths. Three microlitre of the suspended nucleic acid was used in the PCR amplification. (Abdelrahman, 2008)

3.6.3 PCR assays

The amplification was performed in an MJ minicycler (MJ Research Inc. MA USA). The reaction concentration of each reagent in a PCR mixture was 100-500ng of total genomic DNA, 10mM Tris-HCL (PH 8.3) 50 mM KCL, 1.5mM $MgCL_2$. 15-20 pmol each primer, 400 µM each deoxynucleoside triphosphate and 1 U of *Taq* DNA polymerase (Roche Diagnostics., Indianapolis, USA) in a final volume of 25µl as shown in the table 3.3. The PCR conditions are shown in table 3.4.

Table 3.3: Concentration of each reagent in a PCR mixture (total volume 25µl) for the *bla* $_{TEM}$, *bla* $_{SHV}$, *bla* $_{CTX-M}$, *cmlA*, *aadA*

Reagent	Volume (µl): 1 sample	Final concentration
Taq DNA Polymerase (5 U/µl)	0.2	1 U
dNTP (100mM)	0.1	400µM
$MgCL_2$ (25mM)	1.5	1.5 mM
Forward primer (100pmol)	0.2	20pmol
Reverse primer (100pmol)	0.2	20pmol
Total genomic DNA	3.0	100-500ng
PCR buffer 10X	2.5	1X PCR buffer 10mM Tris-HCL (PH 8.3), 50 mM KCL
Sterile double distilled water	17.3	-

Table 3.4: PCR conditions for the bla TEM, bla SHV, bla CTX-M, cmlA, aadA, adapted from Kiratisin *et al.*,2008, Costa *et al.*,2008 and Kikuvi *et al.*,2007 with minor modifications.

Each step of PCR	PCR conditions (^{0}C,minutes)				
	bla TEM	*bla* SHV	*Bla* CTX-M	*AadA*	*cmlA*
Predenaturation	95, 5	95, 5	95, 5	95, 5	94,2
30 cycles of					
Denaturation	95, 1	95, 1	95, 1	95, 1	94, 1
Annealing	50, 1	55, 1	55, 1	60, 1	45, 1
Extension	72, 1.5	72, 1.5	72, 1.5	72, 1	72, 2
Final extension	72, 10	72, 10	72, 10	72, 7	72, 7

3.6.4 Detection of amplified DNA products

The PCR amplification products were analysed by electrophoresis in a 1.5 % agarose gel (Sigma Chemical Co., St. Louis, MO, USA) on horizontal tanks containing 1X Tris-acetate- EDTA as running buffer. Prior to electrophoresis, samples were mixed with loading dye (Promega, Madison, WI, USA). The gel was run in a 1X Tris-acetate-EDTA buffer at a consistent voltage of 100 volts for 30 minutes and a standard TrackIt™ 1 Kb DNA ladder (Invitrogen, Life Technologies) was used a size marker. The gel was stained with ethidium bromide solution (Carlsbad, CA U.S.A 10mg/ml) for 1 minute.

3.6.5 Documentation of gels and confirmation of PCR product size

The gels were visually inspected after illumination by ultraviolet light of 312 nm wavelength on a transluminator. The results were photographically documented using a camera documentation system. The migration distances of DNA bands were compared with those of TrackIt™ 1 Kb DNA ladder (Invitrogen, Life Technologies) to estimate the size of the PCR products.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of *Escherichia coli*

A total of 97 *E. coli* isolates were recovered from the 100 faecal samples of baboons collected and analysed in this study. Of these isolates, thirty six were from group I baboons (n=36) and sixty one *E.coli* isolates from group II baboons (n =64). No *E. coli* isolates were recovered in three of the faecal samples obtained from group II baboons.

4.2 Antimicrobial susceptibility of E. coli isolates

The resistant profile to 16 antimicrobial agents for *E. coli* isolates from both groups of baboons is shown in Table 4.1. Overall, forty nine (50.5%) of 97 *E. coli* isolates were resistant to at least one of 16 antimicrobials agents tested by disk diffusion method. Most of the *E.coli* isolates from group I and group II baboons showed moderate levels of resistance to ampicillin (36.1, 34.4%), sulphamethoxazole (33, 36.1%), amoxyclav (30.6, 26.2%), piperacillin (22.2, 23%), tetracycline (22.2, 19.7 %), streptomycin (11.1, 21.3%), and co-trimoxazole (25, 9.8%) respectively. *E.coli* isolated from group II baboons showed a higher prevalence of resistance to most antimicrobial tested in this study compared with those isolated from group I baboons, however resistance to co-trimoxazole was significantly higher ($P \leq 0.05$) in group I baboons than in isolates from group II baboons. There was no resistance against meropenem and ciprofloxacin observed in *E.coli* isolates from both groups of baboons. No resistance was observed against ceftriaxone, gentamicin, amikacin and ofloxacin in *E.coli* isolated from group I baboons. However 1 (1.6%) isolate from group II baboons showed resistance to these four antimicrobial agents. 1 (2.8%) isolate and 7 (11.5%) isolates from the group I and group II baboons respectively showed resistance to ceftazidime a third generation

cephalosporin. However only 1 (1.6%) isolate from group II baboons showed resistance to ceftriaxone.

Table 4.1: Frequency of antimicrobial susceptibility among ninety seven *Escherichia coli* isolates

	Resistance % (no of resistant isolates)		
Antimicrobial agents	Group I baboons (n=36)	Group II baboons (n=61)	Total (n=97)
Ampicillin	13 (36.1)	21 (34.4)	34 (35.1)
Piperacillin	8 (22.2)	14 (23)	22(22.7)
Amoxyclav	11 (30.6)	16 (26.2)	27 (27.8)
Ceftriaxone	0 (0)	1 (1.6)	1 (1)
Ceftazidime	1(2.8)	7 (11.5)	8 (8.2)
Meropenem	0 (0)	0 (0)	0 (0)
Gentamicin	0 (0)	1 (1.6)	1 (1)
Amikacin	0 (0)	1 (1.6)	1 (1)
Kanamycin	2 (5.6)	0 (0)	2 (2.1)
Streptomycin	4 (11.1)	13 (21.3)	17 (17.5)
Tetracycline	8 (22.2)	12 (19.7)	20 (20.6)
Co-trimoxazole	9 (25.0)	6 (9.8)	15 (15.5)
Sulphamethoxazole	12 (33.0)	22 (36.1)	34 (35.1)
Ciprofloxacin	0 (0)	0 (0)	0 (0)
Ofloxacin	0 (0)	1 (1.6)	1 (1)
Chloramphenicol	2 (5.6)	6 (9.8)	8 (8.2)

Among the aminoglycoside and β-lactam antibiotics tested, levels of resistance were higher in the 'older' classes 17.5% to 35.1% (ampicillin, amoxyclav, piperacillin, streptomycin) compared to the 'newer' classes 1% to 8.2% (ceftazidime, ceftriaxone, meropenem, amikacin, kanamycin and gentamicin). In isolates obtained from group II baboons, only resistance to ampicillin and sulphamethoxazole reached frequencies of greater than 30%. In contrast, in addition to the two antimicrobial agents, isolates obtained from group I baboons showed greater than 30% resistance to amoxyclav.

The phenotypes of resistance exhibited by the 36 *E. coli* isolates from group I baboons and 61 isolates from group II baboons are presented in Table 4.2. A total of 18/36 (50.8%) and 30/61 (49.2%) of the *E. coli* isolates from group I baboons and group II baboons showed susceptibility to all antimicrobials tested in this study.

