CHARACTERIZATION OF AMINOGLYCOSIDE RESISTANT BACTERIAL STRAINS IMPLICATED IN INVASIVE INFECTIONS IN KENYA

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Characterization of Aminoglycoside Resistant Bacterial Strains Implicated in Invasive Infections in Kenya.

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DECLARATION

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

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DEDICATION

To my Family; first to my father Mr. Dominic Maina Ndegwah for enabling and facilitating me through the MSc degree programme; to my mother Alice Nyambura Ndegwa for her kind words of encouragement through out the research; to my brothers Dennis Ndegwa and Davis Wainaina for their love and support and last but not least to my husband Philip Lipesa for his company and enduring support in my life.

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MAY GOD BLESS YOU ALL!

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LIST OF ACRONYMNS AND ABBREVIATIONS

AMEs	Aminoglycoside-modifying enzymes
APHs	Aminoglycoside Phosphotransferases
AACs	Aminoglycoside Acetyltransferases
ANTs	Aminoglycoside Nucleotidyltransferases
AKUH	Aga Khan University Hospital
CTX-M	Cefotaxime-M type beta-lactamases
ERC	Ethical Review Committee
ESBL	Extended spectrum beta-lactamases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
IRT	Inhibitor resistant TEM lactamases
MDR	Multidrug resistant strains
NNIS	National Nosocomial Infections Surveillance
RNA	Ribonucleic acid
SHV	Sulf*h*ydryl variable
OXA	Oxacillin- hydrolyzing abilities
PCR	Polymerase Chain Reaction
UTI	Urinary Tract Infection

ABSTRACT

Aminoglycosides resistance through production of aminoglycoside-modifying enzymes (AMEs) is the most common type of microbial resistance. Possession of AMEs genes in Gram negative bacteria on plasmids, transposons and integrons facilitates the rapid acquisition of drug resistance. The study aimed at characterizing Aminoglycoside resistant strains of *Escherichia*, *Klebsiella*, *Pseudomonas* and *Acinetobacter* implicated in invasive infections in Nairobi, Kenya. The experimental design was a two point cross-sectional design comparing 54 clinical isolates obtained from Kenya Medical Research Institute (KEMRI) laboratory collected in 2001-2006 and 54 clinical isolates from Aga Khan University Hospital (AKUH-new) collected in 2007-2008. The isolates were identified using standard methods, tested for antimicrobial susceptibility to seven aminoglycosides; amikacin, gentamicin, kanamycin, neomycin, streptomycin, tobramycin, and High level Resistance (HLR) spectinomycin using disk diffusion by Kirby Bauer method. They were also tested for Extended spectrum beta-lactamases (ESBL) production by synergy between Ceftazidime and Clavulanate whereby a disk of Augmentin (20 μg of Amoxicillin plus 10 μg of Clavulanic acid) and a disk of Ceftazidime (30 μg) were placed 30 mm apart (center-to-center). Deoxyribonucleic acid (DNA) was extracted by the boiling method. Detection and characterization of AMEs was done by PCR using selected primers. Conjugation experiments were carried out to detect conjugative plasmids using *E. coli* J53 (Sodium azide resistant) and *E. coli* C600 (Rifampicin resistant) as

donors. Results showed an increase in aminoglycosides resistance particularly to naturally derived antibiotics like streptomycin, kanamycin, and Gentamicin either due to their prolonged and continuous use. AKUH- New isolates showed the highest percentages of resistance with 87%, 81% and 69% resistance to streptomycin, kanamycin and Gentamicin compared to KEMRI stored isolates. This may be attributed to lose of the AMEs due to the long storage of the isolates. A large number of *P. aeruginosa* strains (85%) were found to be Multi-drug resistant and showed resistance to Carbapenems. A total of 24 out of 108 (22%) of the clinical isolates tested were found to be ESBLs producers. These were mainly *E.coli* and *Klebsiella* spp. isolates. The genotypic results of the six AMEs amplified by PCR showed the most prevalent AME in the present study was *AAC(6')-Ib-cr* (45.9%), followed by *AAC(3)-II* (30.9%), *AAC(6')-II* (25.9%), *AAC(6')-I* (22.2%), and *AAC(3)-I* (16.3%). Increase in Aminoglycoside resistance by both naturally derived and semi-synthetic antibiotics is alarming. Methods of monitoring their effectiveness should be instituted at various levels of healthcare system in Kenya, to assist in determination of more appropriate chemotherapeutic agents for infection control.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Aminoglycosides are a group of antibiotics that are characterized by the presence of an aminocyclitol ring linked to amino-sugars in their structure. They are particularly active against aerobic and facultative anaerobic Gram-negative bacteria including members of family *Enterobacteriaceae* as well as *Acinetobacter*, *Pseudomonas* and *Enterobacter* genera (Mingeot-Leclercq *et al.*, 1999). They are most frequently used for treatment of invasive infections such as septicaemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections.

The Aminoglycoside-aminocyclitol family was one of the first groups of antibiotics to encounter the challenge of resistance (Wright, 1999). Since their discovery in 1944, they have come along way with bacteria developing even more complex resistance mechanisms of selection (Shakil *et al.*, 2008). Despite the introduction of newer, less toxic antimicrobial agents, aminoglycosides continue to play an important role in the treatment of serious Gram-negative bacterial infections. Aminoglycoside resistance has been reported globally among several problematic Gram-negative pathogens that are often responsible for serious nosocomial infections, including *Acinetobacter* spp. especially *A. Baumannii* and *Pseudomonas aeruginosa*. Members of the family

Enterobacteriaceae have also been reported to show resistance because of their production of extended-spectrum β-lactamase (Slama, 2008).

A strong association between the use of antibiotics and the emergence of antibiotic resistance has been demonstrated in hospital-acquired infections. According to Al Naiemi *et al.* (2006), the prevalence of resistance is highest where antibiotic use is high, especially in intensive care units (ICUs). The incidence of nosocomial infections in ICU is showing a rising trend mainly because of increasing invasive procedures performed in the ICU including; indwelling urinary catheters, sophisticated life support, intravenous fluid therapy, cardiovascular prosthetic devices, implantable orthopaedic prosthesis, and immunosuppressive therapy.

Data obtained from National Nosocomial Infections Surveillance (NNIS) for a study done from 1975 to 2003 indicated that nosocomial infections caused by *Acinetobacter* and *Pseudomonas* spp are on the increase. The incidence of *Acinetobacter* spp. in nosocomial pneumonia increased from 1.5% to 6.9%, in bloodstream infections increased from 1.8% to 2.4%, in surgical site infections from 0.5% to 2.1%, and in urinary tract infections from 0.6% to 1.6%. Importantly, multi-resistant strains of *Acinetobacter* spp. are being isolated with increasing frequency. For instance the incidence of *P. aeruginosa* increased from 9.6% to 18.1% in nosocomial pneumonia, from 9.3% to 16.3% in urinary tract

infection, and from 4.7% to 9.5% in surgical site infection (Slama, 2008). However, it declined slightly from 4.8% to 3.4% in bloodstream infections.

In Africa, benign strains such as *Escherichia coli, Pseudomonas, Acinetobacter, Enterobacter* and *Creseomonas* are developing multiple drug resistance and are now associated with invasive infections particularly among the immune-suppressed individuals (Okeke *et al.,* 2000). Clinical studies illustrate that patients infected with resistant strains of key Gram-negative pathogens have increased mortality, longer hospitalisation, and higher hospital costs than those infected by susceptible strains. Thus, there is need to intensify research in antibiotic therapy and especially in aminoglycoside resistant Gram negative bacteria that cause invasive infections in Kenya.

1.2 Aminoglycosides:

1.2.1 What are Aminoglycosides?

Aminoglycosides are antibiotics obtained from the genus *Streptomyces* (named with the suffix *–mycin*), *Micromonospora* (named with the suffix *–micin)* and *Bacillus* spp. or may be synthesised from naturally occurring aminoglycoside possibly to counter resistance (Table 1).

Table 1: Aminoglycoside antibiotics and their respective source organisms.

Aminoglycoside	Source Organism
Kanamycin	*Streptomyces kanamyceticus*
Streptomycin	*Streptomyces griseus*
Gentamicin	*Micromonospora purpurea*
Spectinomycin	*Streptomyces spectabilis*
Butirosin	*Bacillus circulans*
Tobramycin	*Streptomyces tenebrarius*
Neomycin	*Streptomyces fradiae*
Amikacin	Semi-synthetic derivative of Kanamycin
Netilmicin	Semi-synthetic derivative of Sisomicin
Isepamicin	Semi-synthetic derivative of Gentamicin B

1.2.2 Mode of Action

Aminoglycosides work by binding to the bacterial 16S rRNA on 30S ribosomal unit, inhibiting the translocation of the peptidyl-tRNA from the A (for aminoacyl)-site to the P (for peptidyl)-site causing misreading of mRNA hence leaving the bacterium unable to synthesize proteins vital for its growth. Aminoglycosides possess high affinity for prokaryotic RNA. When the drug binds to the ribosome, the structure is unable to translate mRNA for protein production leading to cell death. Although all aminoglycosides bind to the 30S ribosomal unit and interfere with protein synthesis, the details of interactions for

different classes of aminoglycosides are different (Vakulenko and Mobashery, 2003). In addition, Aminoglycosides inhibit cell respiration and cause potassium leakage of cell membranes. They have an oxygen-dependent transport system and are ineffective against anaerobic bacteria (Mingeot-Leclercq *et al.,* 1999). They are hence effective against Gram negative aerobic or facultative anaerobic bacteria such as *E. coli, Pseudomonas, Acinetobacter* and *Klebsiella*.

The bactericidal activity of aminoglycosides depends more on their concentration than on the duration of bacterial exposure to inhibitory concentrations of antibiotic and is also significantly less dependent on the bacterial inoculum size (Vakulenko and Mobashery, 2003). Significantly, aminoglycosides exhibit the post-antibiotic effect. They continue to kill bacteria even after the aminoglycoside has been removed following a short incubation with the microorganism. Generally, newer aminoglycosides such as gentamicin, tobramycin, amikacin, netilmicin, isepamicin, dibekacin, and arbekacin have broader spectra of activity than the older compounds like streptomycin and Kanamycin.

1.2.3 Clinical Importance of Aminoglycosides

Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the anti-infective armamentarium (Kotra *et al.,* 2000). They are particularly active against aerobic and facultative aerobic Gram-negative bacteria

including members of family *Enterobacteriaceae*, *Acinetobacter*, *Pseudomonas* and *Enterobacter* genera. The most frequent use of aminoglycosides is empiric therapy for serious infections such as septicemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life threatening infections (Mingeot-Leclercq *et al.*, 1999).

Aminoglycosides have been shown to have versatile clinical utility where local resistance patterns influence the choice of therapy (Shakil *et al.*, 2008). Gentamicin, amikacin, and netilmicin are used in meningitis, pneumonia and sepsis. Streptomycin has applications in tularemia, tuberculosis and plague. It is an alternative choice for the treatment of brucellosis. Paromomycin is used against amoebic dysentery. Spectinomycin is used in treatment of gonorrhoea. Neomycin finds its applications in burns, wounds, ulcers, and dermatitis. Among these aminoglycosides gentamicin is the antibiotic used most often because of its low cost and reliable activity against Gram-negative aerobes.

Despite their potential nephrotoxicity, neurotoxicity, ototoxicity and bacterial resistance, aminoglycoside antibiotics remain valuable and sometimes indispensable in the treatment of various infections and prophylaxis in special situations. Aminoglycosides exhibit several characteristics that make them useful

as antimicrobial agents such as predictable pharmacokinetics, little or no allergic reactions, and they often act in synergy with other antibiotics (Vakulenko and Mobashery, 2003 ; Shakil *et al.*, 2008).

1.3 Aminoglycoside Resistance

1.3.1 Epidemiology of Aminoglycoside resistance

The resistance of clinical isolates to aminoglycoside antibiotics vary with the specific drug, the microorganism, its mechanism of resistance, the geographic area and many other factors. Resistance to Aminoglycosides is generally associated with enzymatic modification of the antibiotic. Selection of microorganisms producing aminoglycoside modifying enzymes depends on the amount of antibiotic usage in particular hospitals and the standards of clinical treatment within various countries (Vakulenko and Mobashery, 2003).

A study conducted by SENTRY Antimicrobial Surveillance ProGram in 1997, on analysis of bloodstream infections in the United States hospitals revealed that the four most common Gram-negative bacteria (*E. coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, and *Enterobacter* spp.) were quite susceptible to the Aminoglycosides tested: 96 to 100% to amikacin, 90 to 96% to gentamicin, and 94 to 97% to tobramycin (Schmitz *et al.*, 1999). A similar study performed by European SENTRY proGram showed susceptibility to amikacin, gentamicin, and tobramycin among *E. coli* was 99.6%, 95.4%, and 95.7%, respectively; among

Klebsiella spp., it was 95.6%, 88.1%, and 85.8%, respectively; and among *Pseudomonas aeruginosa* isolates, 91.8%, 78.8%, and 80.8%, respectively. Among clinical isolates belonging to *Acinetobacter* spp., only 58%, 43%, and 60% were susceptible to amikacin, gentamicin, and tobramycin, respectively (Vakulenko and Mobashery, 2003). In a different study conducted on *Acinetobacter baumannii* in 2005, data collected showed high level resistance to Aminoglycosides of 100%, 100%, 100% and 93% for gentamicin, amikacin, streptomycin and kanamycin respectively. However, rates of resistance to tobramycin, netilmicin and neomycin were 86%, 93% and 46% respectively.

Francetić *et al.* (2008) in Croatia, tested 676 Gram negative isolates from blood, urine, and cerebrospinal fluid of patients in ICUs against aminoglycosides. The overall gentamicin resistance of Gram negative bacilli decreased from 42% to 26%, netilmicin resistance decreased from 33% to 20%, whereas amikacin resistance did not significantly change (20% to 19%). However, *E. coli* and *P. aeruginosa* showed a significant reduction and *A. baumannii* a significant increase of amikacin resistance. All Gram negative bacilli tested showed significant reduction in gentamicin and netilmicin resistance during amikacin period, except for *K. pneumoniae* and *Enterobacter*, which showed a non-significant reduction.

More recent surveys (Neonakis *et al.,* 2003 and Fihman *et al.,* 2008) have demonstrated that broadening of aminoglycoside resistance spectra to include most of the clinically available aminoglycosides, such as gentamicin, tobramycin, amikacin and netilmicin occurred in countries and hospitals where these antibiotics are used more extensively.

1.3.2 Mechanisms of Aminoglycoside Resistance

There are four mechanisms of aminoglycoside resistance. It is noteworthy that more than one mechanism may be at play at the same given time in a bacterium in the case of some classes of drug. Reduced uptake or decreased cell permeability due to absence of or alteration in the aminoglycoside transport system, inadequate membrane potential, or modification in the lipo-polysaccharide phenotype. This mechanism is likely to be chromosomally mediated and the level of resistance that is seen is moderate, that is, intermediate susceptibility. This mechanism has been observed in *P. aeruginosa* and in *E. coli* which have an active efflux system (Mingeot-Leclercq *et al.,* 1999).

Resistance to streptomycin can occur by alteration of the ribosomal binding sites by mutation since this agent binds to a single site on the 30S subunit of the ribosome. Resistance to the other aminoglycosides by this mechanism is uncommon since they bind to multiple sites on both ribosomal subunits.

Many aminoglycoside producing organisms express rRNA methylases, which are capable of modifying the 16S rRNA molecule at specific drug binding positions. This mechanism has been reported in members of the family *Enterobacteriaceae* and *P. aeruginosa.* A number of genes encoding such enzymes have been identified from several aminoglycoside producers; *Streptomyces tenjimariensis, Streptomyces tenebrarius* and *Micromonospora purpurea* (Shakil *et al.,* 2008).

Production of aminoglycoside-modifying enzymes (AMEs) is the most common mechanism of Aminoglycoside Resistance and therefore of most clinical importance. The three families of enzymes: Aminoglycoside Acetyltransferases (AACs), Aminoglycoside Nucleotidyltransferases (ANTs) and Aminoglycoside Phosphotransferases (APTs) perform co-factor dependent drug modification in the bacterial cytoplasm. Modified aminoglycosides bind poorly to the ribosome and fail to trigger energy-dependent phase II allowing the bacteria to survive in the presence of the drug.

1.3.3 Association of Aminoglycosides resistance and Extended Spectrum Beta-lactamases (ESBLs)

There is no precise definition of ESBLs. A commonly working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to penicillins, first, second, and third-generation Cephalosporins and Aztreonam by

hydrolysis and which are inhibited by β-lactamase inhibitors such as Clavulanic acid (Paterson and Bonomo, 2005).

There are a variety of ESBL families but of importance are the types that occur in clinical isolates such as TEM type beta-lactamases, CTX-M type beta-lactamases, Sulf*h*ydryl variable (SHV), and Oxacillin- hydrolyzing abilities (OXA) based on their resistance profiles to broad spectrum cephalosporins. TEM and SHV are variants of penicillases whilst CTX-M is a result of chromosomal mutation (Paterson and Bonomo, 2005). TEM-1 is the most commonly-encountered beta-lactamase in Gram-negative bacteria. Inhibitor-resistant β-lactamases (IRT) are not ESBLs but are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes (Bradford, 2001). IRT family are resistant to clavulanic acid and sulbactam and are generally susceptible to cephalosporins such as cefotaxime and cefoxitin. CTX-M family confer resistance to cefotaxime. OXA family confers resistance to cefotaxime and ceftriaxone but provides only marginal protection against ceftazidime. SHV family confers resistant to ceftazidime and cefoxitin.

The prevalence of ESBL enzymes has been increasing in many parts of the world (El Kholy *et al.*, 2003). Infections caused by ESBL-producing isolates are difficult to treat. ESBL production is usually associated with resistance to other classes of antimicrobial agent, such as aminoglycosides and fluoroquinolones.

Outbreaks of *Klebsiella* infections with strains resistant to third-generation cephalosporins have been reported in Kenya without documentation of ESBL production (Musoke and Revathi, 2000). CTX-M enzyme (CTX-M-12) which is plasmid-encoded has been found in *Klebsiella pneumoniae* isolated from cerebrospinal fluid and blood here in Kenya (Kariuki *et al.,* 2001).

1.4 Genetic basis of Aminoglycoside resistance

Antibiotic resistance is a specific type of drug resistance when a microorganism has the ability of withstanding the effects of antibiotics. Antibiotic resistance evolves via natural selection acting upon random mutations, but it can also be engineered by applying evolutionary stress on a population. Once such a gene is generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals) by conjugation, transduction, or transformation. Many antibiotic resistance genes reside on plasmids, facilitating their transfer. Two types of genetic elements, self-transferable conjugative plasmids and transposons facilitate rapid dissemination of resistance genes not only within a given species but also among a variety of bacterial species (Shakil *et al.,* 2008). Antibiotic resistance can also be introduced artificially into a microorganism through laboratory protocols, sometimes used as a selectable marker to examine the mechanisms of gene transfer or to identify individuals that absorbed a piece of DNA that included the resistance gene and another gene of interest.

Aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism. There are over 50 different aminoglycoside-modifying enzymes (AMEs) that have been identified and enzymatic modification result in high-level resistance. The level of resistance produced differs significantly in various microorganisms and individual strains and depends on many factors, including the amount of enzyme produced, its catalytic efficiency, and the type of aminoglycoside.

ESBL-containing plasmids often carry resistance genes for gentamicin and tobramycin (Karam and Heffner, 2000). Furthermore, several of these genes are also included in transposons and integrons which result in rapid dissemination at molecular level (Mingeot-Leclercq *et. al.*, 1999). According to Reyes *et al.* (2003), Class 1 integrons have been isolated from *Enterobacteriaceae* encoding aminoglycoside modifying enzymes AAC(6')-Ib, ANT(2")-I and ANT(3")-I. Type 2 integrons were reported in nosocomial isolates of *Acinetobacter baumannii* indicating mobilization of these elements between bacteria of different genera.

1.5 Aminoglycoside Resistance Genes in Selected Bacteria

The epidemiology of aminoglycoside resistance is becoming more complex, in part because of the multitude of aminoglycoside-modifying enzymes that exist for these antibiotics (Vakulenko and Mobashery, 2003).

The nomenclature is defined as follows: Aac, Ant, or Aph for the type of enzymatic modification, followed by a number in parentheses designating the site of modification and the Roman numerals and letters that follow stand for unique resistance profiles and protein designations (Shaw *et al.*,1993).

1.5.1 Aminoglycoside Acetyltransferases

They comprise four classes of enzymes: Aac(1), Aac(3), Aac(2'), and Aac(6'). They utilize acetyl co-enzyme A as the donor of the acetyl group. They confer resistance to tobramycin, gentamicin, dibekacin, sisomicin, kanamycin, neomycin, paromomycin, and lividomycin.

The most common AMEs in Gram negative pathogens is Aac(6') which confers resistance to kanamycin and amikacin (Davies and Wright, 1997). This enzyme was first discovered in *Pseudomonas* spp. (Shaw *et al.*, 1993). *AAC(6')* gene has however been identified in both Gram-negative and Gram-positive micro-organisms. The gene for *AAC(6')-Ib* has been detected in either transposons or integrons which have facilitated its rapid dissemination in the presence of selective antibiotic pressure among a wide range of microorganisms (Vakulenko and Mobashery, 2003).

Aac(3)s are widely distributed among different genera, including aminoglycoside producers. They are the second most common. The gene for

AAC(3)-Ia was detected on conjugative plasmids and transposons and within gene cassettes in integrons from *Enterobacteriaceae* and *P. aeruginosa* (Vakulenko and Mobashery, 2003). The *AAC(3)-II* is commonly seen in various clinical isolates of Gram-negative bacteria and the frequency of the *AAC(3)-II* phenotype varies among different genera, from 18% in *Pseudomonas* spp. to 60% in some other Gram-negative bacteria. It was demonstrated that 85% of the bacteria that show the *AAC(3)-II* phenotype produce Aac(3)-IIa and only 6% produce Aac(3)-IIb (Shaw *et al.*, 1993). Three other Aac(3)s from clinical isolates, *AAC(3)-III, AAC(3)-IV,* and *AAC(3)-VI,* are uncommon.

Aac(1) and has been identified in animal isolates of *E. coli* J62-1 (Shaw *et al.*, 1993). Probably because Aac(1) enzymes produce no clinically important resistance, the genes for these acetyltransferases have not been cloned, so the distribution of *AAC(1)* among clinical isolates has not been studied. Aac(2) is restricted primarily to *Providencia* and *Proteus* spp. The gene for *AAC(2')-Ia* has been isolated from *Providencia stuartii.* However, this gene has in a few instances been observed in *Pseudomonas* strains (Shaw *et al.*, 1993).

1.5.2 Aminoglycoside nucleotidyltransferases

Comprise five classes; Ant(2"), Ant(3"), Ant(4'), Ant(6), and Ant(9). They utilize ATP as the second substrate and modify aminoglycoside antibiotics by transferring AMP to their hydroxyl group which confers resistance to

tobramycin, gentamicin, dibekacin, sisomicin, kanamycin, streptomycin, and spectinomycin.

ANT(2")-Ia is a widespread gene among all Gram-negative bacteria (Shaw *et al.,* 1993, Vakulenko and Mobashery, 2003) however the frequency of its detection varies from one country to another. *ANT(3")-I* is also wide spread among Gram negative micro-organisms (Mingeot-Leclercq *et al.,* 1999). *ANT(3'')-Ia* gene is commonly used in molecular biology and confers resistance to streptomycin and spectinomycin (Shaw *et al.,* 1993). This gene was detected within transposons (Tn7 and Tn21) and various plasmids in Gram negative bacteria and later detected in Gram-positive bacteria; *Staphylococcus aureus* and *Corynebacterium glutamicum* (Vakulenko and Mobashery, 2003). Also, class I integrons harboring *ANT(3")-I* genes have been identified frequently among various clinical isolates of *Enterobacteriaceae, P. aeruginosa,* and *V. cholerae* (Vakulenko and Mobashery, 2003).

ANT(4')-I has been observed in Gram positive bacteria accounting for 30% in *Staphylococcus* strains (Shaw *et al.,* 1993). *ANT(4')-II* was first isolated in *P. aeruginosa* and has been subsequently observed in *E. coli, Citrobacte*r spp., *Klebsiella* spp., and *Serrati*a spp (Shaw *et al.*, 1993). *ANT(6')-Ia* was identified in almost 50% of *Enterococcus* spp. and later identified in *Bacillus subtilis* (Vakulenko and Mobashery, 2003). *ANT(6')-II* has only been observed in

Pseudomonas strains (Shaw *et al.,* 1993). *ANT(9)-I* gene is specific to *Staphylococcus aureus* (Shaw *et al.,* 1993).

1.5.3 Aminoglycoside Phosphotransferases (kinases)

They utilize ATP as the second substrate and are able to phosphorylate specific hydroxyl groups in all classes of aminoglycoside antibiotics. Seven classes of enzymes, Aph(3'), Aph(2"), Aph(3"), Aph(4), Aph(7''), Aph(6), and Aph(9) have been identified in clinical isolates and aminoglycoside-producing organisms. They confer resistance to kanamycin, neomycin, lividomycin, paromomycin, ribostamycin, butirosin, amikacin, isepamicin gentamicin, tobramycin, and hygromycin.

The largest class of aminoglycoside phosphotransferases are Aph(3') with seven different types; Aph(3')-I to Aph(3')-VII, identified among Gram-negative and Gram-positive bacteria and also aminoglycoside producing microorganisms. (Mingeot-Leclercq *et al.,* 1999). The gene *APH(3')-I* (aph(3')-Ia enzyme) was discovered on transposons Tn*903* in *E. coli*. Subsequently, it was identified on plasmids and transposons in many Gram-negative bacteria, including *K. pneumonia, Salmonella enterica* serovar Typhimurium, other *Salmonella enterica*, *Proteus vulgaris, V. cholerae, Campylobacter jejuni*, and the fish pathogen *Pasteurella piscicida* (Vakulenko and Mobashery, 2003). The high frequency of occurrence of Aph(3')-I and other enzymes producing kanamycin

resistance in bacteria resulted in the clinical obsolescence of the kanamycins. Aph(3')-II is rarely found in clinical isolates and the gene for this enzyme is also located on a transposable element, Tn*5*. The gene for Aph(3')-IIb has been identified in the chromosome of *P. aeruginosa* (Vakulenko and Mobashery, 2003). *APH(3')-III* phosphotransferases were originally isolated from *Staphylococcus aureus* and *Streptococcus faecalis*. Later the gene was identified in *Campylobacter coli*, which became a precedence for antibiotic resistance gene transfer between Gram-positive and Gram-negative bacteria (Vakulenko and Mobashery, 2003). The genes for *APH(3')-IV* and -*V* were detected only in antibiotic-producing microorganisms. *APH(3')-VI* is primarily from *Acinetobacter* spp. and studies show distribution of 83-95% of amikacin-resistant *Acinetobacter* strains demonstrated the presence of *APH(3')-Via*. (Vakulenko and Mobashery, 2003). *APH(3')-VII* distribution of the gene has not been studied.

APH(2") genes have been detected in Gram positive bacteria. However, *APH(2')-Ib*, *-Ic*, and *-Id* are not as widely distributed among clinical isolates (Vakulenko and Mobashery, 2003). *APH(3")-I* has two enzymes: Aph(3")-Ia was identified in a streptomycin-producing strain of *Streptomyces griseus* and Aph(3")-Ib, was cloned from a broad-host-range plasmid, RSF1010, that is widely distributed among Gram-negative bacteria (Shaw *et al.*,1993). Two genes *APH(6)-Ia* and *-Ib* were cloned from streptomycin producers, *S. griseus* and

Streptomyces glaucescens. The third enzyme, Aph(6)-Ic, is encoded by a gene located within transposon Tn*5*, which is found infrequently in Gram-negative bacteria (Shaw *et al.*, 1993). *APH(4), APH(7")* and *APH(9)* do not present any clinical significance.

Över *et al.*, (2001) prevalence of aminoglycoside resistance genes in *Klebsiella, Enterobacter* and *E. coli* showed 96% of the isolates possessed *ANT(2")-I, AAC(3)-II, AAC(6')-I, AAC(6')-III* and *AAC(6')-IV*. While, *P. aeruginosa* showed permeability resistance was most common (66%) followed by *ANT(2")-I, AAC(6")-II, AAC(6')-III* and *AAC(3)-I*. Production of *AAC(3)-I, APH(3')-VI,* and *ANT(3")-I* was reported to be predominant by worldwide surveys on *Acinetobacter* spp., but there were considerable regional differences in their genotypes (Doi *et al.*, 2004). Recently Miró *et al.*, 2008 showed that the most frequent AMEs genes in *Enterobacteriaceae* were *APH(3")-Ib* (61%), *ANT(3")-Ia* (40%), *AAC(3)-IIa* (10%), *APH(3)-Ia* (8%), *AAC(6')-IIb* (4%), *ANT(2")-Ia* (3%) and *AAC(6')-Ic* (0.3%).

Despite the existence of more than 50 AMEs, only several of them such as Ant(2")-I, Aac(6')-I and, to a lesser extent Aac(3)-I, Aac(3)-II, Aac(3)-III, Aac(3)-IV as well as Aac(3)- VI have been selected in Gram-negative bacteria to produce the majority of aminoglycoside resistance (Vakulenko and Mobashery, 2003).

Although aminoglycosides are widely used in treatment of infections and the public health implications of rising levels of resistance, no studies have been done on epidemiology and genetic basis of aminoglycoside resistance in Kenya. This study was designed to determine antimicrobial resistance levels within this important class of antimicrobials

1.6 STATEMENT OF THE PROBLEM

The emergence and spread of antimicrobial resistance determinants has increasing importance worldwide, particularly among nosocomial bacterial pathogens. *A. baumannii*, ESBL-producing *Enterobacteriaceae*, and *P. aeruginosa* are key Gram-negative pathogens that are involved in serious nosocomial infections. Multi-drug resistant strains are particularly problematic leading to increased mortality, longer hospital stays and higher hospital costs over and above the values associated with susceptible strains of these pathogens. Successful treatment requires a 'hit hard and hit fast' approach with an antibiotic that provides coverage of these important Gram-negative organisms, including multi-drug resistant strains.

The large number and diversity of modifying enzymes involved in aminoglycoside resistance further complicate the situation. In addition, it has been suggested that resistance patterns are influenced by clinical usage of specific aminoglycoside drugs. The genes for the aminoglycoside-modifying enzymes are often located on plasmids or transposons together with the genes encoding resistance to other classes of antibacterials, the total consumption of non-aminoglycoside antibiotics can thus significantly influence the epidemiological features of aminoglycoside resistance.

1.7 JUSTIFICATION

The high prevalence of antibiotic resistance is a public health concern that has led to increased interest in studying the ways in which bacteria avoid the effects of antibiotics. Aminoglycoside antibiotics are useful for empirical treatment of febrile neutropenic patients and patients with serious infections caused by aerobic Gram-negative microorganisms, including *Enterobacteriaceae* and *P. aeruginosa*. The emergence of drug-resistant Gram-negative bacterial strains due to their ability to readily accept foreign DNA so as to adapt to and survive in environments that are hostile, has to some extent contributed to the development of invasive infections.

In Kenya, there is paucity of data on aminoglycoside resistance in invasive infections although they form the mainstay drugs in our hospitals for treatment of serious nosocomial infections by *Acinetobacter* spp., *P. aeruginosa*, and ESBL- producing *Enterobacteriaceae*. There is therefore need to study the mechanisms and epidemiology of aminoglycoside resistance to understand the emergence and dissemination of resistant bacteria particularly in hospital settings.

1.8 HYPOTHESES

1. The rate of Aminoglycoside resistance in the genera *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* implicated in invasive infections in Kenya is on

the increase.

2. The genera *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* implicated in invasive infections in Kenya have mobile genetic determinants of antibiotics such as integrons, transposons and conjugative plasmids responsible for Aminoglycoside resistance.

1.9 OBJECTIVES

1.9.1 General Objective

To characterize aminoglycoside resistant strains of the genera *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* implicated in invasive infections in Nairobi, Kenya.

1.9.2 Specific Objectives

1. To determine *in-vitro* antimicrobial susceptibility patterns of *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* isolated from invasive infections at selected hospitals in Kenya.

2. To determine the production of extended spectrum beta-lactamases by these bacteria.

3. To determine the presence of aminoglycoside-modifying enzymes in these bacterial pathogens.

4. To determine the presence of mobile genetic determinants of antibiotics resistance.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study design

The study was a two point cross-sectional comparison of old stored and new isolates. The stored isolates were obtained from Kenya Medical research Institute (KEMRI) laboratory and were collected between 2001-2006 at two hospitals, Kenyatta National Hospital (KNH- old) and Aga Khan University Hospital (AKUH-old). New isolates were collected from Aga Khan University Hospital (AKUH-new) in 2007-2008.

2.2 Ethical consideration

This study did not involve sampling patients from the hospital directly. All consecutive clinically significant *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* bacterial isolates were collected from AKUH laboratory and KEMRI, CMR stored isolates. All isolates transferred for use in this study contained only laboratory numbers; no names of patients were contained in these records. Permission to carry out the study was obtained from Kenya Medical Research Institute (KEMRI) Scientific Committee and Ethical Review Committee (Appendix C); and Aga Khan University Hospital (AKUH) Scientific and Ethical Review Committees.

2.3 Sample size determination

The sample size was determined using the Fisher formula (1925), as shown below.

$$n = \frac{Z^2\alpha/2\ P\ (1\text{-}P)}{d^2}$$

Where;
n= minimum sample size
Z= Standard normal derivative that corresponds to 95% confidence interval (1.96)
α = the level of significance (95%)
P = Expected prevalence of Micro-organism of interest (*E. coli*= 6%, *Klebsiella* spp. =5%, *P. aeruginosa*= 3% and *Acinetobacter baumannii*= 1.5%)
d^2= Absolute precision (0.05)

Therefore, total sample size (n) was **108** where; 41 isolates were *E. coli*, 35 *Klebsiella*, 21 *P. aeruginosa* and 11 *A. baumannii.*

2.4 Strain collection and selection

A total of 108 isolates were collected over the period of 2001-2008 from two sources- KEMRI Laboratory and Aga Khan University Hospital (Table 2). Specimen sources included; Intravenous blood, Urine, Sputum, Tracheal aspirates, Pus swabs, cerebral spinal fluid (CSF), Catheters, and High Vaginal Swabs (HVS). All the 108 isolates were sub cultured onto MacConkey plates and incubated aerobically at 35-37^{o}C for at least 24 h, in preparation for the susceptibility testing. Isolates from AKUH were also sub cultured on

MacConkey plates and incubated aerobically at 35-37^{o}C for at least 24 h, in preparation for the susceptibility testing.

Table 2: Clinical isolates of *E. coli, Klebsiella* spp., *A. baumannii* and *P. aeruginosa* collected from selected Kenyan Hospitals.