Table 4.2: Phenotypes of resistance detected among the *E. coli* isolates recovered from baboons

	Group I baboons		Group II baboons	
No. of antimicrobials	No. of isolate (%)	Most frequent pattern (%)	No. of isolate (%)	Most frequent pattern (%)
Susceptible	16(50.8%)	-	30 (49.2%)	-
1	2 (5.6%)	AMC	1 (1.6%)	AMC
1	1 (2.8%)	AMP	1(1.6%)	AMP
1	1(2.8%)	TET	3 (4.9%)	SMX
1			1 (1.6%)	CAZ
2	1(2.8%)	TET-SMX	1 (1.6%)	PIP-OFL
2	1(2.8%)	SXT-SMX	1 (1.6%)	AMC-CTR
2	1(2.8%)	AMP-CAZ	1 (1.6%)	AMC-SMX
2			1 (1.6%)	CAZ-CHL
3	1(2.8%)	AMP-AMC-CHL	1 (1.6%)	AMP-CAZ-SMX
3	1(2.8%)	AMP-TET-SMX	1 (1.6%)	AMC-CAZ-SMX
3			1 (1.6%)	AMP-AMC-CAZ
4	1(2.8%)	AMP-PIP-AMC-TET-SMX	1 (1.6%)	AMP-STR-SXT-SMX
4	2 (5.6%)	AMP-PIP-AMC-SXT-SMX	1 (1.6%)	AMP-AMC-TET-CHL
4			1 (1.6%)	AMP-PIP-STR-SXT
4			2 (3.3%)	AMP-PIP-TET-SMX
5			1 (1.6%)	AMP-AMC-AMK-SXT-SMX
5			1 (1.6%)	AMP-PIP-AMC-GEN-SMX
5			1 (1.6%)	AMP-AMC-STR-TET-SMX
5			1 (1.6%)	AMP-PIP-AMC-STR-SMX
6	1(2.8%)	AMP-PIP-AMC-KAN-SXT-SMX	2 (3.3%)	AMP-PIP-AMC-STR-TET-SMX
6	1(2.8%)	AMP-KAN-STR-SXT-SMX-CHL	1 (1.6%)	AMP-PIP-AMC-KAN-TET-SMX
6	1(2.8%)	AMP-PIP-AMC-TET-SXT-SMX	1 (1.6%)	AMP-PIP-AMC-STR-SMX-CHL
6			1 (1.6%)	AMP-PIP-CAZ-STR-TET-SMX
6			1 (1.6%)	AMP-STR-TET-SXT-SMX-CHL
7	3 (8.3%)	AMP-PIP-AMC-STR-TET-SXT-SMX	1 (1.6%)	AMP-PIP-AMC-STR-TET-SMX-CHL
7			1 (1.6%)	AMP-PIP-KAN-TET-SXT-SMX-CHL
8			1 (1.6%)	AMP-PIP-AMC-CAZ-STR-TET-SXT-SMX

AMP, Ampicillin; AMC, amoxyclav; PIP, Piperacillin; CTR, Ceftriaxone; CAZ, Ceftazidime; MRP,Meropenem; GEN,Gentamicin; AMK, Amikacin; KAN, Kanamycin; STR, streptomycin; TET, tetracycline; SXT, Co-trimoxazole; SMX, sulphamethoxazole; CIP, ciprofloxacin; OFL, ofloxacin; CHL, Chloramphenicol

Overall thirty nine different resistance patterns were observed. Only amoxyclav and ampicilin (5.1%) resistance phenotype were found to be common in isolates from the two groups of baboons. The percentage of strains showing multiresistance (resistance to two or more subclasses of antimicrobial agents) was 38.9% and 41% of *E. coli* isolates from group I baboons and group II baboons, respectively. Although sulphamethoxazole resistance was the most frequently observed among *E. coli* isolates (4.9%) from group II baboons, combined resistance to ampicillin-piperacillin-amoxyclav-streptomycin-tetracycline-co-trimoxazole-sulphamethoxazole was the most common among isolates (8.3%) from group I baboons. The resistance spectra of the group II baboons varied more than those from group I baboons however, no significant difference was observed in the patterns of multiresistance between the isolates from group I and group II baboons.

4.3 Genetic characterization of resistance genes using PCR

Ampicillin resistant *E.coli* isolates were screaned for beta-lactamase encoding genes (TEM, SHV and CTX-M), streptomycin resistant *E.coli* isolates were screened for the presence of *aadA1* or *aadA2* and chloramphenicol resistant *E.coli* isolates were screened for the presence of *cmlA*. Table 4.3 shows resistant genes detected among the resistant *E.coli* isolates from baboons.

4.3.1 Ampicillin resistance genes

Using specific primers, PCR was carried out on the genomic DNA of thirty seven ampicillin resistant *E. coli* isolates for the presence of genes encoding TEM, SHV and CTX-M β-lactamases. Twenty eight (75.7%) of the thirty seven ampicillin *E.coli* isolates were positive for at least one of the three β-lactamase genes tested. The majority of the strains showed (21 of 37) positive amplification for *bla* $_{TEM}$. This was followed by *bla* $_{SHV}$ (positive in 19 of 37) and *bla* $_{CTX-M}$ (5 strains). 1 (7.1%) and 4 (17.4%) of these *E.coli* isolates from group I and group II baboons respectively harboured the gene encoding CTX-M β-lactamase. It was interesting to note that *bla*

Table 4.3: Resistance genes detected among antimicrobial resistant *E.coli* isolates from baboons

	Group II baboons				Group I baboons			
Phenotype of resistance		Genes detected				Genes detected		
	Number of isolates with this phenotype	Genes	Number of isolates	Percentage of resistant gene detected	Number of isolates with this phenotype	Genes	Number of isolates	Percentage of resistant gene detected
Ampicillin	23	*bla* TEM	13	56.5%	14	*bla* TEM	8	57.1%
		bla SHV	11	47.8%		*bla* SHV	8	57.1%
		bla CTX-M	4	17.4%		*bla* CTX-M	1	7.1%
streptomycin	16	*aadA1 or aadA2*	5	31.3%	4	*aadA1 or aadA2*	0	0%
chloramphenicol	6	*cmlA*	4	66.7%	2	*cmlA*	1	50%

CTX-M genes was frequently detected in *E.coli* isolates from group II baboons (4 of 23) as compared to group I baboons (1 of 14). The 931bp *bla* TEM is shown in figure 4.1.

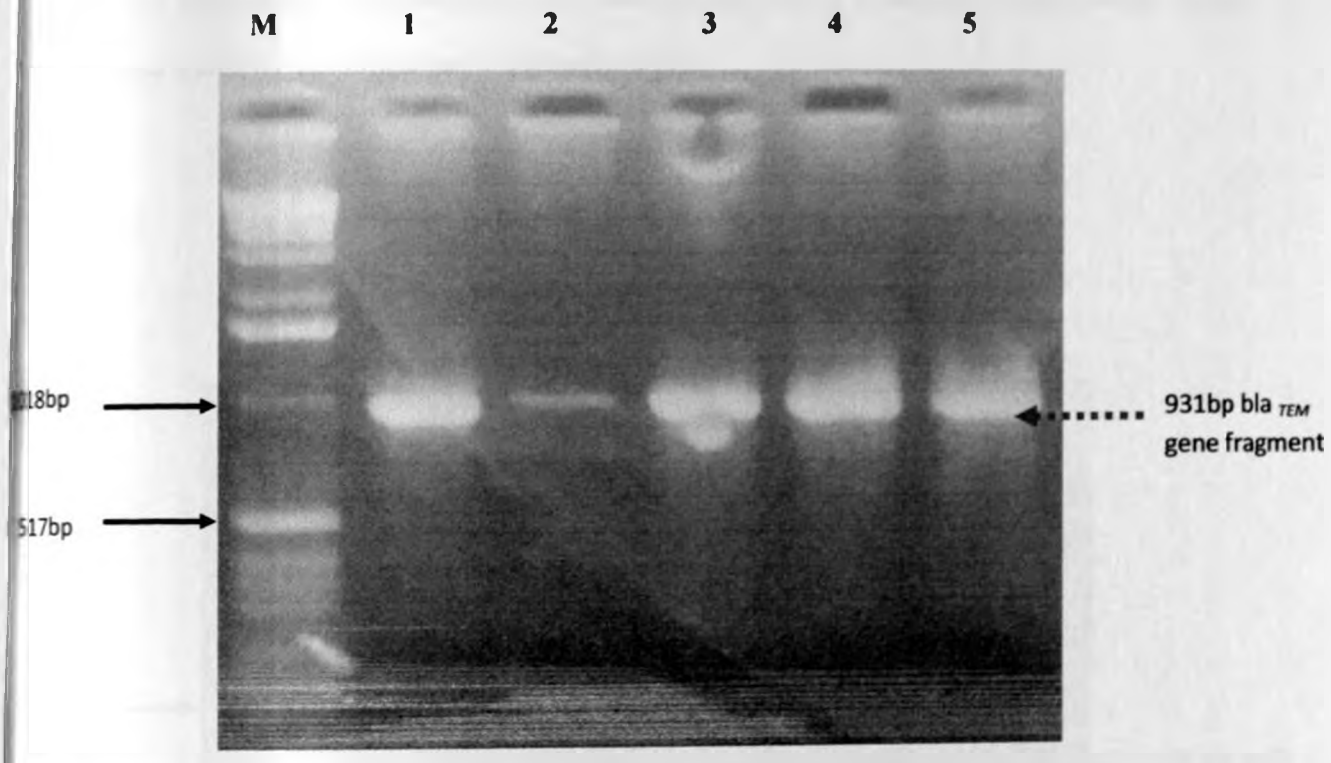


Figure 4.1: PCR amplicons obtained with bla TEM primers. Lane M contains the TrackIt™ 1 Kb DNA ladder (Invitrogen) of which the sizes of some fragment are given on the left hand side. Lane 1 (Aw 33b), 2 (Aw26a), 3 (Aw11d), 4 (Ac 11d), 5 (Ac 10d). The 931*bla* TEM gene.

4.3.2 Chloramphenicol resistance genes.

A total of 8 *E.coli* isolates showed the chloramphenicol phenotype of resistance. Of these one *E.coli* isolate was from group I baboons and seven from group II baboons. The *cmlA* gene, which is a non-enzymatic chloramphenicol resistance gene, was detected in 5 of 8 resistant *E.coli* isolates. Figure 4.2 shows a positive amplification of 698bp *cmlA* gene.

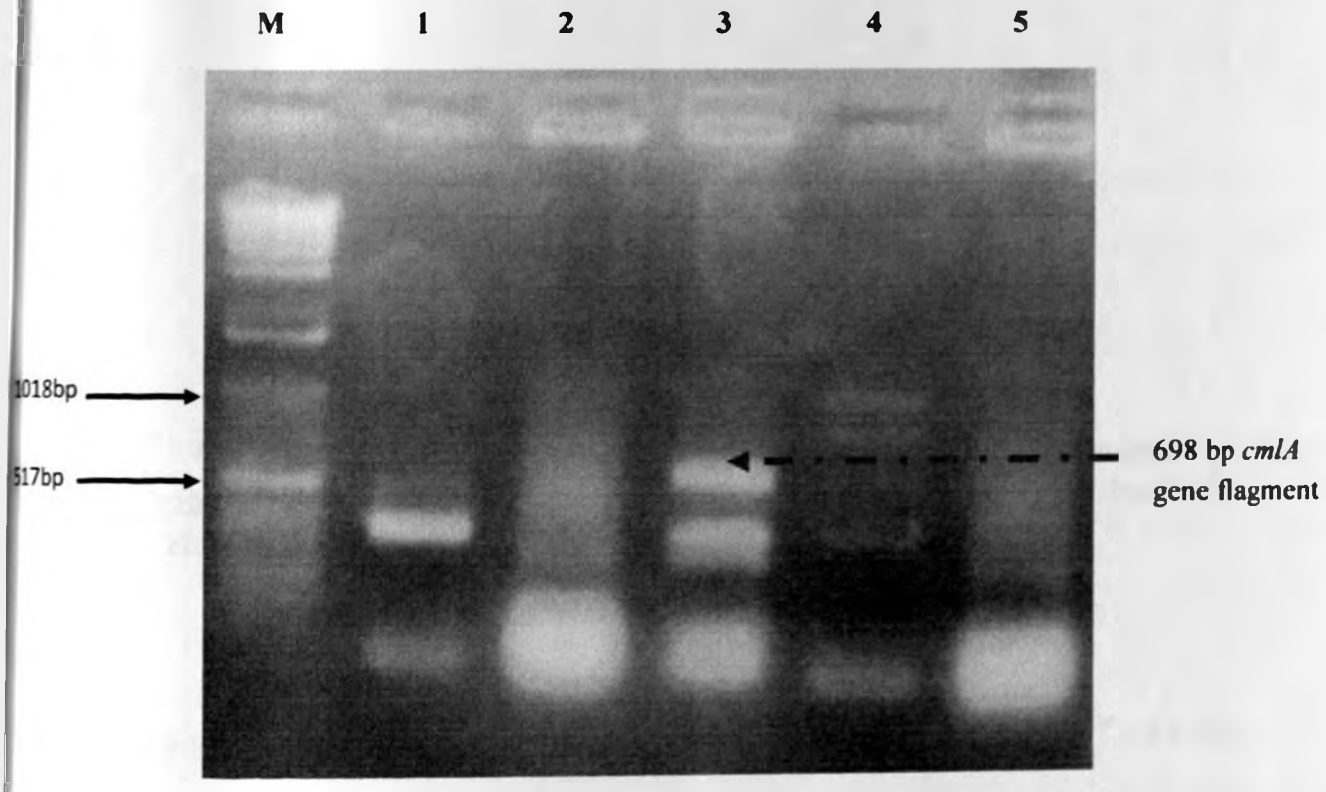


Figure 4.2: PCR amplicons obtained with cmlA primers. Lane M contains the TrackIt™ 1 Kb DNA ladder (Invitrogen) of which the sizes of some fragment are given on the left hand side. Lane 1 (Ac 43c), 2 (Ac34a), 3 (Ac30c), 4 (Ac 57a), 5 (Aw6a). The 698 bp *cmlA* gene.

4.3.3 Streptomycin resistance genes

The *aadA1* or *aadA2* gene, encoding an amino glycoside adenylyltransferase that modifies streptomycin, was detected in 5 of the 20 streptomycin resistant isolates of this study. No gene was amplified in group I baboons as all the five genes were detected in *E.coli* isolates from group two baboons. Figure 4 shows a positive amplification of 282bp *aadA* gene.