Clinical Isolate	Urine	Blood	Tracheal aspirates	Sputum	Pus swabs	Others*	Total
Specimen Source: Aga Khan University Hospital -new isolates (2007-2008)							
E. coli	16	-	-	-	-	-	**16**
Klebsiella spp.	5	3	1	1	1	1	**12**
A. baumannii	3	-	3	-	1	-	**7**
P. aeruginosa.	8	3	5	-	1	2	**19**
						Total	**54**
Specimen Source: Aga Khan University Hospital -old (2001-2006)							
E. coli	10	-	-	-	2	-	**12**
Klebsiella spp.	-	4	-	2	2	-	**8**
A. baumannii	-	-	-	-	-	-	**-**
P. aeruginosa	-	-	-	-	1	-	**1**
						Total	**21**
Specimen Source: Kenyatta National Hospital-old (2003-2006)							
E. coli	13	-	-	-	-	-	**13**
Klebsiella spp.	11	-	3	-	-	1	**15**
A. baumannii	4	-	-	-	-	-	**4**
P. aeruginosa	1	-	-	-	-	-	**1**
						Total	**33**

Others* - Cerebral spinal fluid (CSF), Catheters, and High Vaginal Swabs (HVS).

2.4 Laboratory procedures

2.4.1 Identification of bacteria

All the 108 isolates were tested for their biochemical characteristics using five tube method media (Oxoid Ltd., Basingstoke, United Kingdom) of Triple sugar iron agar, Sulphur indole motility agar, Simmon's citrate agar, MRVP broth and urea agar (Appendix A) and their reaction to oxidase reagent (1% dimethyl-p-phenylene-diamine dihydrochloride). Gram stain tests were also done to confirm morphological characteristics of the isolates.

Organisms once identified were stocked in 1ml capacity vials containing Mueller Hinton broth (Oxoid Ltd., Basingstoke, United Kingdom) containing 15% (v/v) glycerol and stored at -70°C until analysed.

2.4.2 Antibiotic susceptibility testing

Overnight cultures of all the 108 isolates were obtained and used to test for susceptibility to seven aminoglycosides. The aminoglycosides used in the study were amikacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), streptomycin (10 µg), tobramycin (10 µg) obtained from Oxoid Limited United Kingdom and High level Resistance (HLR) spectinomycin (300 µg) obtained from Rosco Diagnostica, Denmark.

The tests were carried out using the disc diffusion by Kirby bauer method on Mueller Hinton Agar and incubated at 37°C for 18-24 h. Disk susceptibility tests were interpreted according to the guidelines provided by the manufacture's

interpretation charts. *E. coli* ATCC 25922 with known MICs was used as control organism. Other antibiotics from various families were also tested for purposes of resistance profiling (Table 3).

2.4.3 Detection of Extended spectrum Beta-Lactamases producing bacteria

The presence of ESBLs in *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* spp. was detected by double disk diffusion test in which synergy between ceftazidime and clavulanate was sought. In this test a disk of augmentin (20 μg of amoxicillin plus 10 μg of clavulanic acid) and a disk of ceftazidime (30 μg) were placed 30 mm a part (center-to-center). A clear-cut extension of the edge of the ceftazidime inhibition zone toward the disk containing clavulanic acid was then interpreted as synergy, which suggests the production of ESBL (Neo-sensitabs Susceptibility testing 19th Edition, 2007/2008). The double-disk synergy test was considered positive when decreased susceptibility to ceftazidime was combined with synergy between ceftazidime and augmentin (Jarlier *et. al.,*1988). Repeat experiments were done whereby in each repeat, the cephalosporin was replaced with a representative drug from 1st, 2nd, 3rd and 4th generation cephalosporin plus a monobactam (Table 3).

Table 3: Antibiotic families and their drug concentrations used to test for resistance in the study.

Antibiotic Family	Antibiotics tested (Concentration)
Aminoglycosides:	Amikacin (30 µg), Gentamicin (10 µg), Kanamycin (30 µg), Neomycin (30 µg), Streptomycin (10 µg), Tobramycin (10 µg)
Tetracyclines:	Tetracycline (30 µg), Minocycline (30 µg)
Quinolones:	Norfloxacin(10 µg), Ciprofloxacin (5 µg), Nalidixic acid (30 µg)
Chloramphenicol:	Chloramphenical (30 µg)
Sulphonamides:	Trimethoprim (5 µg), Sulfafurazole (300 µg)
Nitrofurans:	Nitrofuratonin (300 µg)
Penems & Carbapenems:	Meropenem (10 µg), Imipenem (10 µg) and Imepenem Cilastatin (20 µg)
Oxazolidinones:	Linezoid (30 µg)
Lincosamides:	Lincomycin (30 µg)
Beta-lactam:	Augmentin (30 µg) (20 µg of Amoxicillin plus 10 µg of Clavulanic acid)
Mono-bactam:	Aztreonam(30 µg)
Cephalosporins:	
1st Generation	None used.
2nd Generation	Cefoxitin- FOX (30 µg) and Cefuroxime-CU/CXM (30 µg)
3rd Generation	Cefotaxime-CTX (30 µg), Ceftazidime CAZ/ CEZDI (30 µg), Ceftriaxone CRO/CETRX(30 µg)
4th Generation	Cefepime-FEP (30 µg)

2.4.4 DNA Extraction by boiling method

An 18-24 h single colony of each isolate was suspended in 1 ml of sterile distilled water, which was then heated at 95°C for 10 min. After heating, centrifugation was done at 14,000 rpm for 6 min at 4°C. The DNA-containing supernatant was extracted and used as the source of template for further PCR amplification experiments.

2.4.5 Polymerase Chain Reaction and sequencing

Selected isolates were tested for AMEs by using the PCR primers listed in Table 4 based on their aminoglycoside resistance profiles. Class 1 and 2 integrons on all 108 isolates were also detected using selected primers (Table 4).

PCR amplification reactions was performed in a volume of 25 µl containing 12 µl of Qiagen PCR Master Mix (Qiagen GmbH, Hilden, Germany), 1.0 µM concentrations of each primer, 6 µl of PCR water and 5 µl of DNA template at the PCR cycles shown (Table 4). PCR products were analysed by gel electrophoresis at 100V for 1½ h in a 2% agarose gel stained in Ethidium bromide. Bioline Hyperladder 1 was used as the standard marker.

Table 4: Selected Aminoglycoside resistance genes and Integrons sequences detected by Polymerase Chain Reaction.

AME genes (Aminoglycoside Resisted)	F/ R Primer	PCR cycle
aac(6')-I (Tob, Amk, Kan)	TATGAGTGGCTAAATCGAT/ CCCGCTTTCTCGTAGCA	94°C 15 min, (94°C 45s, 55°C 45 s, 72°C 45s) 34 cycles, 72°C 10 min
aac(6')-II (Tob, Amk, Kan)	CGCTTGTTGATTTGCTGCT GTTCGC/TTGAAACGACCT TGACCTTCCG	94°C 15 min, (94°C 45s, 55°C 30s, 72°C 1 min) 30 cycles, 72°C 10 min
aac(6')-1b-cr (Tob, Amk, Kan+ NA, Cip, Nor)	TTGCGATGCTCTATGAGTG GCTA/ CTCGAATGCCTGG CGTGTTT	94°C 5 min, (94°C 45s, 55°C 45s, 72°C 1 min) 36 cycles, 72°C 10min
aac(3)-I (Gen, Tob)	AGCCCGCATGGATTTGA/ GGCATACGGGAAGAAGT	94°C 15min, (94°C 1 min, 55°C 1 min, 72°C 1 min) 30 cycles,72^0C 10 min
aac(3)-IIa (Gen, Tob)	GCTAAACTCCGTTACC/ TAGCACTGAGCAAAGCC	94°C 15 min, (94°C 45s, 60°C 30s, 72°C 90s) 30 cycles, 72°C 10 min
ant(4')-IIb (Tob, Kan)	GAGAACCCATATGCAACA TACTATCGCC/ TAGAATTCT AGCGCGCAC TTCGCTCTTC	94°C 15 min, (94°C 1min, 58°C 30s, 72°C 1 min) 35 cycles, 72°C 10 min

Integrons:		
Int-I	GTTCGGTCAAGGTTCTG/ GCCAACTTTCAGCACATG	94°C 5 min, (94°C 30s, 50°C 30s, 72°C 90s) 35 cycles, 72°C 10 min
Int- II	ATGTCTAACAGTCCATTTT/ AAATCTTTAACCCGCAAAC	94°C 5 min, (94°C 30s, 50°C 30s, 72°C 90s) 35 cycles, 72°C 10 min

KEY: Tob- Tobramycin, Kan- Kanamycin, Gen- Gentamicin, Amk- Amikacin, NA- Nalidixic acid, Cip- Ciprofloxacin, Nor- Norfloxacin

2.4.6 Conjugation Experiments

Resistant organisms (donor) and *E. coli* C600 (recipient) or *E. coli* J53 (recipient) were cultured onto solid Mueller-Hinton agar and incubated at 37°C overnight. The organisms were then suspended in normal saline to Macfarland 0.5 standard and inoculated at a ratio of 1:3 (Donor: Recipient) in 3ml Mueller Hinton broth (Oxoid Ltd) in bijou bottles and incubated in an aerobic incubator at 37°C overnight. Using a sterile inoculating wire loop each of the mixtures were sub-cultured onto: MacConkey agar containing 30 µg/ml ampicillin and 30 µg/ml rifampicin for *E. coli* C600 or 0.3 µg/ml sodium azide for *E. coli* J53 divided into 3 sections to select for transconjugants, donor and the recipient. Antibiotic susceptibility was repeated using the disk diffusion method so as to confirm transferred resistance genotypes.

2.4.7 Plasmid studies

2.4.7.1 Harvesting and Extraction

Bacteria found resistant to at least 3 aminoglycosides antibiotics were selected for plasmid analysis using the protocol (Sambrook *et. al.*,1989) described below. Bacteria stored at -80°C were revived by sub culturing on Mueller Hinton agar and incubated at 37°C for 24 h aerobically. A single bacterial colony was transferred into 2 ml of Mueller Hinton broth medium in a loosely capped 15 ml tube. The culture was incubated at 37°C with vigorous shaking overnight. A volume of 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at 13,000xg for 30 secs at 4°C in a microfuge. The supernatant was removed by aspiration and the dry pellet was re-suspended in 100µl of ice cold solution I (Appendix B) by gentle agitation. Bacterial cell wall was digested by addition of 200µl of freshly prepared solution II (Appendix B) and on gentle shaking 150µl of ice cold solution III was used to precipitate cell debris plus protein material (Appendix B). In a microfuge the solution was centrifuged at 13,000g for 5 mins at 4°C and the supernatant was transferred to a fresh tube. The double stranded DNA was precipitated using 2 volumes of ethanol at room temperature, vortexed and allowed to stand for 2 mins at room temperature. Centrifugation was then done at 13,000g at 4°C in a microfuge for 5 mins and the supernatant was removed by gentle aspiration. The tube was then placed in an inverted position to dry the DNA. The pellet was then rinsed using 1 ml of 70% ethanol at 4°C, the supernatant was removed and the pellet allowed to air dry for 10 mins. Plasmid DNA was then re-dissolved in 50 µl of TE (pH 8.0)

containing DNAse free pancreatic RNAse (20 μg/ml).

2.4.7.2 Electrophoresis and Photography

Agarose gel (1%) containing 0.05% Ethidium bromide was prepared with wells and once set it was mounted in the electrophoresis tank and Trisborate EDTA (TBE) electrophoresis buffer (Appendix B) added to cover the gel to a depth of about 1mm. The samples of DNA were mixed with gel-loading buffer. Slowly 25μl of the mixture was loaded into the wells using a disposable micropipette. Plasmid DNA was separated by electrophoresis at 100 volts DC for 3 h. Plasmids from standard strain *E. coli* 39R861 (NCTC 50192) and *E.coli* V517 were used as size markers. Voltage was switched off when the bromophenol blue and xylene cyanol FF had migrated the appropriate distance through the gel. Visualization of the bands was done on an UV trans-illuminator (UVP Inc., San Gabriel, Calif.) and photography of the gel was done using a transmitted illumination camera fitted with a Polaroid film.

CHAPTER THREE

3.0 RESULTS

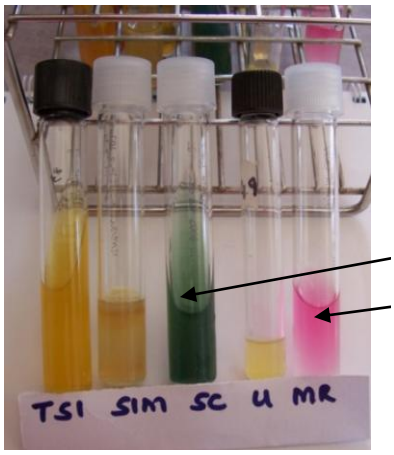
3.1 Biochemical testing

The identity of the 108 clinical isolates was confirmed by five tube biochemical method and Gram stain test. The general characteristics of the isolates were as shown below (Table 5 and Plate 1).

Table 5: Biochemical tests and Gram stain results for bacterial isolates collected from stored KEMRI isolates (2001-2006) and Aga Khan University Hospital-new (2007-2008).

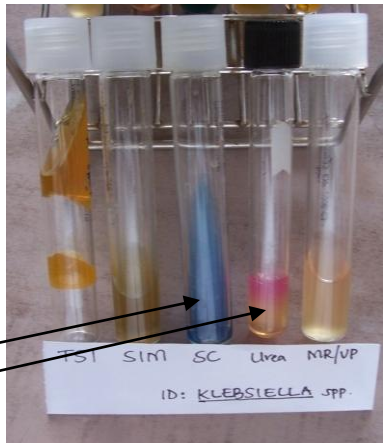
Micro-	**Gram**	**TSI**			**SIM**		**SC**		**U**	**OX**	**MR-VP**	
Organism	**Stain**	Gas	Slant	Butt	H_2S	Indole	Mot	Cit	Urease	Oxidase	MR	VP
E. coli	G-ve	+	+	+	-	+	+	+	-	-	+	-
Klebsiella spp.	G-ve	+	+	+	-	-	+	-	-	-	+/-	+/-
A. baumannii	G-ve	-	-	-	-	-	-	+	-	-	+	-
P. aeruginosa.	G-ve	-	-	-	-	-	-	+	-	+	-	-

TSI- Triple sugar iron, SIM- Sulphur Indole motility, SC- Simmons Citrate, U-Urea, MR-VP- Methyl red- Vogues Proskeur.



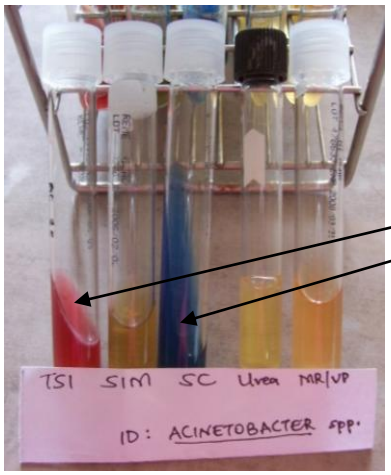
Citrate +ve
Methyl red +ve

Plate 1a: Biochemical testing results of *E. coli*.



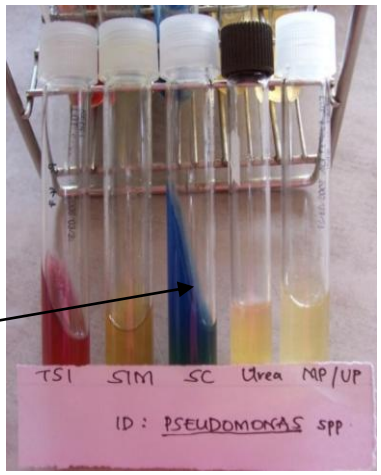
Citrate +ve
Urea –ve

Plate 1b: Biochemical testing results of *Klebsiella* spp.



TSI -ve
Citrate +ve

Plate 1c Biochemical testing results of *A. baumannii*.



Citrate +ve
Oxidase +ve

Plate 1d: Biochemical testing results of *P. aeruginosa*.

KEY: TSI- Triple sugar Iron, SIM- Sulphur Indole Motility, SC- Simmons Citrare, U- Urea, MR-VP- Methyl Red- Voges Proskauer Media.

Plate 1: Biochemical tests results for *E. coli, Klebsiella* spp., *A. baumannii* and *P. aeruginosa* isolates collected from stored KEMRI isolates (2001-2006) and Aga Khan University Hospital-new (2007-2008).

3.2 Antibiotic susceptibility testing

3.2.1 Aminoglycoside susceptibility testing

The Standard control micro-organism *E. coli* ATCC 25922 showed susceptibility to the seven aminoglycosides used in the study (Plate 2).