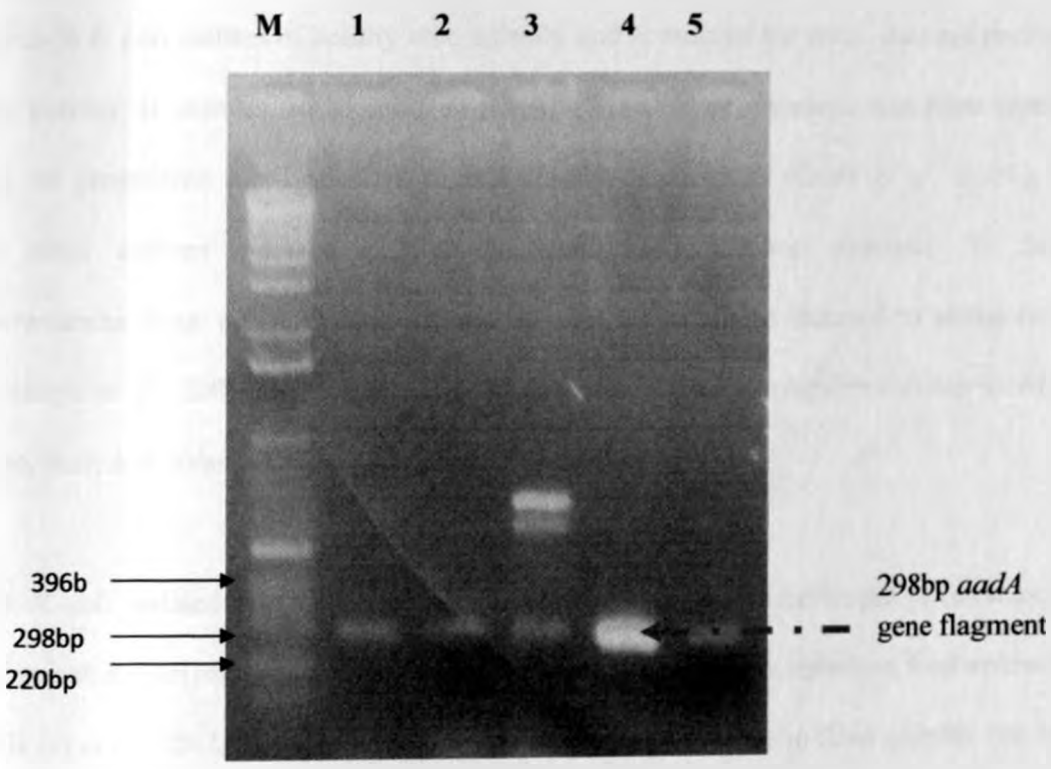


Figure 4.3: PCR amplicons obtained with *aadA* primers. Lane M contains the TrackIt™ 1 Kb DNA ladder (Invitrogen) of which the sizes of some fragment are given on the left hand side. Lane 1 (Ac 57a), 2 (Ac40a), 3 (Ac30c), 4 (Ac 39b), 5 (A34a). The 298 bp *aadA* gene.

CHAPTER FIVE

5.0 DISCUSSION

Escherichia coli are the commonest microflora of the intestinal tract of a wide variety of animals and also of humans. This microorganism is usually non-pathogenic however, it is frequently implicated in animal and human infections that require the use of antibiotics (Paterson and Bonono, 2005; Barreto *et al.*, 2009). There are few data which exist in the literature about the susceptibility to antibiotics in *E. coli* isolates of healthy wild animals, and in most of the cases, data are restricted to a small number of animals. In addition, differences in levels of resistance had been reported depending on geographic localization of studied wildlife populations (Costa *et al.*, 2008). For example, some authors detected a high prevalence of antibiotic resistance in faecal *Enterobacteriaceae* from wild and domestic rodents that had not been exposed to antibiotics in Kenya (Gakuya *et al.*, 2001) while others did not detect resistance among *Enterobacteriaceae* of wild moose, deer, and voles in Finland (Osterblad *et al.*, 2001).

In general, *E. coli* isolated from both groups of baboons showed low percentages of resistance to chloramphenicol, kanamycin, gentamicin, which compares with previous reports in food animals in Kenya (Kikuvi *et al.*,2007a; Ole-Mapenay, 2007). Chloramphenical use in food animals has been banned in Kenya (Ole-Mapenay, 2007); however there still exist restricted use mainly in topical application for treatment of ophthalmic condition in dogs. The most common resistance observed in this study was to ampicillin, sulphamethoxazole followed by amoxyclav, piperacillin tetracycycline, streptomycin and co-trimoxazole. This finding is in agreement with the results of previous studies, (Barreto *et al.*, 2009; Emacar *et al.*, 2010) which have shown a common occurrence of resistance to these antimicrobials in *E. coli* isolates from both healthy children and HIV patients taking antibiotics, although the prevalence of resistance in this study is much lower than those of the

Emacar *et al.*, (2010) isolates from HIV patients. The high frequency of resistance to these antimicrobials in baboons may be associated with the contact of these animals with humans during feeding and cleaning. Other authors have also suggested a possible correlation between the level of antibiotic resistance in bacteria from animals and the level of contact of these animals with humans, suggesting that the current prevalence of antimicrobial resistance found in fecal animal bacteria may be of anthropogenic nature (Costa *et al.*, 2008).

Although limited data are available for comparison, bacterial isolates from group II baboons were more resistant than those from group I baboons against most antimicrobials tested in this study. Similarly, the frequency of resistance was higher in isolates from baboons feeding on human refuse compared to those living in undisturbed ecosystem in the wild in an older study done in Kenya which unfortunately did not use standard susceptibility testing methods (Routman *et al.*, 1985). Since the isolates included in this study originated from baboons captured from various locations throughout the country, they may be considered to be representative and epidemiologically unrelated. However, group I baboons tested in this study could not be simply assumed to be wild baboons, because they had stayed in captivity for a period of less than a month being fed on a diet of Purina monkey chow (no less than 5% protein), fruit, and water. Therefore the direct effect of diet could not be eliminated. Further studies should be carried out to obtain more data on antimicrobial resistance among both pathogenic and indicator bacteria from wild baboons.

When compared to livestock in Kenya, the prevalence of resistance observed in this study was generally similar to those reported in healthy cattle but lower than in healthy pigs and poultry (Kikuvi *et al.*, 2007a). Especially, resistance against antimicrobials commonly used as feed additives or used for a long time in livestock animals such as tetracycline, ampicillin, and

streptomycin was lower in both groups of baboons than in pigs and poultry: 19.7-22.2%, 34.4-36.1%, and 11.1-21.3%, in baboons, whereas 40%, 50.5%, 25.7% in pigs and 34% 32%, 34% in poultry, respectively. However, *E. coli* isolates from both groups of baboons showed higher resistance levels against chloramphenicol (8.2%), which has been banned for use in food animals in Kenya compared with 4.4% in cattle as reported by Ole-Mapenay, 2007. Nonetheless, resistance against chloramphenicol (8.2%) kanamycin (1%), and co-trimoxazole (15.5%) was still much lower in baboons, compared with 20%, 12%, and 30% in poultry, respectively (Kikuvi *et al.*, 2007a). The low level of resistance in *E. coli* isolates in this study to chloramphenicol, ceftazidime, kanamycin, amikacin, gentamycin and ceftriaxone are in line with the observation by Nys *et al.* (2004) that resistance in newer antibiotic is emerging.

There was no resistance observed in *E. coli* isolates for ciprofloxacin and meropenem. These results agrees with the finding by Gakuya *et al.* (2001) in a study of antimicrobial resistance in *E.coli* isolates from wild and domestic rats. Probably this could be due to the fact that these two antibiotic are used a second line of treatment in Kenya and in most cases they are usually not available. Compared to other food producing animals in other parts of the world like Bagladesh which reported a much higher frequency of resistance of 82% to ciprofloxacin (Akond *et al.*, 2009), this study showed complete susceptibility of *E.coli* isolates. Studies in Korea of *E.coli* isolates from dogs have also reported resistance of up to 16% resistance to cipfloxacin (Nam *et al.*, 2009).

Unexpectedly, resistance to co-trimoxazole was significantly higher ($p \leq 0.05$) in group I baboons as compared to group II baboons. Although group I baboons were in captivity for a period of less than a month resistance could have been acquired by interaction of resistant commensal bacteria from the environment since these baboons were captured from the Aberdare national park where

there is interaction with food producing animals and pastoralist who may have been exposed to antimicrobial agents.

In this study, a pattern of multiresistances in *E. coli* isolates from both group I baboons and group II baboons were similar. Also, one isolate from group II baboons showed resistance to 8 out of 16 antimicrobials tested, whereas three isolate from group I baboons were resistant to 7 antimicrobials. A previous study reported that the prevalence of multiresistance in *E. coli* isolates from baboons feeding on human refuse was significantly higher than that observed in strains originating from baboons in the wild (Routman *et al.*, 1985). The investigator also documented that the reason for the elevated resistance prevalence in strains from baboons feeding on human refuse is due to close contact with humans which could make this resistance to be of anthropogenic nature. When wild baboons are captured from the wild and brought to the Institute of Primate Research, they are first kept in group cages and later in individual cages. During the holding period in cages, wild baboons could have been exposed to bacteria from cohabitated baboons or from cleaners, who may have contained resistance genes that could be transmitted horizontally among baboons in the same place.

Resistance was encoded by genes that are normally widespread in *Enterobactericeae* and are known to be commonly located on transposons, which are mobile DNA elements that play an important role in transmission and dissemination of antimicrobial determinants (Akond *et al.*, 2009). Ampicillin resistance genes in *E. coli* isolates observed in this study were largely associated with TEM and SHV ß-lactamase genes, with only five isolates positive for CTX-M ß-lactamase genes. This agrees with other reports that TEM and SHV ß-lactamase genes are the most prevalent in ampicillin resistant *E. coli* of animal origin, as well as being commonly reported in human *E. coli* isolates of hospital origin (Brinas *et al.*, 2002). Further, this study identified 9 isolates as resistant to

cephalosporins (i.e. potential ESBL producers), of which five were positive for CTX-M ß-lactamase genes. Extended spectrum beta-lactamase (ESBL) resistance genes have previously been reported in wild animals and pets by Costa *et al.*, (2008) in Portugal. Reports of CTX-M12 ESBL in Kenya are also documented by Kariuki *et al.*, (2001). ESBL-targeted drugs are being used more frequently, but may result in mutations of TEM and SHV β-lactamase genes, as well as the widely prevalent CTX-M types (Paterson and Bonomo, 2005). For the potential ESBL producers more identification and confirmation is required and further genotypic analysis is needed. The present observation showed that *E. coli* resistance genes from baboons are similar to those found in other animals and humans; however these need further investigation, specifically by sequencing the TEM, SHV and CTX-M β-lactamase PCR products.

It is important to indicate that the *E. coli* isolates showed in general lower levels of resistance to aminoglycosides (with the exception of streptomycin) and as referred by others, the *aadA1* and *aadA2* genes were detected in 5 (25%) of 20 streptomycin resistant *E .coli*. The primers used in this study were able to detect either of the two *aadA* genes. These genes were only detected in group II baboons' isolates perhaps suggesting that group I baboons had not yet acquired the resistance mechanism to streptomycin since they had been in captivity for a short period. Although the gene *aadA1* or *aadA2* was not amplified in the remaining 15 streptomycin resistant isolates, other mechanisms of streptomycin resistance, such as the production of APH (3")-I or APH (6)-I phosphoryltranferase (Vakulenko and Mobashery, 2003) cannot be excluded.

All the chloramphenicol resistant isolates were of the MDR phenotype, suggesting that resistance to chloramphenicol is likely to be part of a multiple resistance system. The non-enzymatic chloramphenicol resistance gene (*cmlA*) which also confers resistance to florfenicol was identified

by PCR in 5 (62.5%) of the 8 resistant isolates. While no cmlA chloramphenicol resistance mechanism was identified in the three remaining resistant isolates, suggesting that other mechanism of chloramphenicol resistance such as the chloramphenicol acetyltranferase (*cat*) responsible for enzymatic inactivation of the drug or *flo* genes that encodes efflux pump may be involved. The use of chloramphenicol in veterinary medicine in food animals in Kenya has been banned. However restricted use is carried out mainly in topical application as a treatment for ophthalmic conditions in dogs and cats, it is hardly ever used systemically. Chloramphenicol resistance was almost exclusively found in group II baboons-derived samples indicating that, chloramphenicol resistance has most probably been co-selected through linked trimethoprim and ampicillin resistance genes. The resistance genes could also have been of anthropogenic nature due to existence of close contact of these animals with humans.

A limitation of the study described here is that it was only possible to answer the question of whether the genes were present. It has, however,made it possible sample and do susceptibility testing to the numerically major commensal bacteria in the gastrointestinal tract and later amplify resistance gene in resistant isolates; noting that the existence of a resistance gene in a bacterial strain does not prove that the strain is resistant. Attempt was not made to quantify the resistance genes because it is the presence of these genes rather than their abundance that is important for predicting resistance potential of the population. Exposure to antibiotics would quickly increase the up-regulation of gene expression which may lead to production of more enzyme for phenotypic resistance to be expressed. What is not clear is how these genes came to be present in the first place. In the case of the captive animals, one possible explanation would be indirect spread of resistance genes from human caretakers or from the diet, but this explanation does not work in the case of the "wild" baboons. Another possibility is that there are selective pressures other than administered

antibiotics that select for the acquisition and maintenance of β-lactamases, *cmlA* and *aadA* genes. However, although there are antibiotic-producing organisms in soil, the level of antibiotics in most soils is far too low to act as a significant selective pressure (Jeters *et al.*, 2009).

A question that could not be answered by this type of study is whether the resistance genes found were genetically linked to a mobile element, a linkage that suggests that the gene could move readily within the animal once it was present. Studies have shown that most of resistant genes are commonly found on mobile genetic elements and on conjugation experiments they are easily transferred to donor bacteria (Ahmed *et al.*, 2010). Although this question could not be answered, the explanation of resistance genes in captive baboons as being due to indirect spread of resistance genes from human caretakers or from the diet this shows that the IPR baboons may be either recipients or sources of the zoonotic transmission of MDR antibiotic resistance to human caretakers. A wider study is therefore needed to further try to identify the enviromental factors that may be involved in the spread of resistance genes, whether the observed resistance could be transferred between bacteria, the role of plasmid and integron.

5.1 CONCLUSIONS

1. A moderate to high percentage of resistance of between 15.5- 35.1% to ampicillin, streptomycin, tetracycline, piperacillin, amoxyclav, co-trimoxazole, sulphamethoxazole was observed in *E.coli* isolates from baboons in this study.
2. Resistance to aminoglycoside is generally low except for streptomycin in *E.coli* isolates from baboons in Kenya.
3. Resistance to ceftazidime and ceftriaxone third generation cephalosporin is emerging in commensal bacteria
4. Multidrug resistant *E.coli* is commonly found in IPR captive baboons.
5. Captive baboons at IPR harbour β-lactamase genes, *cmlA* and *aadA* resistant genes in their gastrointestinal tract and thus they may be a potential reservoir for zonotic transmission of resistant multidrug resistance gene to human caretaker.

5.2 RECOMMEDATIONS

1. Minimal contact with captive baboons should be maintained to prevent possible horizontal transfer of resistant commensal bacteria between baboons and humans.