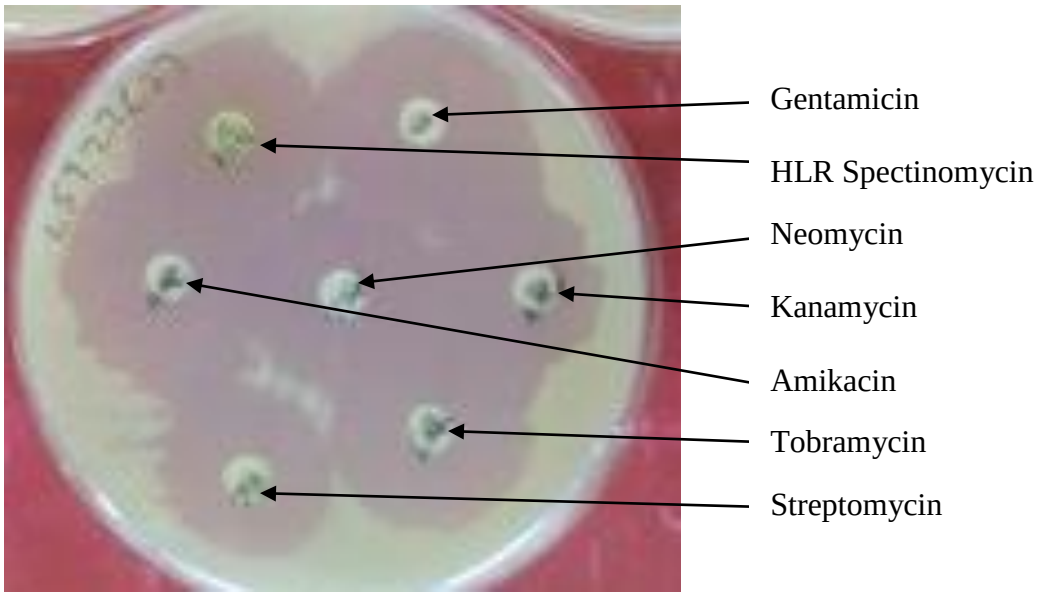


Plate 2: *E. coli* ATCC 25922 (Standard control organism) showing susceptibility to the seven aminoglycoside antibiotics used in the study.

The clinical isolates of *E. coli*, *Klebsiella* spp. and *A. baumannii* generally showed susceptibility to amikacin and HLR spectinomycin and relative resistance to the kanamycin, tobramycin, streptomycin, gentamicin and neomycin (Plate 3).

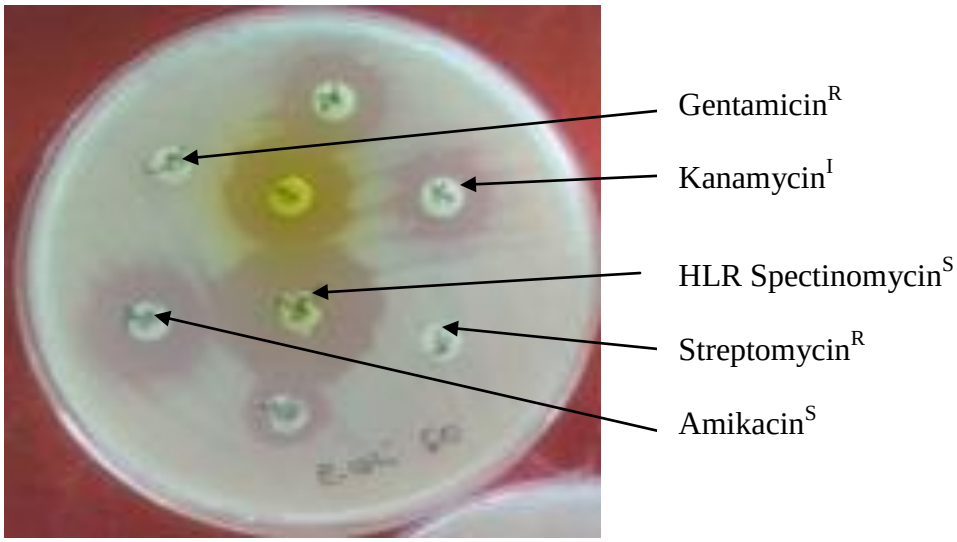


Plate 3a: *E. coli* isolate from urine sample collected from Aga Khan University Hospital in 2008 showed resistance to gentamicin, streptomycin and kanamycin, and susceptibility to amikacin and HLR spectinomycin.

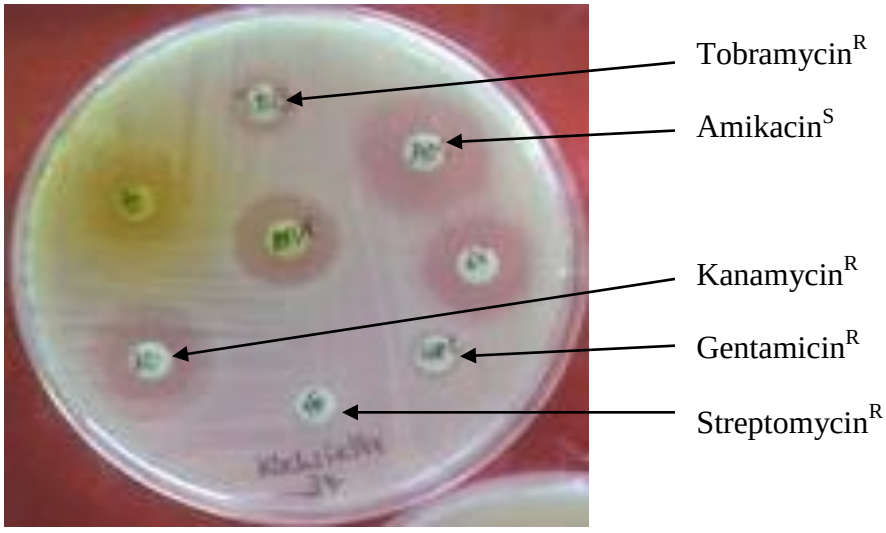


Plate 3b: *Klebsiella* spp. from sputum collected from Aga Khan University Hospital in 2008 showed resistance to gentamicin, streptomycin, kanamycin and tobramycin, and susceptibility to amikacin.

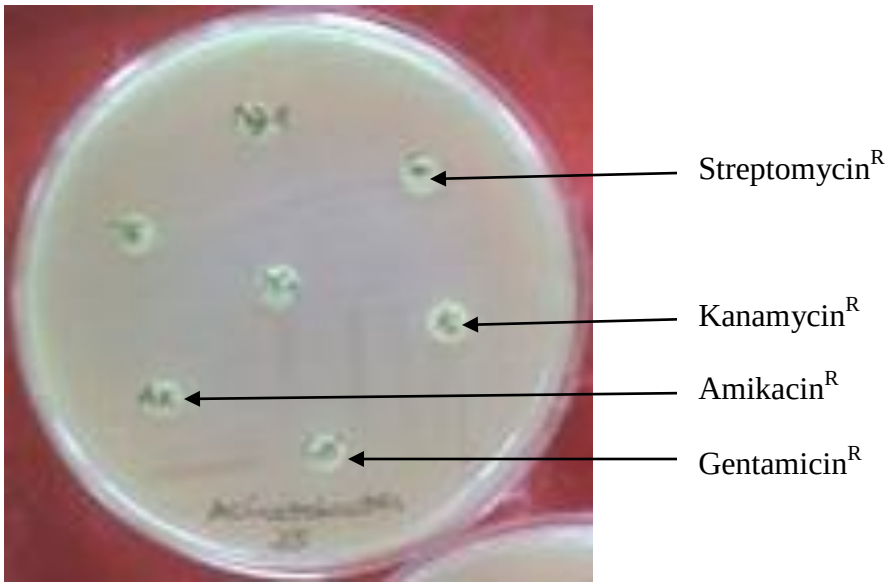


Plate 3c: MDR *A. baumannii* collected from tracheal aspirates from Aga Khan University Hospital in 2007 showing resistance to amikacin, kanamycin, gentamicin and streptomycin.

Plate 3: *E. coli, Klebsiella* spp. and *A. baumannii* isolates showing resistance to aminoglycoside antibiotics.

P. aeruginosa tested for aminoglycoside resistance were observed to include 85% Multi-drug resistant (MDR) *strains* from both Aga Khan University Hospital-new (2007-2008) and from Stored KNH-old and AKUH-old laboratory isolates (2001-2006). They were also tested against carbapenems including: meropenem (10µg), imipenem (10µg) and imepenem cilastatin (20µg); they showed resistance to these antibiotics (Plate 4).

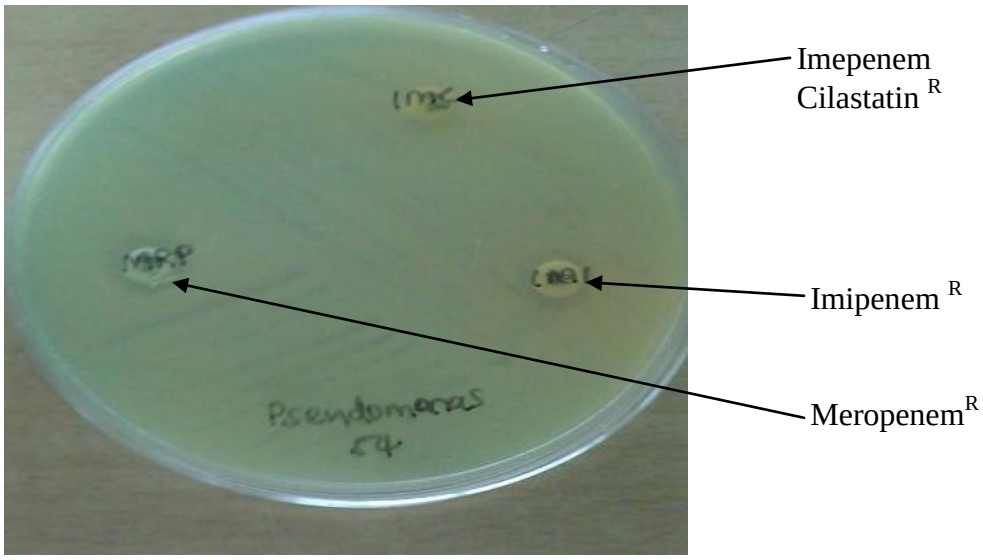


Plate 4a: MDR *P. aeruginosa* from AKUH- new isolates collected 2007 showing resistance to meropenem, imipenem and imepenem cilastatin.

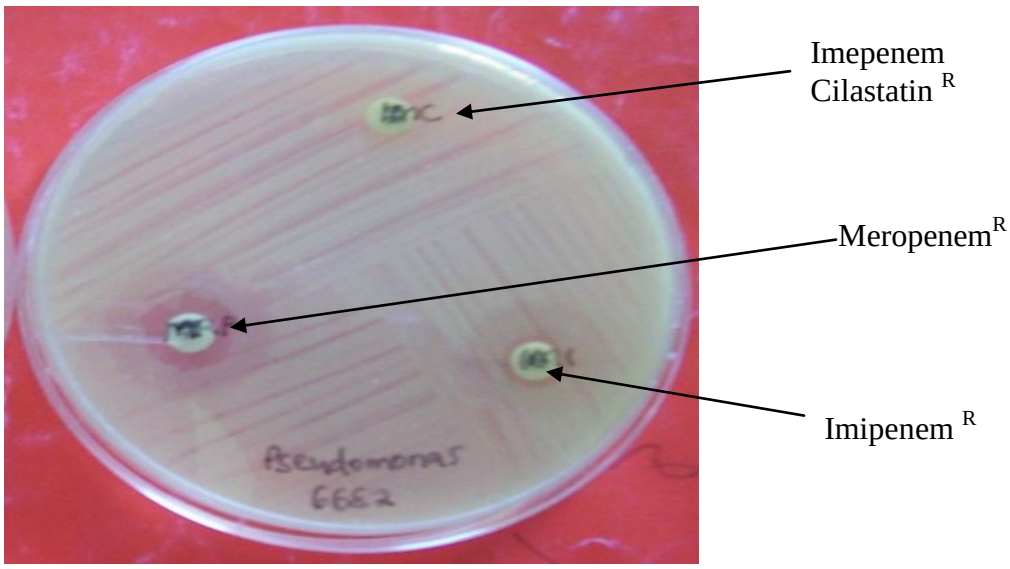


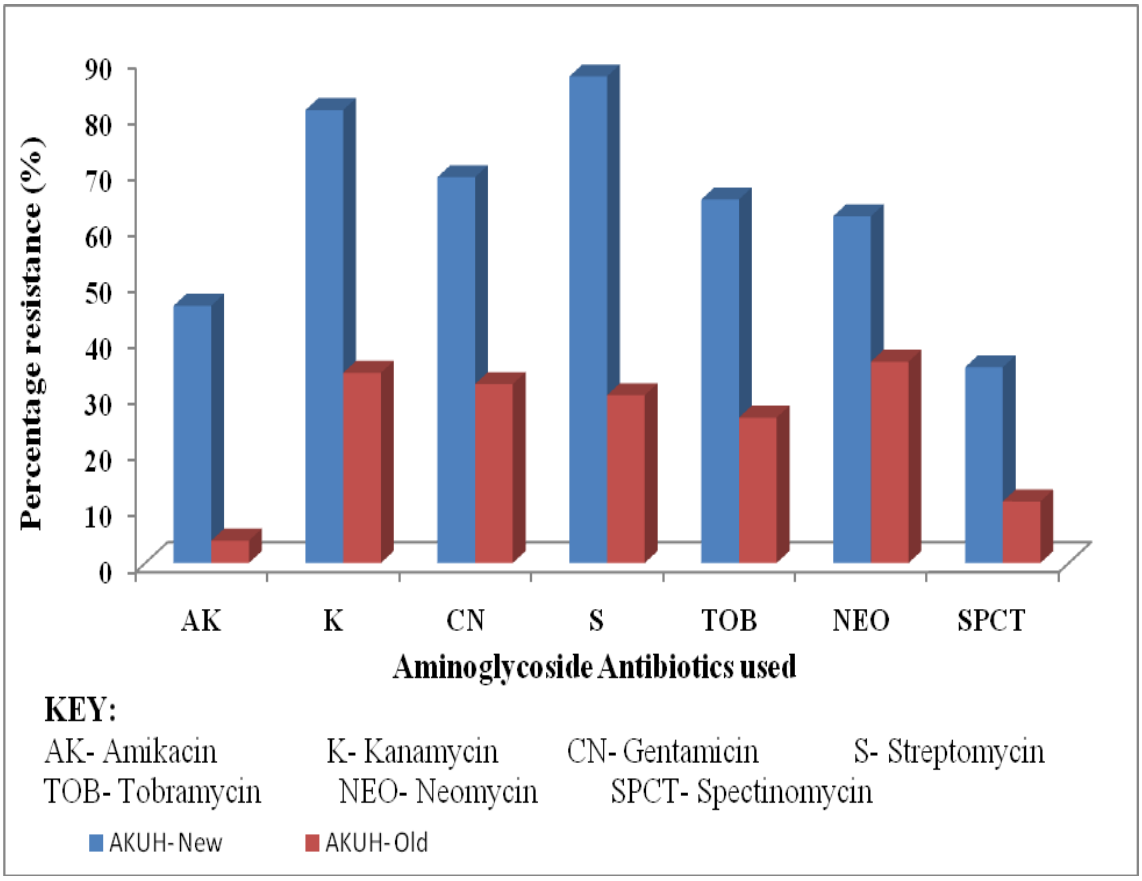
Plate 4b: MDR *P. aeruginosa* from AKUH- old isolates collected 2005 showing resistance to meropenem, imipenem and imepenem cilastatin

Plate 4: MDR *P. aeruginosa* isolates showing resistance to carbapenems.

Overall, the isolates from Aga Khan University Hospital (AKUH- New) collected in 2007-2008 showed 87%, 81%, 69%, 65%, 62%, 46% and 35% resistance to streptomycin, kanamycin, gentamicin, tobramycin, neomycin, amikacin and spectinomycin respectively (Figure 1). The AKUH- old isolates collected in 2001- 2006 showed 36%, 34%, 32%, 30%, 26%, 11% and 4% resistance to neomycin, kanamycin, gentamicin, streptomycin, tobramycin, spectinomycin and amikacin respectively.

There was an increase in resistance by the four Gram negative bacteria from Aga Khan University Hospital isolates collected over eight years (2001-2008) to the seven aminoglycoside antibiotics. Kanamycin increased by 68%, amikacin by 40%, streptomycin by 57%, gentamicin by 37%, tobramycin by 39%, neomycin by 26% and HLR spectinomycin by 24% .

Figure 1: Antibiotic resistance of isolates from AKUH- new (2007-2008) and AKUH-old laboratory (2001-2006) to seven Aminoglycoside antibiotics.