2. Human caretakers of these baboons should always wear protective clothing when handling baboons in captivity.

3. There is a need to establish awareness on importance of baboons as a risk to human health especially the handlers.

4. There is need for a detailed epidemiological and molecular studies on acquisition of resistance genes and their distribution and whether or not some of the genes are associated with known mobile elements

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7.0 APPENDICES

Appendix 1: Zone diameter interpretive standards breakpoints for Enterobactericiae

Antimicrobial agents	Zone diameter nearest whole mm			
	Disk content(ug)	Resistance	intermediate	susceptible
Ampicillin	10	≤13	14-16	≥17
Piperacillin	100	≤17	18-20	≥21
Amoxyclav	30	≤13	14-17	≥18
Ceftriaxone	30	≤13	14-20	≥21
Ceftazidime	30	≤14	15-17	≥18
Meropenem	10	≤13	14-15	≥16
Gentamicin	10	≤12	13-14	≥15
Amikacin	30	≤14	15-16	≥17
Kanamycin	30	≤13	14-17	≥18
Streptomycin	10	≤11	12-14	≥15
Tetracycline	30	≤11	12-14	≥15
Ciprofloxacin	5	≤15	16-20	≥21
Ofloxacin	5	≤12	13-15	≥16
Co-trimoxazole	25	≤10	11-15	≥16
Sulphamethoxazole	100	≤12	13-16	≥17
Chloramphenicol	30	≤12	13-15	≥16

Source: Clinical Laboratory Stardand Institute (CLSI) 2008.

Appendex 2: antimicrobial resistance antibiograms for group II baboons

Sampling date	Sample ID	AMP	PIP	AMC	Ceftri	CAZ	AMK	GEN	AMK	KAN	STR	TET	SXT	SMX	CIP	OFL	CHL
19.10.09	Ac1b	17S	20I	17I	30S	23S	27S	19S	18S	18S	16S	19S	27S	15I	29S	23S	21S
19.10.09	Ac2a	24S	24S	22S	30S	25S	31S	25S	25S	23S	19S	22S	30S	23S	42S	30S	25S
19.10.09	Ac3a	19S	21S	14I	24S	19S	26S	21S	23S	20S	19S	21S	25S	14I	39S	25S	21S
19.10.09	Ac4a	6R	18I	12R	14I	22S	24S	25S	6R	22S	13I	12I	6R	6R	16I	18S	25S
19.10.09	Ac5b	20S	22S	18S	26S	20S	26S	22S	21S	21S	20S	21S	25S	17S	38S	27S	23S
19.10.09	Ac6d	23S	6R	21S	28S	20S	30S	21S	22S	20S	22S	23S	28S	21S	35S	6R	25S
19.10.09	Ac7d	20S	21S	17I	28S	24S	26S	23S	21S	21S	19S	22S	33S	22S	42S	35S	21S
19.10.09	Ac8b	19S	20I	18S	29S	19S	26S	21S	20S	19S	16S	21S	28S	6R	37S	29S	22S
19.10.09	Ac9a	19S	22S	18S	26S	21S	32S	20S	20S	17I	16S	20S	30S	19S	34S	30S	23S
19.10.09	Ac10d	6R	16R	12R	28S	19S	29S	11R	21S	21S	17S	21S	16S	6R	31S	36S	22S
19.10.09	Ac11d	6R	14R	11R	26S	19S	28S	22S	21S	20S	10R	9R	12I	6R	28S	29S	6R
19.10.09	Ac12c	21S	20I	17I	22S	17I	26S	22S	19S	21S	19S	22S	28S	15I	29S	24S	21S
19.10.09	Ac15c	16I	19I	20S	23S	18S	22S	24S	21S	20S	19S	21S	23S	19S	31S	22S	19S
19.10.09	Ac17b	18S	21S	20S	30S	20S	23S	20S	20S	18S	18S	20S	28S	19S	31S	23S	23S
19.10.09	Ac18d	19S	23S	25S	24S	22S	31S	21S	24S	18S	20S	21S	31S	18S	38S	29S	23S
19.10.09	Ac19c	16I	18I	22S	21S	19S	27S	18S	23S	22S	18S	16S	20S	21S	35S	31S	24S

19.10.09	Ac20c	6R	15R	13R	26S	17I	26S	23S	19S	21S	8R	10R	13I	6R	37S	30S	26S
19.10.09	Ac21d	20S	20I	14I	25S	19S	30S	21S	20S	19S	17S	20S	25S	18S	36S	29S	22S
19.10.09	Ac22a	16I	21S	21S	25S	17I	38S	21S	22S	19S	16S	20S	28S	18S	28S	22S	22S
19.10.09	Ac23a	23S	19I	16I	28S	18S	23S	20S	22S	17I	20S	24S	29S	17S	32S	24S	22S
19.10.09	Ac24a	6R	14R	16I	27S	17I	25S	21S	24S	18S	10R	8R	10R	6R	26S	23S	6R
19.10.09	Ac25a	6R	6R	11R	21S	20S	24S	21S	17S	19S	10R	8R	18S	6R	36S	30S	23S
19.10.09	Ac26a	9R	16R	14I	31S	20S	30S	24S	22S	21S	12I	9R	16S	6R	35S	26S	24S
19.10.09	Ac27b	17S	19I	19S	28S	22S	25S	21S	19S	19S	20S	19S	22S	18S	39S	34S	21S
19.10.09	Ac28a	17S	24S	8R	6R	17I	20S	21S	16I	21S	20S	19S	31S	15I	35S	26S	19S
19.10.09	Ac29b	26S	24S	19S	22S	24S	20S	26S	24S	22S	20S	18S	26S	16S	28S	24S	19S
19.10.09	Ac30c	6R	14R	10R	30S	24S	18S	20S	16I	18S	10R	12I	16S	6R	30S	20S	6R
19.10.09	Ac31c	19S	22S	15I	22S	21S	29S	27S	22S	21S	18S	18S	29S	20S	36S	28S	22S
19.10.09	Ac32c	19S	24S	18S	25S	19S	30S	24S	20S	22S	22S	19S	25S	19S	36S	33S	25S
19.10.09	Ac33b	6R	23S	14I	16I	6R	34S	25S	25S	22S	20S	20S	20S	6R	35S	31S	21S
19.10.09	Ac34a	6R	19I	13R	27S	24S	35S	24S	24S	23S	14I	8R	21S	12I	41S	33S	6R
19.10.09	Ac35b	15I	19I	13R	18S	14R	26S	21S	19S	20S	20S	15S	17S	6R	25S	19S	16I

19.10.09	Ac36b	22S	24S	18S	27S	24S	32S	26S	28S	25S	23S	22S	28S	22S	36S	29S	23S
21.10.09	Ac37a	20S	24S	18S	30S	22S	25S	23S	22S	22S	24S	20S	28S	12I	35S	28S	22S
21.10.09	Ac38b	23S	22S	21S	27S	24S	30S	22S	21S	26S	15S	24S	28S	22S	33S	33S	23S
21.10.09	Ac39b	20S	20I	12R	30S	24S	34S	27S	21S	25S	12I	12I	16S	6R	34S	32S	21S
21.10.09	Ac40b	6R	16R	25S	32S	22S	36S	26S	23S	24S	9R	22S	10R	14I	35S	24S	25S
21.10.09	Ac41a	20S	19I	16I	22S	17I	24S	20S	20S	19S	16S	20S	26S	19S	26S	23S	21S
21.10.09	Ac42a	17S	21S	16I	22S	18S	26S	20S	20S	22S	16S	20S	25S	17S	27S	24S	23S
21.10.09	Ac43c	26S	25S	22S	23S	6R	26S	26S	25S	26S	25S	26S	36S	25S	33S	28S	6R
21.10.09	Ac44b	24S	25S	22S	28S	22S	32S	27S	26S	19S	19S	23S	29S	27S	33S	30S	20S
21.10.09	Ac45a	6R	13R	6R	15I	17I	24S	19S	18S	19S	10R	10R	14I	6R	26S	23S	22S
21.10.09	Ac46d	6R	15R	11R	27S	14R	18S	18S	18S	19S	11R	9R	6R	6R	28S	25S	21S
21.10.09	Ac47b	12R	22S	18S	22S	16I	24S	22S	20S	21S	21S	18S	24S	22S	26S	22S	16I
21.10.09	Ac48b	20S	21S	19S	24S	22S	28S	22S	18S	19S	16S	20S	24S	20S	33S	25S	22S
21.10.09	Ac49d	13R	28S	22S	23S	20S	30S	24S	24S	22S	8R	13I	8R	6R	29S	26S	18S
21.10.09	Ac50d	14I	20I	13R	25S	19S	25S	22S	19S	23S	12I	13I	26S	16S	30S	24S	18S
21.10.09	Ac51c	6R	22S	6R	20S	14R	28S	27S	25S	22S	23S	18S	24S	19S	31S	25S	21S
21.10.09	Ac52c	6R	14R	16I	26S	20S	26S	22S	18S	18S	12I	11R	12I	6R	29S	26S	23S

21.10.09	Ac53a	17S	19I	16I	25S	18S	24S	22S	18S	21S	15S	19S	24S	17S	23S	20S	20S

21.10.09	Ac54b	6R	17R	14I	23S	6R	27S	22S	18S	20S	9R	11R	12I	6R	32S	26S	23S
21.10.09	Ac55c	6R	19I	12R	26S	19S	23S	21S	21S	18S	11R	6R	13I	6R	29S	27S	24S
21.10.09	Ac56c	20S	22S	19S	25S	20S	30S	20S	21S	19S	17S	22S	28S	21S	32S	25S	22S
21.10.09	Ac57a	6R	19I	14I	25S	20S	26S	23S	21S	20S	9R	7R	9R	6R	32S	24S	6R
21.10.09	Ac58c	19S	22S	17I	25S	19S	25S	22S	24S	20S	16S	19S	26S	14I	27S	25S	22S
21.10.09	Ac59c	18S	20I	19S	21S	6R	26S	19S	18S	17I	15S	21S	28S	17S	34S	27S	23S
21.10.09	Ac60b	18S	24S	23S	27S	19S	24S	18S	19S	20S	22S	19S	18S	22S	36S	31S	24S
21.10.09	Ac61c	20S	22S	22S	26S	21S	33S	22S	23S	20S	16S	19S	22S	6R	33S	26S	22S
21.10.09	Ac62b	18S	21S	18S	24S	19S	25S	20S	20S	19S	15S	19S	26S	20S	27S	23S	22S
21.10.09	Ac63a	6R	17R	11R	30S	19S	27S	23S	24S	21S	10R	13I	11I	6R	35S	24S	24S
21.10.09	Ac64b	16I	20I	18S	24S	20S	22S	20S	18S	20S	20S	24S	16S	8R	34S	22S	16I
E. COLI	25922	20S	28S	23S	33S	24S	29S	22S	22S	20S	19S	22S	38S	20S	39S	32S	19S
E. COLI	25922	18S	24S	20S	31S	26S	30S	20S	22S	20S	19S	22S	33S	22S	40S	36S	22S
E. COLI	25922	20S	25S	20S	32S	27S	32S	21S	23S	19S	19S	23S	31S	23S	38S	34S	23S

Key. R-Resistance; I- Intermediate; S-susceptible

Source: Author results (2010)

Appendix 3: Antimicrobial resistance antibiograms for group I baboons

sampling date	Sample ID	AMP	PIP	AMC	Ceftri	CAZ	MR	GEN	AMK	KAN	STR	TET	SXT	SMX	CIP	OFL	CHL
19.10.09	Aw1b	6R	20I	22S	28S	20S	36S	24S	23S	24S	22S	12I	15I	13I	41S	33S	26S
19.10.09	Aw2a	17S	23S	17I	38S	21S	35S	18S	20S	20S	18S	10R	16S	23S	38S	33S	26S
19.10.09	Aw3a	19S	20I	21S	28S	26S	32S	26S	19S	23S	18S	22S	32S	19S	41S	31S	24S
19.10.09	Aw4a	23S	23S	11R	23S	21S	23S	22S	20S	16I	22S	20S	29S	23S	32S	37S	23S
19.10.09	Aw5a	6R	17R	13R	30S	21S	29S	35S	15I	13R	15S	17S	9R	6R	37S	35S	37S
19.10.09	Aw6a	11R	19I	13R	20I	15I	28S	22S	20S	21S	15S	12I	13I	20S	28S	24S	12R
19.10.09	Aw7a	8R	14R	9R	29S	20S	37S	28S	27S	21S	16S	11R	11I	6R	39S	34S	22S
19.10.09	Aw8a	16I	21S	22S	22S	22S	29S	19S	21S	21S	16S	18S	22S	21S	39S	28S	19S
19.10.09	Aw9a	22S	23S	19S	31S	24S	32S	22S	24S	22S	26S	17S	23S	20S	41S	33S	18S
19.10.09	Aw10b	18S	25S	18S	24S	18S	28S	19S	20S	19S	18S	13I	26S	19S	32S	27S	21S
19.10.09	Aw11a	6R	19I	18S	32S	25S	31S	24S	25S	22S	16S	8R	13I	6R	33S	31S	26S
19.10.09	Aw12a	21S	25S	23S	31S	24S	32S	23S	24S	21S	20S	23S	30S	23S	41S	28S	26S
19.10.09	Aw13a	16I	20I	13R	24S	20S	30S	21S	19S	19S	17S	15S	27S	20S	36S	39S	21S
19.10.09	Aw14a	20S	20I	20S	17I	17I	23S	22S	24S	22S	21S	18S	21S	20S	25S	22S	19S
19.10.09	Aw15a	21S	24S	20S	29S	23S	29S	25S	21S	21S	19S	23S	29S	20S	34S	31S	27S
19.10.09	Aw16a	18S	34S	19S	29S	21S	26S	21S	19S	20S	18S	21S	29S	20S	38S	29S	22S
19.10.09	Aw17a	20S	22S	17I	25S	21S	15I	20S	18S	18S	18S	21S	28S	18S	41S	26S	22S
19.10.09	Aw18b	18S	21S	18S	28S	21S	25S	21S	23S	20S	19S	10R	23S	7R	32S	25S	19S