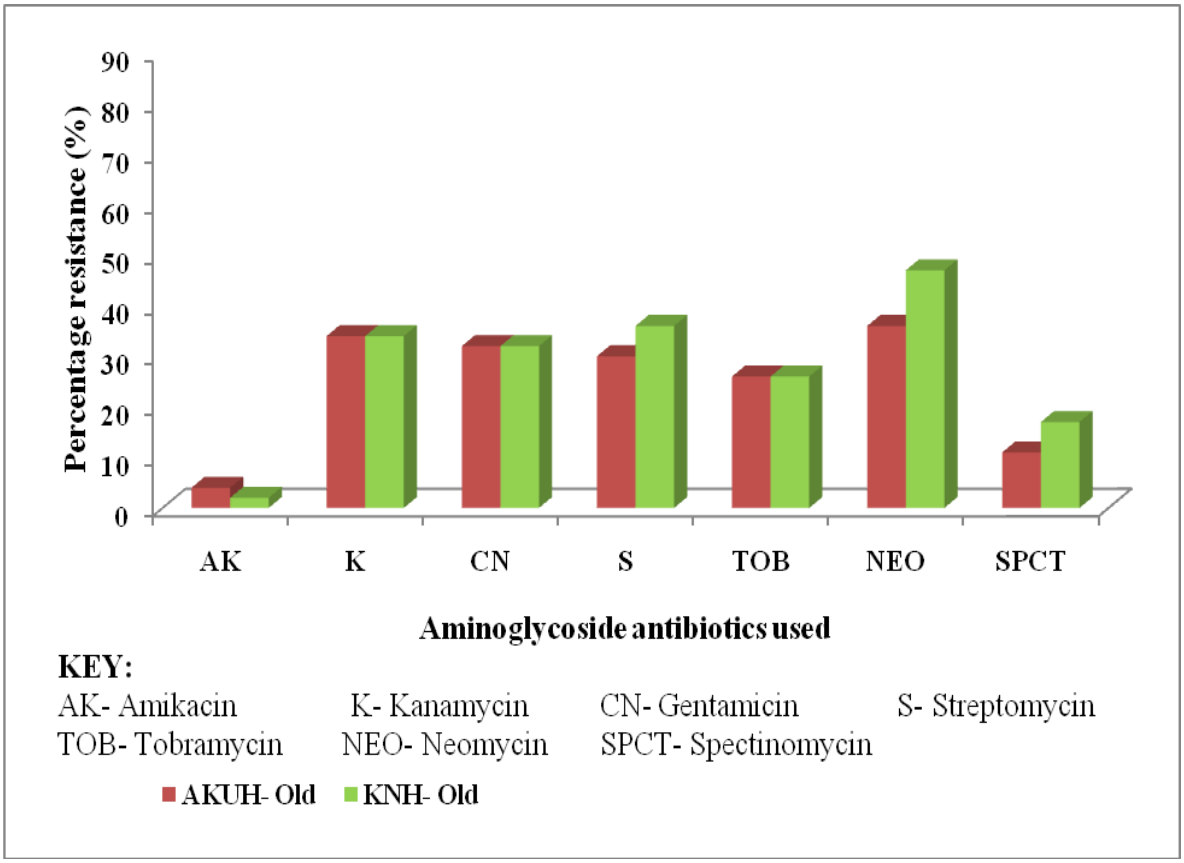


There was no significant difference ($p>0.05$) in the percentage resistance of aminoglycoside antibiotics between Aga Khan University Hospital- New isolates and Aga Khan University Hospital- Old isolates.

The KNH- old isolates showed 47%, 36%, 34%, 32%, 26%, 17% and 2% resistance to neomycin, streptomycin, kanamycin, gentamicin, tobramycin, spectinomycin and amikacin respectively (Figure 2). These isolates had similar percentage resistance for kanamycin, gentamicin and tobramycin. KNH- Old

isolates had a higher percentage of resistance to the aminoglycoside antibiotics than AKUH- Old isolates.

Figure 2: Antibiotic resistance of isolates from AKUH-old laboratory (2001-2006) and Stored KNH-old to seven Aminoglycoside antibiotics.



There was no significant difference ($p>0.05$) in the percentage resistance of aminoglycoside antibiotics between Aga Khan University Hospital- Old isolates and Kenyatta National Hospital- Old isolates.

3.2.2 Presence of Extended Spectrum Beta-Lactamases (ESBLs): ESBLs were detected by double disk diffusion method and interpreted based on resistance to Monobactam – Azetronam, Broad-spectrum Cephalosporins such as ceftazidime, cefotaxime, cefuroxime and cefriaxone and suceptibility to cefoxitin (Plate 5). A key hole or ghost zone between clavulanic acid (augumentin) and any of the broad-spectrum cephalosporins indicated the presence of ESBL production (Neo-sensitabs- Susceptibility testing 19th Edition User's Guide. 2007/2008).

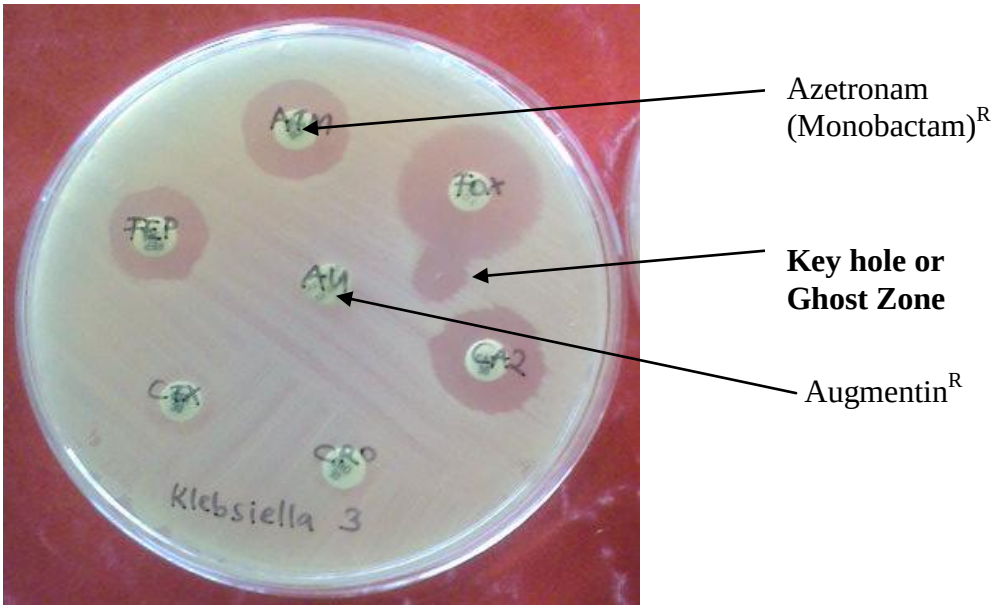
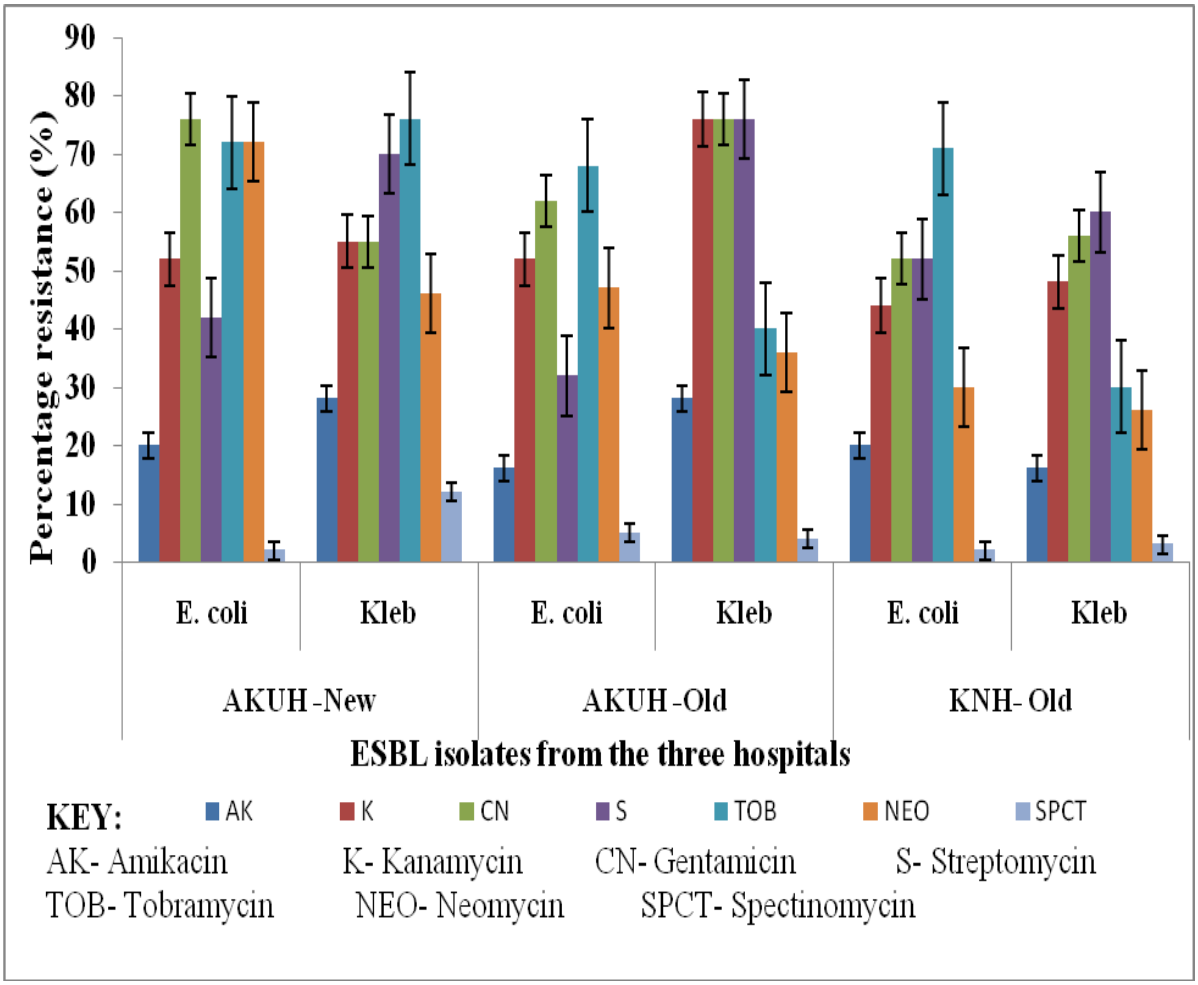


Plate 5: ESBL *Klebsiella* spp. isolated from Aga Khan University Hospital in 2007.

A total of twenty-four out of 108 (22%) of the clinical isolates tested were found to be ESBLs producers. These were only *E. coli* and *Klebsiella* spp. isolates. A total of 18 Stored KEMRI isolates collected in 2001-2006 were ESBLs producers, including ten *E. coli* and eight *Klebsiella* spp. while six AKUH New isolates were ESBLs producers, including two *E. coli* and four *Klebsiella* spp. ESBL *Klebsiella* spp. were generally more resistant to Aminoglycoside antibiotics than ESBL *E. coli* (Figure 3). AKUH- New ESBL isolates were seen to have the highest percentage resistance to the seven aminoglycosides compared to AKUH- Old and KNH-Old ESBL isolates.

A variety of Extended Spectrum Beta-Lactamases (ESBLs) families were also detected; these included Inhibitor resistant TEM beta-lactamases (IRT), CTX-M type beta-lactamases, Sulf*h*ydryl variable (SHV) and Oxacillin- hydrolyzing abilities (OXA) based on their resistance profiles to broad spectrum cephalosporins- ceftazidime, cefotaxime, cefuroxime and cefriaxone. Majority of the isolates belonged to IRT family which are resistant to clavulanic acid and sulbactam and are generally susceptible to cephalosporins such as cefotaxime and cefoxitin (Plate 6).

Figure 3: ESBL *E. coli* and *Klebsiella* spp. from the three hospitals showing percentage resistance to seven aminoglycoside antibiotics used in study.



There was no significant difference ($p>0.05$) in the percentage resistance of ESBL *E. coli* and *Klebsiella* spp. from the three hospitals showing percentage resistance to seven aminoglycoside antibiotics used in study.

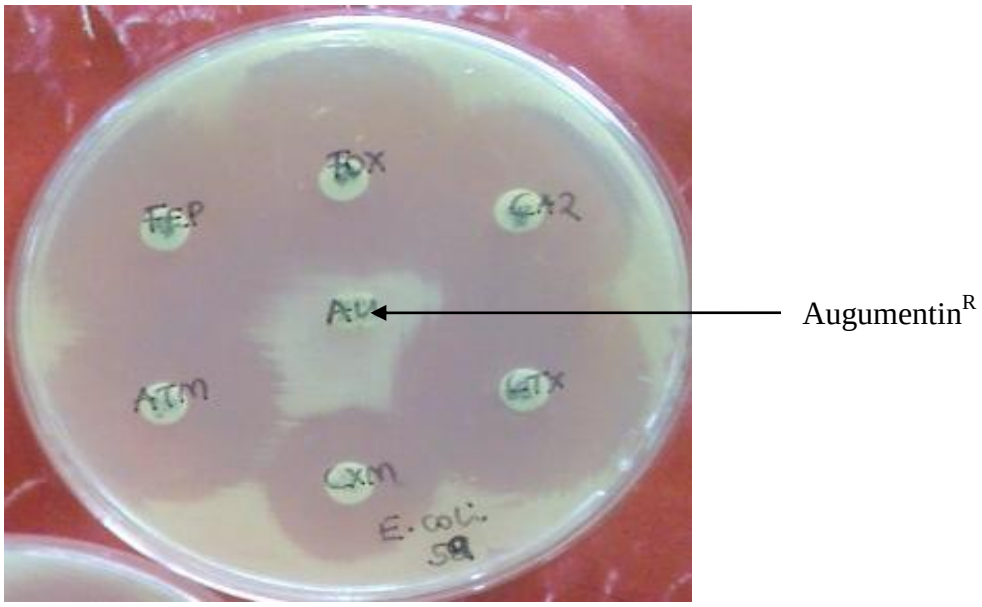


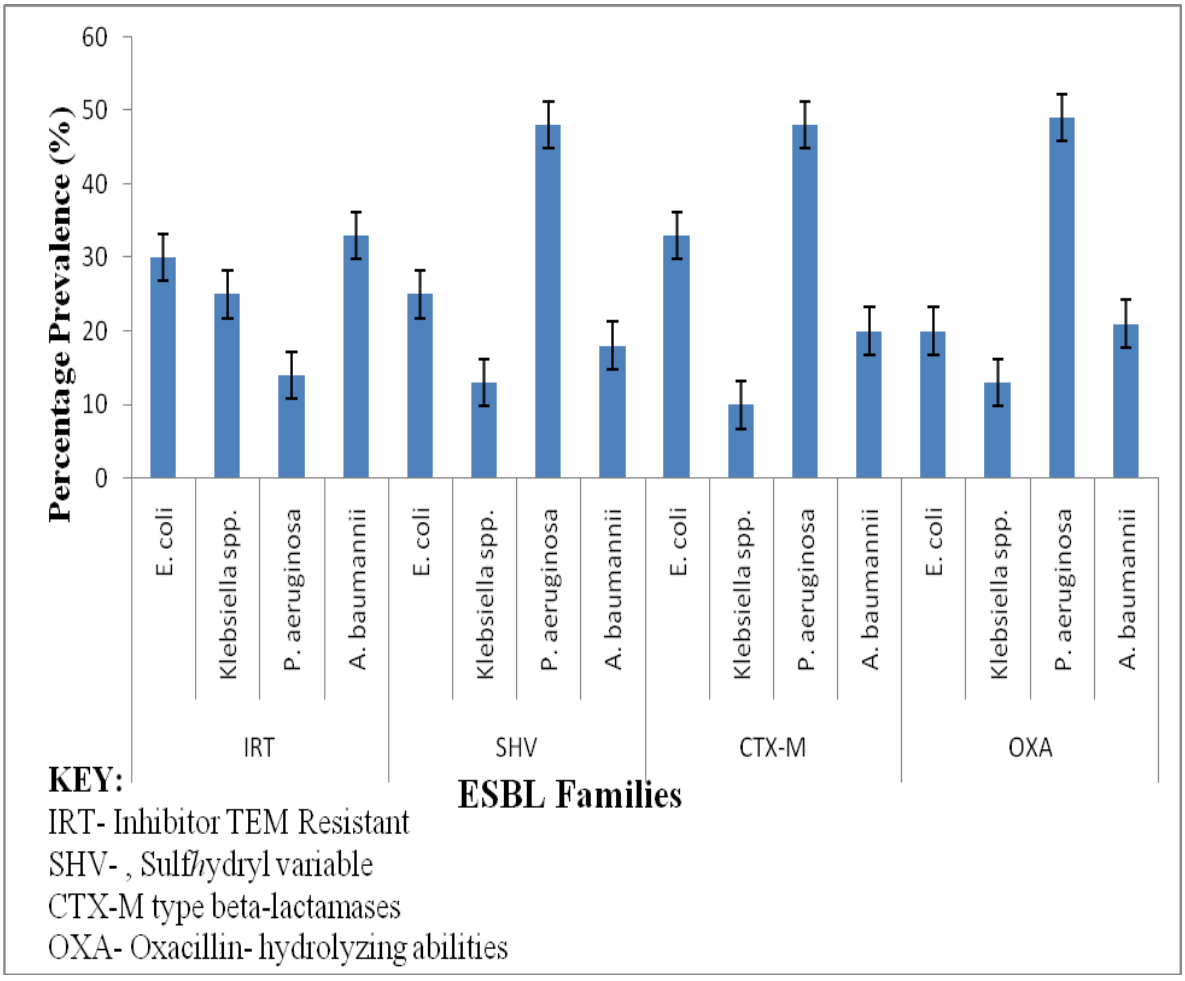
Plate 6: Inhibitor resistant TEM lactamases (IRT) type *E. coli* collected from Aga Khan University Hospital in 2007 showing resistance to Augmentin and susceptible to Cephalosporins.

The isolates from AKUH- new in 2007-2008 were found to be majorly IRT β-lactamases family followed by CTX-M, OXA and finally SHV as shown in Figure 4. MDR *P. aeruginosa* obtained were found to be in several ESBL families including SHV, CTX-M and OXA showing a high prevalence of about 46%.