19.10.09	Aw19a	16I	21S	15I	28S	21S	27S	22S	22S	21S	23S	21S	9R	6R	36S	27S	24S
19.10.09	Aw20b	13R	21S	18S	21S	14R	32S	23S	25S	21S	20S	17S	20S	23S	25S	22S	15I
19.10.09	Aw21a	17S	21S	17I	25S	22S	31S	20S	26S	21S	20S	21S	18S	18S	34S	31S	22S
19.10.09	Aw22b	20S	18I	17I	29S	17I	24S	20S	18S	17I	16S	22S	29S	18S	32S	28S	21S
19.10.09	Aw23b	18S	21S	16I	23S	18S	24S	23S	18S	18S	16S	13I	25S	16S	38S	26S	21S
19.10.09	Aw24a	20S	23S	20S	16I	19S	26S	19S	17S	17I	17S	19S	27S	17S	36S	24S	22S
19.10.09	Aw25b	19S	21S	17I	22S	18S	37S	23S	21S	21S	17S	19S	26S	19S	33S	25S	23S
19.10.09	Aw26a	6R	16R	7R	28S	19S	26S	22S	20S	21S	11R	11R	9R	6R	33S	37S	22S
19.10.09	Aw27a	6R	15R	12R	29S	18S	23S	22S	19S	20S	11R	9R	9R	6R	30S	24S	24S
19.10.09	Aw28a	6R	14R	12R	27S	18S	32S	23S	19S	20S	12I	13I	10R	6R	39S	15I	22S
19.10.09	Aw29a	16I	19I	16I	23S	18S	33S	17S	18S	17I	16S	19S	24S	15I	42S	29S	21S
19.10.09	Aw30a	11R	23S	17I	36S	18S	32S	21S	17S	6R	8R	12I	6R	6R	27S	26S	6R
19.10.09	Aw31a	6R	15R	8R	30S	20S	32S	24S	19S	18S	19S	19S	8R	6R	37S	30S	22S
19.10.09	Aw32a	22S	21S	17I	30S	18S	36S	21S	21S	23S	19S	23S	33S	22S	40S	33S	26S
19.10.09	Aw33a	6R	17R	10R	33S	19S	36S	23S	21S	20S	10R	10R	9R	6R	39S	35S	22S
19.10.09	Aw34a	17S	21S	19S	26S	18S	27S	20S	21S	19S	16S	21S	24S	19S	37S	24S	21S
19.10.09	Aw35a	18S	20I	18S	26S	19S	26S	20S	20S	19S	17S	22S	29S	18S	42S	32S	24S
19.10.09	Aw36a	9R	17R	11R	27S	21S	26S	24S	22S	24S	13I	11R	6R	6R	29S	27S	21S
E. coli	25922	19S	24S	20S	32S	24S	28S	21S	20S	19S	18S	22S	39S	22S	40S	27S	19S
E. coli	25922	19S	25S	20S	30S	23S	28S	19S	21S	19S	19S	23S	31S	19S	41S	33S	22S

Key: R-Resistance; I- Intermediate; S-susceptible

Source: Author results (2010)

Appendix 4: The *Bla* TEM *Bla*SHV, *Bla* CTX-M, *cmlA* and *aadA1* or *aadA2* genes results of Escherichia coli isolates from group II baboons analysed by PCR in this study

Sample No.	*Bla* TEM	*Bla*SHV	*Bla* CTX-M	*cmlA*	*aadA1* or *aadA2*
Ac4a	-ve	+ve	-ve		
Ac10d	+ve	+ve	-ve		
Ac11d	+ve	+ve	-ve	-ve	-ve
Ac20c	+ve	+ve	-ve		-ve
Ac24d	+ve	+ve	-ve	-ve	-ve
Ac25a	+ve	+ve	-ve		-ve
Ac26a	+ve	+ve	-ve		
Ac28a	+ve	+ve	+ve		
Ac30c	+ve	+ve	-ve	+ve	+ve
Ac33b	+ve	-ve	+ve		
Ac34a	+ve	-ve	-ve	+ve	+ve
Ac35b	-ve	+ve	-ve		
Ac39b					+ve
Ac40b	+ve	-ve	-ve		+ve
Ac43c				+ve	
Ac45a	+ve	-ve	-ve		-ve
Ac46d	+ve	-ve	+ve		-ve
Ac47b	-ve	-ve	-ve		
Ac49d	+ve	-ve	-ve		-ve
Ac51c	-ve	+ve	-ve		
AC52c	-ve	-ve	-ve		
Ac54b	-ve	-ve	+ve		-ve
Ac55c	-ve	-ve	-ve		-ve
Ac57a	-ve	-ve	-ve	+ve	+ve
Ac59c					
Ac63a	-ve	-ve	-ve		-ve

Source: Author PCR results (2010)

Appendix 5: The *Bla* TEM *Bla*SHV, *Bla* CTX-M, *cmlA* and *aadA1* or *aadA2* genes results of Escherichia coli isolates from group I baboons analysed by PCR in this study

Sample No	*Bla* TEM	*Bla* SHV	*Bla* CTX-M	*cmlA*	*aadA1 or aadA2*
Aw1b	-ve	+ve	-ve		
Aw5a	+ve	+ve	-ve		
Aw6a	+ve	-ve	-ve	+ve	
Aw7a	+ve	+ve	-ve		
Aw11a	+ve	+ve	-ve		
Aw13a	-ve	-ve	-ve		
Aw20b	-ve	+ve	+ve		
Aw26b	+ve	+ve	-ve		-ve
Aw27a	-ve	-ve	-ve		-ve
Aw28a	+ve	+ve	-ve		
Aw30a	-ve	-ve	-ve	-ve	-ve
Aw31a	+ve	+ve	-ve		
Aw33a	+ve	-ve	-ve		
Aw36a	-ve	-ve	-ve		-ve

Source: Author PCR results (2010)

Appendix 6: PCR assays amplification conditions.

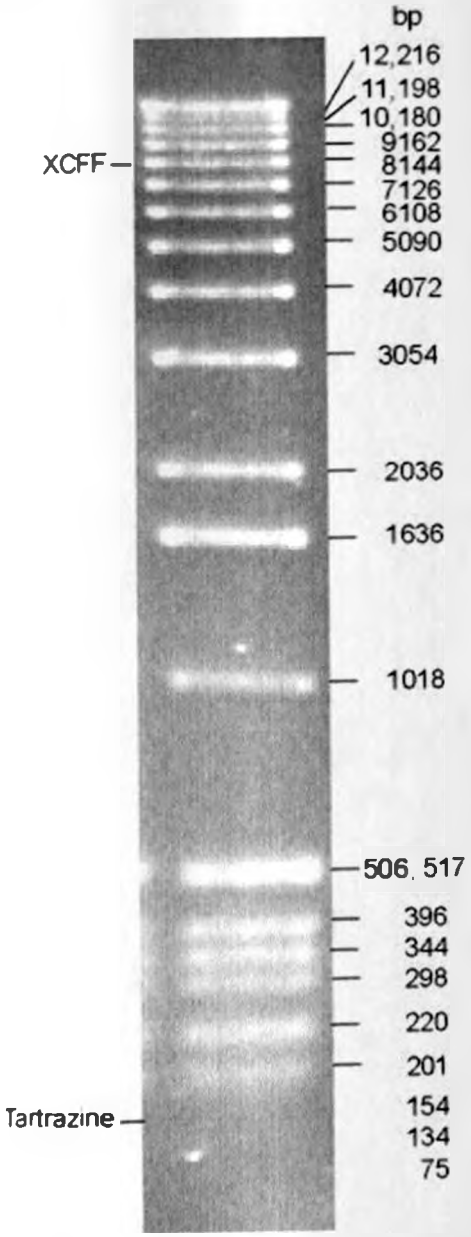
Each step of PCR	PCR conditions (^{0}C,minutes)				
	TEM	SHV	CTX-M	AadA	cmlA
Predenaturation	95, 5	95, 5	95, 5	95, 5	94,2
30 cycles of					
Denaturation	95, 1	95, 1	95, 1	95, 1	94, 1
Annealing	50, 1	55, 1	55, 1	60, 1	45, 1
Extension	72, 1.5	72, 1.5	72, 1.5	72, 1	72, 2
Final extension	72, 10	72, 10	72, 10	72, 7	72, 7

Source: Author PCR conditions (2010)

Appendix 7: The TrackIt™ 1 Kb DNA Ladder (5 µl) was analyzed on a 1% TAE agarose gel

The TrackIt™ 1 Kb DNA Ladder (5 µl) was analyzed on a 1% TAE agarose gel (10 cm x 6.3 cm). The gel was visualized and imaged on a UV transilluminator equipped with a camera. The migration of tracking dyes is indicated in the figure below.

Note: The 1636 bp band and bands ranging in size from 75-500 bp are derived from pBR322.



Source: Invitrogen™ life technologies.