The stored AKUH-old (2001-2006) and KNH-old (2003-2006) ESBL isolates were found also to be majorly IRT β-lactamases family followed by CTX-M (Figure 5). OXA and SHV had the same number of isolates. *E. coli* from CTX-M

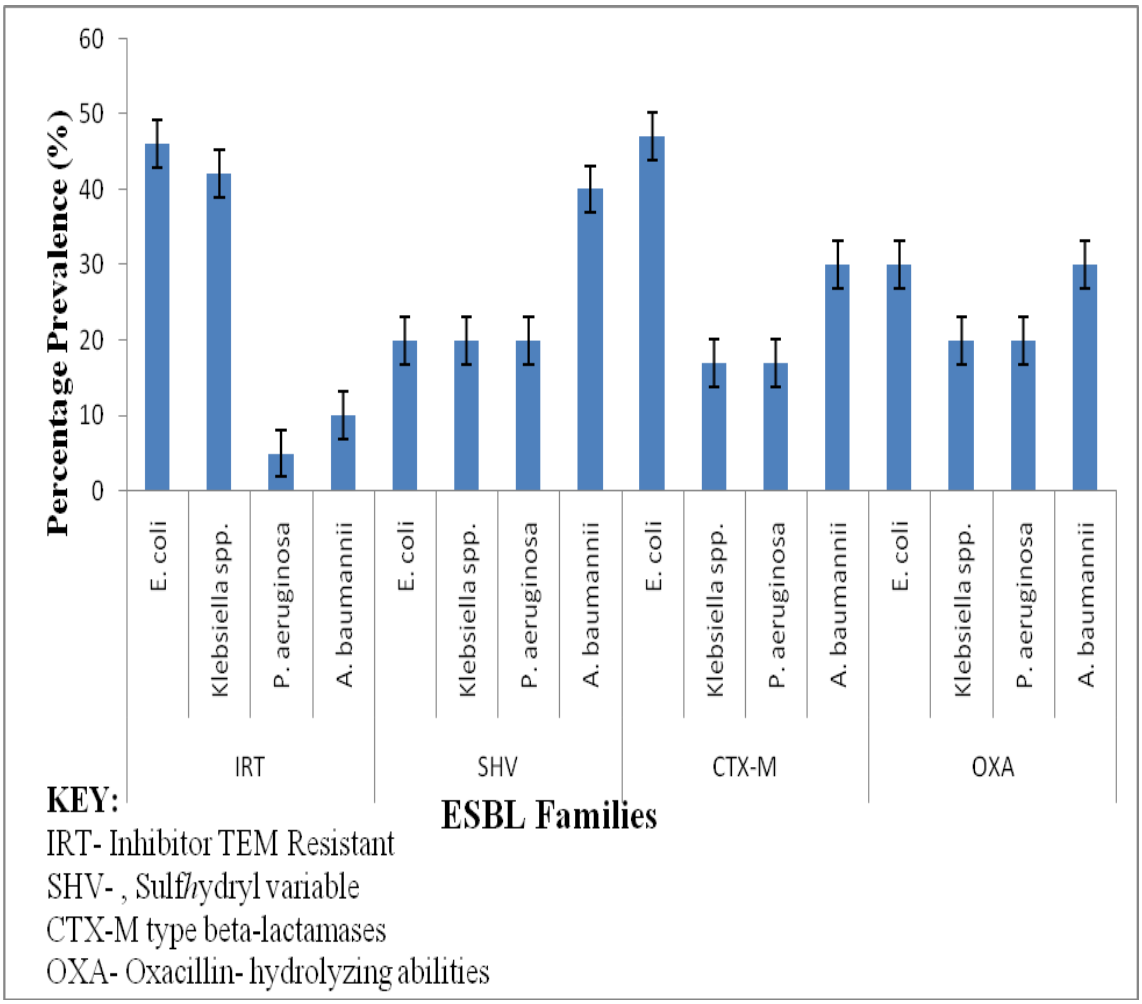
were seen to be the most widespread 46% while *P. aeruginosa* from IRT were the least widespread 5%.

Figure 4: ESBL families detected from the Aga Khan University hospital (AKUH-new) isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa* collected during 2007-2008.



There was no significant difference ($p>0.05$) in the percentage prevalence of ESBL families from Aga Khan University hospital- New isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa* collected during 2007-2008.

Figure 5: ESBL families detected from the stored AKUH-old (2001-2006) and KNH-old (2003-2006) isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa*.



There was no significant difference ($p>0.05$) in the percentage prevalence of ESBL families from the stored AKUH-old (2001-2006) and KNH-old (2003-2006) isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa*.

3.3 Detection of mobile genetic determinants

3.3.1 Detection of conjugative plasmids

Two recipients were used for conjugation experiments *E. coli* C600 (resistant to rifampicin) and *E. coli* J53 (resistant to sodium azide). A total of 25 isolates resistant to ampicillin and to at least three aminoglycoside antibiotics were selected randomly from the antibiotic profiles. These included eight *E. coli*, five *Klebsiella* spp., seven *P. aeruginosa* and five *A. baumannii*. Selection of transconjugants was done on MacConkey agar supplemented with 30µg/ml of rifampicin and 30µg/ml ampicillin for *E. coli* C600 and 0.3µg/ml of sodium azide and 30µg/ml ampicillin for *E. coli* J53. After conjugation process the *E. coli* C600 recipient had ten transconjugants (40%) comprising of three *E. coli*, four *Klebsiella* spp., one *P. aeruginosa* and two *A. baumannii*, while the *E. coli* J53 recipient had only two transconjugants (8%) comprising of one *E. coli* and one *Klebsiella* spp. both from Aga Khan University Hospital- New isolates.

Antibiotic susceptibility testing was done on the transconjugants and they were all found to be resistant to rifampicin and ampicillin but susceptible to aminoglycoside antibiotics as observed in examples in Plates 7a and 7b of *E. coli* 5875 from stored isolates of Kenyatta National Hospital in 2003.

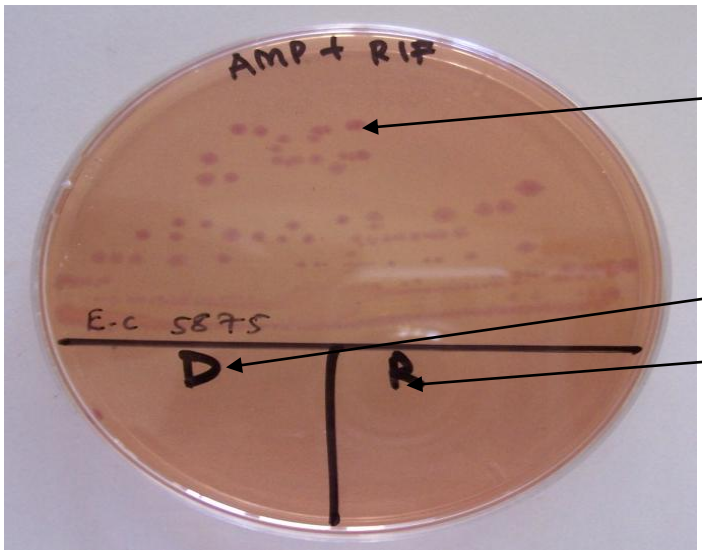


Plate 7a: Transconjugant observed (pink colonies) from *E. coli* 5875 from stored isolates of Kenyatta National Hospital-old in 2003 and *E. coli* C600 recipient.

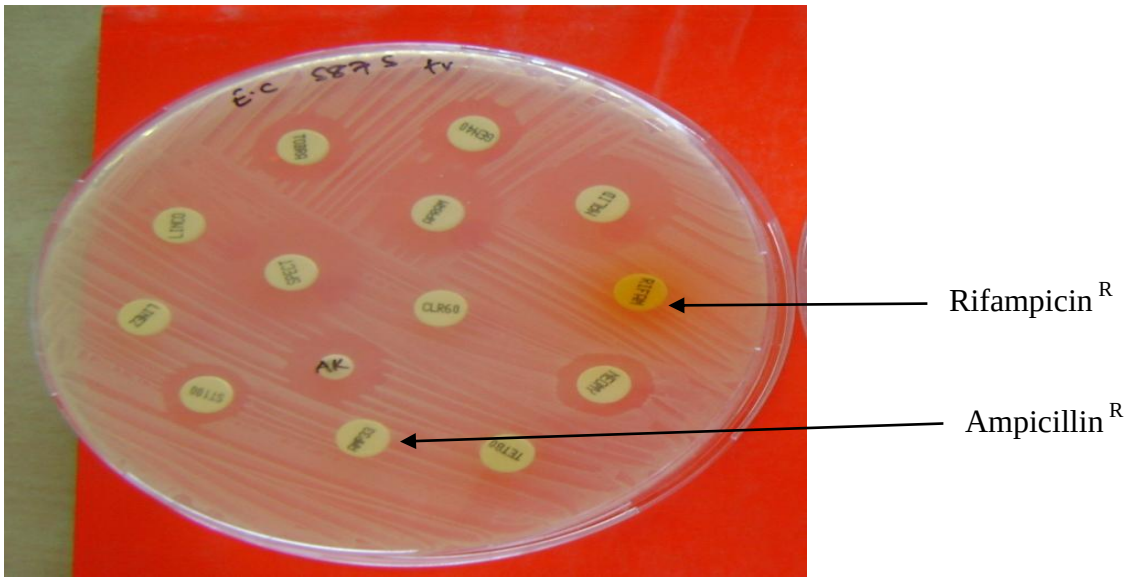


Plate 7b: Transconjugant showing resistance to Ampicillin and Rifampicin antibiotics and Susceptibility to all the seven aminoglycosides antibiotics tested.

Conjugative plasmids were extracted from transconjugants and their donors for plasmid analysis using the protocol (Sambrook *et al.,* 1989) for *E. coli* C600 transconjugants and a Qiagen Miniprep Kit was used for *E. coli* J53 transconjugants. Plasmids from standard strain *E. coli* 39R861 and *E.coli* V517 were used as size markers. Gel photograph of plasmid DNA transferred during conjugation experiments showed they were approximately 60kb (Plate 8).

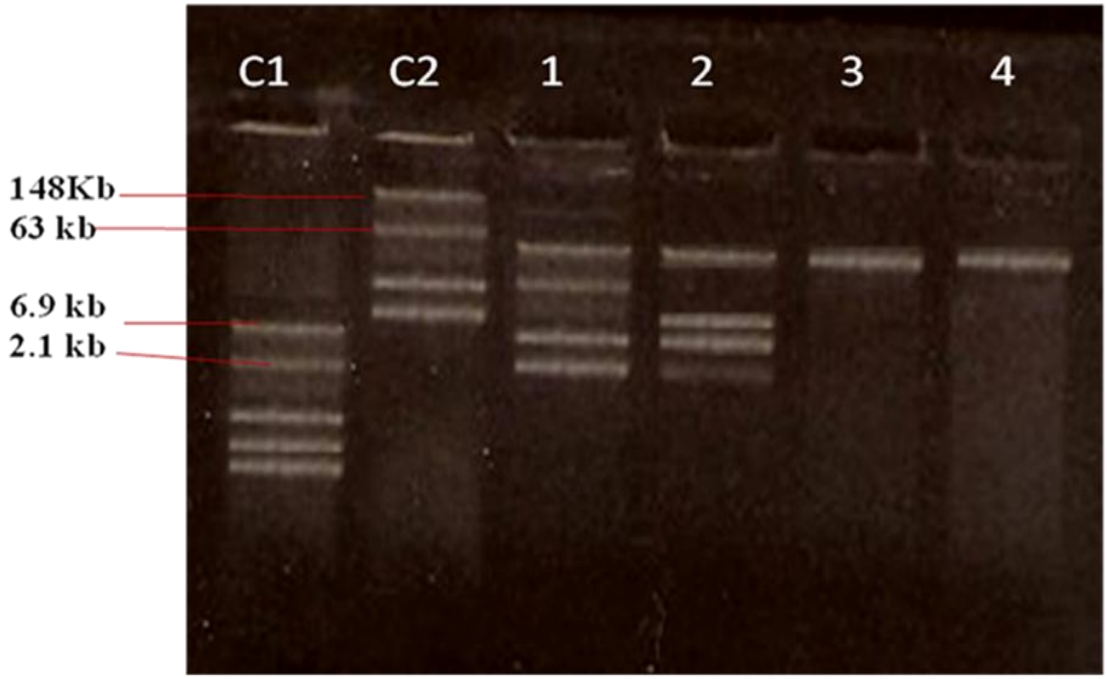


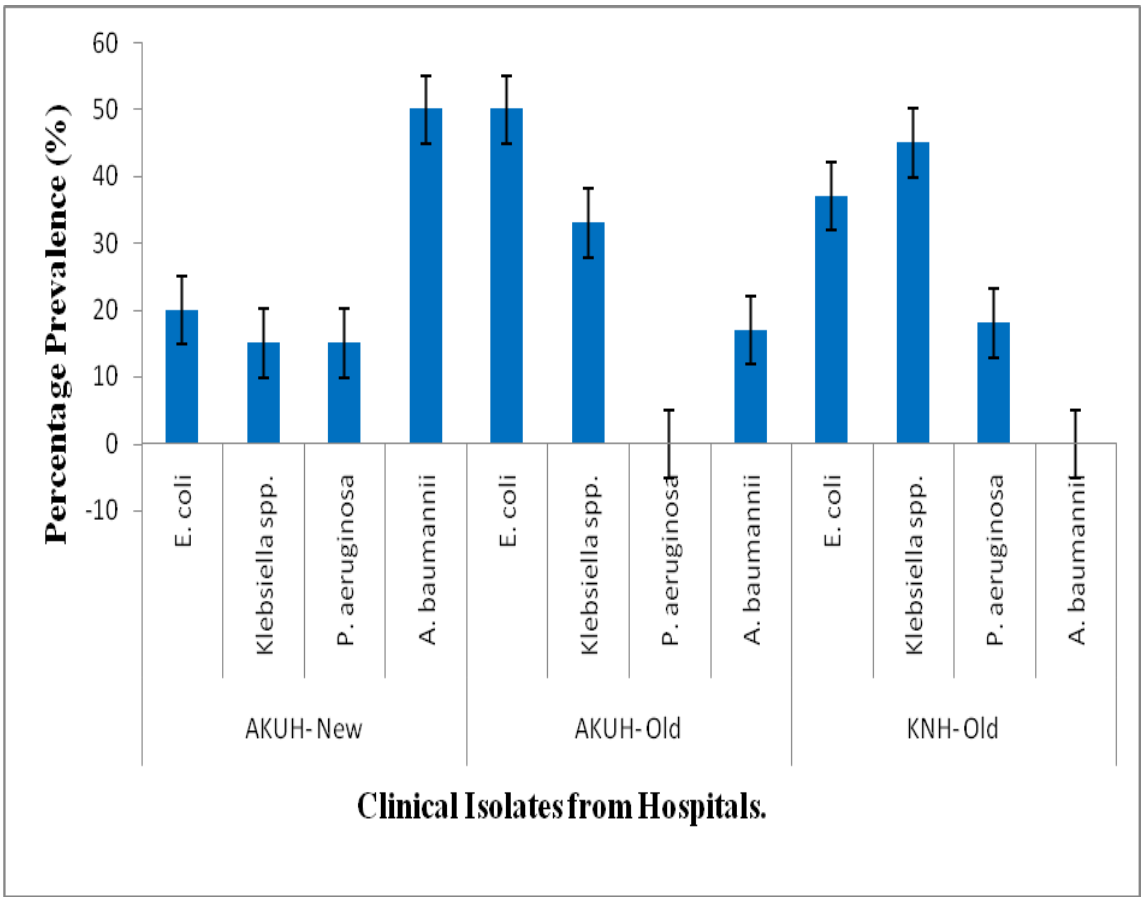
Plate 8: The Ampicillin resistant plasmid transferred during conjugation process by *E. coli* J53 was approximately 60Kb.
Lanes: C1. *E. coli* V517, C2. *E. coli* 39R861, 1. *E. coli* 61, 2. *Klebsiella* spp. 7, 3. Transconjugant *E. coli* 61, and 4. Transconjugant *Klebsiella* spp. 7.

3.3.2 Detection of Class I and II Integrons

This was done by PCR amplification of the Class I and II integrons primers. All the 108 isolates collected from stored KEMRI laboratory and AKUH-new were tested for class I integrons. A total of 37 isolates out of 108 (34%) were found to harbour the class 1 integrons. AKUH-new isolates collected in 2007-2008 had 20 Class 1 Integrons positive isolates where 50% were *P. aeruginosa* (Figure 6). These isolates showed 95% resistance to kanamycin and streptomycin antibiotics. All of these isolates belonged to IRT family of ESBLs (Table 6).

Stored KEMRI isolates had 17 Class 1 Integrons positive isolates. AKUH-old had 50% positive isolates of *E. coli* while Kenyatta National Hospital (KNH-old) had 45% *Klebsiella* spp. No Positive tests for *A. baumannii and P. aeruginosa* were detected from AKUH-old and KNH-old isolates respectively (Table 7). Isolates from AKUH-old collected in 2001-2006 showed 100% resistance to kanamycin and gentamicin. All of these isolates also belonged to IRT family of ESBLs

Figure 6: Class 1 Integrons genes detected from isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa* from the three selected hospitals.



There was no significant difference ($p>0.05$) in the percentage prevalence of Class 1 integrons genes detected from isolates of *E. coli*, *Klebsiella* spp., *A. baumannii* and *P. aeruginosa* from the three selected hospitals.

Table 6: Twenty Class I Integrons positive isolates of *E. coli, Klebsiella* spp., *A. baumannii* and *P. aeruginosa* from Aga Khan University Hospital collected in 2007-2008.

Micro-organism	ESBL Families	Aminoglycoside antibiotics profile						
		AK	K	CN	S	TOB	NEO	SPCT
Aga Khan University Hospital- New isolates (2007-2008)								
E. coli 18	IRT, CTX-M	I	R	S	I	R	S	S
E. coli 21	IRT, CTX-M	S	R	S	R	R	S	S
E. coli 23	All*	I	R	R	R	R	S	S
E. coli 61	All*	S	R	R	I	R	S	S
Klebsiella spp. 8	All*	R	R	R	R	R	S	R
Klebsiella spp. 9	IRT, CTX-M	S	S	S	R	S	S	S
Klebsiella spp. 14	IRT	I	R	R	R	R	I	S
A. baummanii 27	All*	R	R	R	R	S	R	S
A. baummanii 28	All*	R	R	R	R	S	R	S
A. baummanii 35	All*	R	R	R	S	S	R	I
P. aeruginosa 51	All*	R	R	R	R	R	R	S
P. aeruginosa 54	All*	R	R	R	R	R	R	S
P. aeruginosa 70	All*	R	R	R	R	R	S	S
P. aeruginosa 77	All*	R	R	R	R	R	R	S
P. aeruginosa 78	All*	R	R	R	R	R	R	R
P. aeruginosa 79	All*	R	R	R	R	R	R	S
P. aeruginosa 80	All*	R	R	R	R	R	R	R
P. aeruginosa 81	All*	R	R	R	R	R	R	R
P. aeruginosa 85	All*	R	R	R	R	R	R	R
P. aeruginosa 88	All*	R	R	R	R	R	R	S

- All* represents four ESBL families namely; IRT, CTX-M, SHV and OXA.
- IRT- Inhibitor resistant TEM lactamases**,** CTX-M type beta-lactamases, SHV- Sulf*h*ydryl variable and OXA-Oxacillin- hydrolyzing abilities.
- S= Susceptible, R= Resistant, AK- Amikacin, K- Kanamycin, CN- Gentamicin, S- Streptomycin, TOB- Tobramycin, NEO- Neomycin, SPCT- Spectinomycin,

Table 7: Seventeen Class I Integrons positive isolates of *E. coli, Klebsiella* spp., *A. baumannii* and *P. aeruginosa* obtained from stored KEMRI isolates collected in 2001-2006.

Micro-organism	ESBL Families	Aminoglycoside antibiotics profile						
		AK	K	CN	S	TOB	NEO	SPCT
Stored KEMRI isolates (2001-2006)								
Aga Khan University Hospital- Old isolates (2001-2006)								
E. coli 6680	IRT, CTX-M	S	I	R	S	S	S	S
E. coli 6686	IRT	S	R	R	R	R	R	S
E. coli 6301	IRT, CTX-M	S	R	R	S	S	R	R
Kleb spp. 1662	IRT, CTX-M	S	R	R	R	S	R	S
Kleb spp. 1671	IRT, CTX-M	S	R	R	R	R	R	S
P. aeruginosa 6682	All*	R	R	R	R	S	R	R
Kenyatta National Hospital (KNH)- Old isolates (2003-2006)								
E. coli 5875	All*	S	R	R	R	R	R	S
E. coli 6687	IRT, CTX-M	S	R	R	R	R	R	S
E. coli 6703	IRT, CTX-M	S	R	R	R	R	R	S
E. coli 6763	IRT	S	S	S	S	S	R	R
Kleb spp. 5313	IRT	S	S	S	S	S	R	S
Kleb spp. 5419	IRT, CTX-M	S	R	R	R	R	R	S
Kleb spp. 5422	IRT, CTX-M	S	S	S	S	R	S	S
Kleb spp. 5423	IRT	S	R	S	R	S	R	S
Kleb spp. 5424	IRT	S	S	S	S	S	R	S
A. baummanii 6364	All*	S	R	R	R	S	R	S
A. baummanii 6365	All*	S	R	S	R	R	R	R

- All* represents four ESBL families namely; IRT, CTX-M, SHV and OXA.
- IRT- Inhibitor resistant TEM lactamases**,** CTX-M type beta-lactamases, SHV- Sulf*h*ydryl variable and OXA-Oxacillin- hydrolyzing abilities.
- S= Susceptible, R= Resistant, AK- Amikacin, K- Kanamycin, CN- Gentamicin, S- Streptomycin, TOB- Tobramycin, NEO- Neomycin, SPCT- Spectinomycin.

The PCR products of class 1 integrons were observed by gel electrophoresis under UV illumination and seen to weigh approximately 953kb against the Bioline hyper ladder 1 marker (Plate 9).

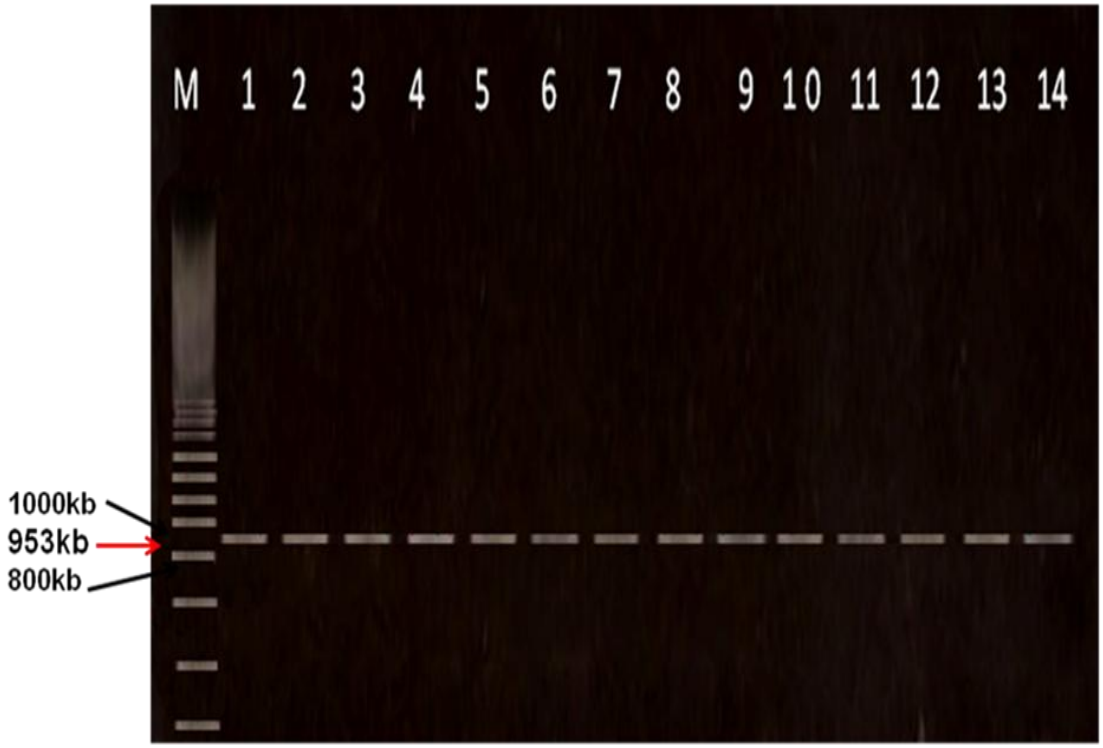


Plate 9: Class 1 integrons positive isolates. Class 1 integrons was observed as a 953 Kb band.
Lanes: M- DNA size marker-bioline hyperladder I. 1. *E. coli* 18, 2. *E. coli* 21, 3. *E. coli* 61, 5. *E. coli* 5875, 5. *E. coli* 6680, 6. *Klebsiella* spp. 8, 7. *Klebsiella* spp. 9, 8. *Klebsiella* spp. 14, 9. *Klebsiella* spp. 5423, 10. *Klebsiella* spp. 1671, 11. *A. baumannii* 35, 12. *A. baumannii* 6364, 13. *P. aeruginosa* 51, and 14. *P. aeruginosa* 77.

3.4 Detection of Aminoglycoside Modifying Enzymes (AMEs)

3.4.1 Phenotypic Characterisation of Aminoglycoside Resistance Mechanisms

According to Livermore *et al.*, 2001, it is possible to determine the presence of AMEs by testing the susceptibility of isolates against a range of clinically available aminoglycosides as a pattern of resistance emerges which is unique to a specific enzyme. All the 108 clinical isolates used in the study. Based on this phenotypic interpretation *P. aeruginosa* had the most AME genes and seen to be conferring 81% resistance by impermeability (Table 8). *E. coli* was seen to posses 44% of *AAC(3)-IV* gene and only 2% of *AAC(6')* gene. *Klebsiella* spp. was also observed to posses 37% of *APH(3')* gene.

3.4.2 Genotypic Characterisation of Aminoglycoside Resistance Mechanisms

A total of Six Aminoglycoside Modifying Enzymes (AMEs) were detected based on their resistance to selected Aminoglycosides determining the number of isolates per primer. The results show that majority of the MDR *P. aeruginosa* contain 83% of the Aminoglycoside Modifying Enzymes genes tested while *A. baumannii* isolates contained the only 16% AME genes. Some of the isolates were found to contain more than one AME gene and are of great interest (Table 9).

Table 8: Phenotypic Characterisation of Aminoglycoside resistance profiles of *E. coli*, *Klebsiella* spp. and *P. aeruginosa*.

Test M.O	GEN	TOB	AMK	KAN	NEO	Gene present	Frequency
E. coli	S	S	S	S	S	Classical*	-
	R	S	S	S	S	*AAC(3)-I*	5%
	R	R	S	R	S	*AAC(3)-II*	5%
	R	R	S	r	R	*AAC(3)-IV*	44%
	S/r	R	R	R	R	*AAC(6')*	2%
	R	R	S	R	R	*ANT(2')*	5%
	S	S	R	R	S	*APH (3')*	-
Klebsiella spp.	S	S	S	S	S	Classical	-
	R	S	S	S	S	*AAC(3)-I*	3%
	R	R	S	r	S	*AAC(3)-II*	3%
	S/r	R	R	R	R	*AAC(6')*	6%
	R	S	S	R	S	*ANT(2')*	-
	S	S	S	R	R	*APH(3')*	37%
P. aeruginosa	S	S	S	S	S	Classical	-
	R	S	S	R	R	*AAC(3)-I*	-
	R	R	S	R	R	*AAC(3)-III*	81%
	S/r	R	R	R	R	*AAC(6')*	86%
	R	R	S	R	R	*AAC(6')-II*	81%
	R	R	S	R	R	*ANT(2')*	81%
	S	S	S	R	R	*APH(3')*	10%
	R	R	R	R	R	Impermeability	81%

*Classical= Historic phenotype of the species, without acquired resistance

S= Susceptible, R= Resistant, r= reduced zones but likely to remain susceptible at standard breakpoints (Livermore *et al.*, 2001)

AK-Amikacin, KAN-Kanamycin, GEN-Gentamicin, TOB-Tobramycin and NEO-Neomycin.

Table 9: Presence of Six Aminoglycoside Modifying Enzymes in *E. coli, Klebsiella* spp., *A. baumannii* and *P. aeruginosa* isolates.

AME genes	Amg resistance Conferred	No. tested	Positive M.O for AME genes				PCR product
			E.coli	*Kleb*.spp	*Pseudo*	*A. bau*	
AAC(6')-I	Tob, Amk, Kan	**27**	-	2	5	-	**1,100bp**
AAC(6')-II	Tob, Gen, Kan	**27**	1	-	5	-	**1,507bp**
AAC(6')-1b-cr	Tob, Gen, Kan+ NA, Cip, Nor	**37**	5	8	7	2	**509bp**
AAC(3)-I	Gen, Tob	**55**	2	2	5	-	**227bp**
AAC(3)-IIa	Gen, Tob	**55**	6	5	6	-	**300bp**
ANT(4')-IIb	Tob, Kan	**27**	-	-	-	-	-

KEY: M.O- Micro-organisms, KAN-Kanamycin, AMK- Amikacin, GEN-Gentamicin, TOB-Tobramycin, NA- Nalidixic Acid, CIP-Ciprofloxacin and NOR-Norfloxacin.

PCR products for *AAC(6')-1b-cr* and *AAC(3)-IIa* genes showed they amplified at 509bp and 300bp (Plate 10 and 11) respectively. *AAC(6')-1b-cr* confers resistance to Aminoglycosides; kanamycin, tobramycin and amikacin, and Quinoloes; nalidixic acid, ciprofloxacin and norfloxacin. *AAC(3)-IIa* confers resistance to gentamicin and tobramycin. Isolates with these resistance profiles were selected from both stored KEMRI isolates collected in 2001-2006 and AKUH- new collected in 2007-2008. *AAC(6')-1b-cr* gene had the largest number of micro-organisms which tested positive (60%).

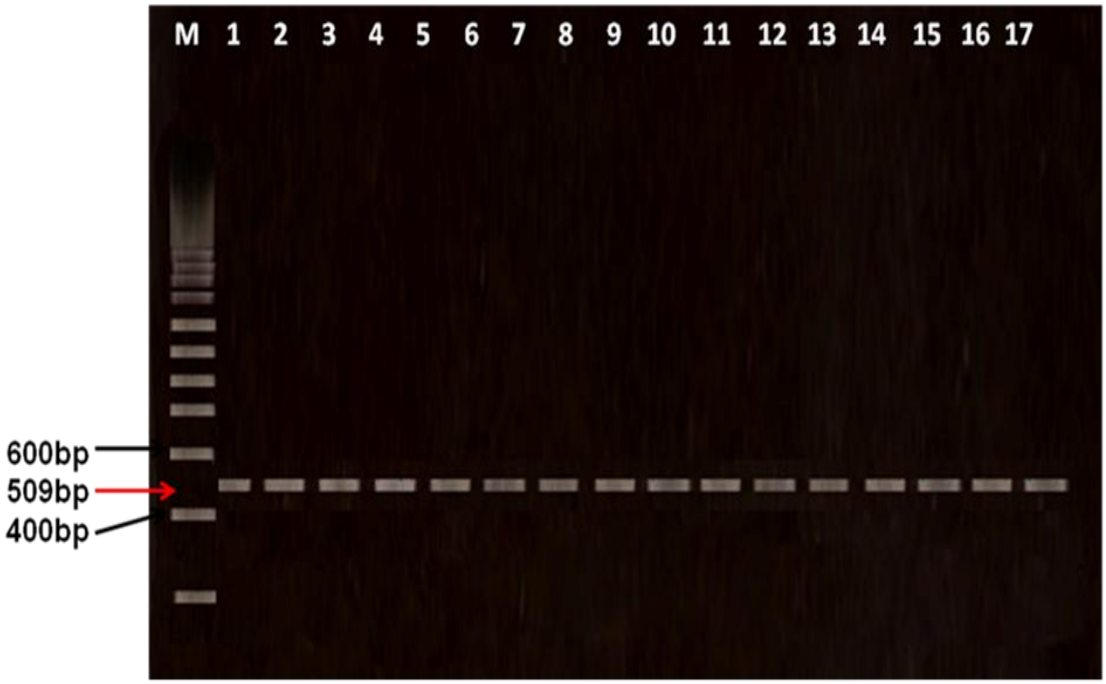


Plate10: *AAC(6)-1b-cr* gene PCR products. The resistance gene was amplified at 509 Kb.

Lanes: M- DNA size marker-bioline hyperladder I. 1. *E. coli* 18, 2. *E. coli* 21, 3. *E. coli* 61, 4. *E. coli* 5875, 5. *E. coli* 6303, 6. *E. coli* 6701, 7. *Klebsiella* spp. 2, 8. *Klebsiella* spp. 8, 9. *Klebsiella* spp. 34, 10. *Klebsiella* spp. 6681, 11. *Klebsiella* spp. 6308, 12. *P. aeruginosa* 43, 13. *P. aeruginosa* 63, 14. *P. aeruginosa* 81, 15. *P. aeruginosa* 6682, 16. *P. aeruginosa* 5876, and 17. *A. baumannii* 5801.

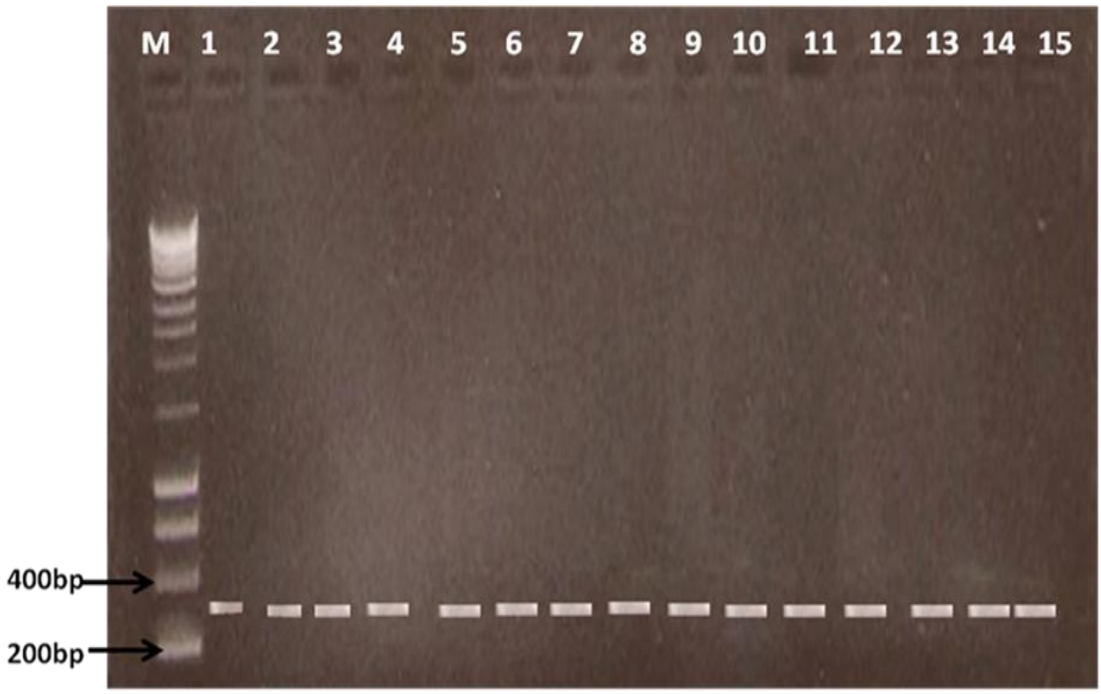


Plate11: *AAC(3)-1Ia* gene PCR products. The resistance gene was amplified at approximately 300bp.

Lanes: M- DNA size marker-bioline hyperladder I. 1. *E. coli* 18, 2. *E. coli* 21, 3. *E. coli* 6680, 4. *E. coli* 6698, 5. *E. coli* 6699, 6. *Klebsiella* spp. 8, 7. *Klebsiella* spp. 9, 8. *Klebsiella* spp. 1667, 9. *Klebsiella* spp. 1671, 10. *Klebsiella* spp. 5422, 11. *P. aeruginosa* 43, 12. *P. aeruginosa* 51, 13. *P. aeruginosa* 64, 14. *P. aeruginosa* 65, and 15. *P. aeruginosa* 5876.

CHAPTER FOUR

4.0 DISCUSSION

The present study involved a collection of One hundred and eight (108) Gram negative clinical isolates of *E. coli*, *Klebsiella* spp., *Acinetobacter baumannii* and *Pseudomonas aeruginosa* which were tested for resistance to seven Aminoglycoside antibiotics. The four bacterial species were obtained from various clinical specimens including; Urine, Blood, Sputum, Pus swabs, tracheal aspirates and other specimens (includes isolates from cerebral spinal fluid (CSF), High Vaginal Swabs (HVS), nose swabs and catheters). Results from this study show a remarkable increase in resistance by older naturally derived aminoglycoside antibiotics like streptomycin, kanamycin, and gentamicin. Overall the isolates collected from AKUH-New in 2007-2008 showed a higher level of resistance to aminoglycosides than AKUH- old isolates collected in 2001-2006. The study also revealed the presence of mobile genetic determinants of antibiotics, including class 1 Integrons and conjugative plasmids responsible for Aminoglycoside resistance.

Antimicrobial resistance among enteric Gram negative bacteria is fast becoming a global public health concern with rapid increase in multidrug resistant organisms. Resistance to Aminoglycoside group of antibiotics is an important clinical problem given that these antibiotics are widely used at present in the treatment of severe nosocomial infections in Kenyan Hospitals. In Kenya,

aminoglycosides most widely used clinically are gentamicin, streptomycin and kanamycin which showed the greatest percentages of resistance. AKUH- New isolates showed the highest percentages of resistance with 87%, 81% and 69% resistance to streptomycin, kanamycin and gentamicin respectively. AKUH- old isolates showed 30%, 34% and 32% resistance to streptomycin, kanamycin and gentamicin and finally KNH-old isolates showed 36%, 34% and 32% resistance to streptomycin, kanamycin and gentamicin.

Miró *et al.,* (2008) in Spain during a period of 3 months 803 *Enterobacteriaceae* isolates were tested against aminoglycosides and the isolates were most resistant to streptomycin 42.6%, followed by kanamycin 12.8% and gentamicin 8.4%. These high levels of resistance to streptomycin, gentamicin and kanamycin may be attributed to their prolonged and continuous use. These older compounds have been in use since 1944 to date and consequently micro-organisms have developed more potent resistance mechanisms due to increasing resistance to older agents. There was need to develop newer semi-synthetic compounds of aminoglycosides in 1970s like amikacin, debikacin, isepamicin and netilmicin to counter resistance by the older naturally derived compounds. Since then the pace of development of new aminoglycosides has markedly slowed down hence their usage in the past four decades has resulted in even higher levels of resistance to both the older and newer drugs.

Amikacin a semi-synthetic derivative of kanamycin in the present study, AKUH-New isolates showed 46% resistance to amikacin, AKUH- old isolates showed 4%, and KNH- old isolates showed 2% resistance. Miró *et al.*, (2008) in Spain showed 0.2% resistance to amikacin. Amikacin resists attacks by most bacterial inactivating enzymes, this is accomplished by the L-hydroxyaminobuteroyl amide (L-HABA) moiety attached to N-3 which inhibits acetylation, phosphorylation and adenylation in the distant amino sugar ring (Vakulenko and Mobashery, 2003). In addition, studies show that more than 80% of gentamicin resistant members of *Enterobacteriaceae* are sensitive to amikacin (Kotra *et al.;* 2000). Similar studies in Europe showed that amikacin exhibited activity against Gram-negative bacilli superior to those of gentamicin and tobramycin (Schmitz *et al.*, 1999). This is the case in the present study possibly due to the fact that Kenyan Hospitals use gentamicin as a first-line antibiotic and amikacin as second line antibiotic (Hart and Kariuki, 1998). This was also evident in analysis of Aga Khan University Hospital isolates collected over 8 years (2001-2008). Results showed an increase in resistance of seven Aminglycoside antibiotics tested to the four Gram negative bacterial species - kanamycin showed 68% increase in resistance, amikacin showed 40% increase, streptomycin 57%, and gentamicin 37%. To prevent the development of bacterial resistance to this very powerful antibiotic, its use should be effectively regulated.

Resistance to High Level Spectinomycin was also remarkably lower than for other aminoglycosides. AKUH- new isolates showed 35% resistance to Spectinomycin, AKUH- old isolates showed 11%, and KNH- old isolates showed 17% resistance. This is probably due to the high drug concentration normally used in clinical management of infections. The killing potential of aminoglycosides is concentration dependent and increases with increasing concentrations of the antibiotic. The bactericidal activity of aminoglycosides depends more on their concentration than on the duration of bacterial exposure to inhibitory concentrations of antibiotic and is also significantly less dependent on the bacterial inoculum size (Vakulenko and Mobashery, 2003).

Multi-drug resistant (MDR) *P. aeruginosa* were observed from KEMRI stored isolates and AKUH-new isolates. However, the AKUH-new *P. aeruginosa* were seen to be susceptible to Neomycin and HLR Spectinomycin while the stored KEMRI isolates were susceptible to only HLR Spectinomycin. These findings are of great concern. Carbapenems are a class of beta-lactam antibiotics naturally derived from *Streptomyces cattleya* and have a broad spectrum of antibacterial activity. They are usually the last line of antibiotic treatment drugs and include: Meropenem (10µg), Imipenem (10µg) and Imepenem Cilastatin (20µg). The MDR *P. aeruginosa* were resistant to all these antibiotics. MDR *P. aeruginosa* obtained from AKUH- New isolates in 2007-2008 when phenotypically characterized for ESBLs were found to belong to several ESBL families

including SHV, CTX-M and OXA showing a high prevalence of about 46%. These observations may be attributed to the fact that *P. aeruginosa* is intrinsically resistant to many structurally unrelated antimicrobial agents (Mesaros *et al.,* 2007) because of the low permeability of its outer membrane (1/100 of the permeability of *E. coli* outer membrane) and the constitutive expression of various efflux pumps with wide substrate specificity (Livermore, 1984 and Livermore, 2001).

The emergence of ESBLs among *Enterobacteriaceae,* mainly *K. pneumoniae* and *E. coli,* has become a growing world-wide problem (Markovska *et al.,* 2008). ESBL detection is not commonly carried out in many microbiology units in developing countries including Kenya. This could be attributed to lack of awareness and/or lack of resources and facilities to conduct ESBL identification. However, more recently, some private hospitals like Aga Khan University Hospital, Gertrude's Children Hospital, Mater Hospital, and Nairobi Hospital have started detection of ESBLs in Gram negative bacteria establishing a platform for further research in evaluation of ESBLs in Kenya.

The high rate of resistance noted among the isolates in the present study, although few in numbers, is of serious concern. Twenty four out of the 108 (22%) of the *Enterobacteriaceae* were ESBL producing *E. coli* and *Klebsiella* spp. ESBL *Klebsiella* spp. were seen to be generally more widespread and

resistant to Aminoglycoside antibiotics than ESBL *E. coli.* In a study carried out in Tanzania (2009) species specific ESBLs rate among *K. pneumonia* and *E. coli* were 63.7% and 24.4% respectively (Mshana *et. al.,* 2009). More than 75% of the studies carried out address ESBL-producing infections with *K. pneumoniae*. The predilection of ESBLs for *K. pneumoniae* has never been clearly explained (Paterson and Bonomo, 2005, Mshana *et al.,* 2009). ESBL production is usually associated with resistance to other classes of antimicrobial agents, such as aminoglycosides, fluoroquinolones and trimethoprim (El Kholy *et al.,* 2003, Ndugulile *et al.,* 2005). ESBL *Klebsiella* spp. were generally more resistant to Aminoglycoside antibiotics than ESBL *E. coli* and AKUH- New hospital ESBL isolates were seen to have the highest percentage resistance to the seven aminoglycosides compared to AKUH- Old and KNH-Old ESBL isolates.

Four ESBL families were detected phenotypically based on their resistance profiles to broad-spectrum cephalosporins. About 45% of the isolates belonged to IRT family which are resistant to clavulanic acid and sulbactam and are generally susceptible to cephalosporins such as cefotaxime and cefoxitin. IRTs are variants of the TEM-1 or TEM-2 β-lactamases. These enzymes were at first given the designation IRT for inhibitor-resistant TEM β-lactamase; however, all have subsequently been renamed with numerical TEM designations

There are at least 19 distinct IRT beta-lactamases (Paterson and Bonomo, 2005). Point mutations that lead to the inhibitor-resistant phenotype occur at a few

specific amino acid residues within the structural gene for the TEM enzyme, Met-69, Arg-244, Arg-275, and Asn-276. The sites of these amino acid substitutions are distinct from those that lead to the ESBL phenotype. IRT β-lactamases have been found mainly in clinical isolates of *E. coli, K. pneumoniae, Klebsiella oxytoca, P. mirabilis, and Citrobacter freundii* (Chaïabi *et. al.,* 1999).

Mobile genetic elements among the nosocomial bacteria were detected in this study and these included conjugative plasmids and integrons. Conjugation phenomena frequently involve mobile plasmids or conjugative transposons, which encode their ability to move copies of themselves from one bacterial cell to another and are widespread in the bacterial world (Clewell and Francia, 2004; Lawley *et al.,* 2004). Conjugation experiments were done in the present study using two recipients; *E. coli* C600 resistant to Rifampicin and *E. coli* J53 resistant to Sodium azide. Twenty (20) transconjugants were obtained and all were resistant to Ampicillin. The conjugative plasmid conferring Ampicillin resistance weighed about 60Kb. Antibiotic susceptibility testing was done on the transconjugants as confirmatory test of resistance conferred by the conjugative plasmid and all the transconjugants were found to be resistant to both Rifampicin and Ampicillin.

In MDR Gram-negative bacteria, integrons play a very important role due to their high capability for transferring antimicrobial resistance genes (Poole,

2005). An Integron is defined as a genetic element that possesses a site, *attI*, at which additional DNA, in the form of gene cassettes, can be integrated by site-specific recombination. Gene cassettes are discrete genetic elements which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome. Class 1 and 2 integrons are the most frequently identified integrons in Gram negative bacteria (Fluit and Schmitz, 2004). A total of 37 isolates out of 108 (34%) were found to harbour the class 1 integrons. No class 2 integrons were detected. Although no further examination was done to determine the type of gene cassettes, the most frequently detected gene cassettes are dihydrofolate reductase (*dfrA)* and aminoglycoside adenyltransferase (*aadA)* (Hussein, 2009). Aminoglycoside adenyltransferase gene cassettes are responsible for aminoglycoside resistance and they include; *aadA1, aadA2*, *aadA5* and *aadA12* which confer resistance to streptomycin and spectinomycin and *aadB* confers resistance to gentamicin, kanamycin and tobramycin. The gene cassette profile *aadA1* has been detected worldwide from clinical *E. coli* and *K. pneumoniae* isolates (White *et al.,* 2001). Several AMEs like Aac(6')-Ib-cr (Fihman *et al.,* 2008), Aac(3) (Poole, 2005) and Aac(6')-I (Neonakis *et al.,* 2003) have been found in a gene cassette located inside class 1 Integron.

The presence of AMEs was determined both phenotypically and genotypically. The present study confirms that there exists a link between the type of

aminoglycoside used and the kind of aminoglycoside resistance mechanism that will prevail. Notable about the phenotypic characterisation was MDR *P. aeruginosa* which was seen to harbour numerous AME genes and confers 81% resistance by impermeability. The percentage incidence of *P. aeruginosa* most prevalent AMEs was as follows; Aac(6')-II 18.39% and Ant(2")-I 11.87% while impermeability played the most part in conferring resistance with 26.15% incidence (Poole, 2005). The occurrence of numerous AME genes in MDR *P. aeruginosa* was of great interest and this was because more than one mechanism of aminoglycosides resistance could be at play at the same given time. *P. aeruginosa* has a very active efflux system causing the reduction of intracellular concentration of aminoglycosides by the outer membrane changes in permeability and inner membrane decrease in transport leading to trapping the drug. The presence of AMEs in integrons and transposons may explain the multi-drug resistance of many aminoglycoside-resistant *P. aeruginosa* isolates (Poole, 2005).

The genotypic results of the six AMEs amplified by PCR showed that the most widespread AME in the present study were *AAC(6')-Ib-cr* (45.9%), followed by *AAC(3)-II* (30.9%), *AAC(6')-II* (25.9%), *AAC(6')-I* (22.2%), and *AAC(3)-I* (16.3%). No Ant(4')-IIb enzyme was detected. Molecular characterisation of aminoglycosides resistance in Spain on *Enterobacteriaceae, AAC-3-IIa* gene had

a 10% prevalence with *AAC-6'-Ib* at 3.8%, and *AAC-6'-Ic* at 0.3%. Twenty two percent of the strains presented more than one enzyme (Miró *et al.,* 2008).

The AME Aac(6')-Ib-cr was the most widespread enzyme in the present study occurring at a frequency of 22% in *Klebsiella* spp., 19% in *P. aeruginosa,* 14% in *E. coli* and 5% in *A. baumannii*. The discovery of the *cr* variant of the aminoglycoside-(6)-N-acetyltransferase (*AAC[6']-Ib-cr*) gene confers resistance against two unrelated classes of antibiotics-aminoglycoside and quinolone- by changing two amino acids Trp102Arg and Asp179- Tyr. The clinical appearance of *AAC(6')-Ib-cr*, including its molecular and phenotypic characteristics, and its association with other antibiotic resistance genes have not been clarified to date (Shin *et al.,* 2009) . Moreover, there has been no recent study on the prevalence of *AAC(6')-Ib-cr* in isolates from Kenya. The *AAC(6')-Ib-cr* gene has been found in a gene cassette located inside class 1 integron (Fihman *et al.,* 2008). This shows that the gene is capable of rapid horizontal transfer between *Enterobacteriaceae* isolates.

Aac(6')-I enzyme is also quite important as it has gained attention for conferring resistance to naturally occurring Kanamycins and Tobramycin as well as to their semi-synthetic derivatives, Amikacin and Neitlmicin. A positive correlation between increased Amikacin use and the occurrence of enzyme-mediated resistance has been described (Schmitz *et al.,* 1999). Amikacin a semi-synthetic

aminoglycoside has an acylated N-1 group which makes it a poor substrate for a number of modifying enzymes. The low incidence of Aac(6')-I in Italian and German hospitals reflects the low percentage of Amikacin usage (15.8% and less than 10% of the total aminoglycosides usage, respectively) (Neonakis *et al.,* 2003). In Kenya, hospitals are extensively using Amikacin in treatment of severe nosocomial infections thus increasing the level of resistance by 40% over the years as reported in this study. This could be attributed to the presence of this enzyme which is not only highly transferable as it is located within integrons and transposons but has been seen to co-exist very frequently with other antibiotic-inactivating enzymes such as ESBLs (Neonakis *et al.,* 2003).

The future of unravelling more mechanisms of Aminoglycoside resistance lies in the determination of the 3D atomic structure of AMEs by X-crystallography. Four AMEs crystal structures have been reported namely; Aac(3), Aac(6'), Ant(4') and Aph(3')-IIIa (Neonakis *et al.,*2003). This information has been useful in the application of structural modifications of aminoglycosides resulting in reduction of the modified antibiotic to bind to the target RNA due to unfavourable steric and/or electrostatic interactions. Attempts to make semi-synthetic aminoglycosides that circumvent resistance enzymes have been done although these await further experimentation (Kotra *et al.,* 2000).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

1. The clinical isolates of genera *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* showed very high levels of resistance to the older naturally derived aminoglycoside antibiotics like streptomycin, kanamycin, and gentamicin. The study results also revealed that the clinical isolates collected from AKUH-New in 2007-2008 showed a higher level of resistance to aminoglycosides than AKUH- old isolates collected in 2001-2006. This may be attributed to their prolonged and continuous use in Kenyan Hospitals as first line antibiotics.

2. The clinical isolates used in this study do posses mobile genetic determinants including class 1 integrons and conjugative plasmids responsible for Aminoglycoside resistance.

3. The high presence of ESBL mediated resistance which has been poorly reported in Africa was detected in the test Gram negative bacteria. In this study 24 (22%) of the *Enterobacteriaceae* were ESBL producing and were shown to have cross-resistance to aminoglycosides on plasmids.

4. Presence of AMEs was established both phenotypically and genotypically. The genes detected were *AAC(6')-Ib-cr* (45.9%), followed by *AAC(3)-II* (30.9%), *AAC(6')-II* (25.9%), *AAC(6')-I* (22.2%), and *AAC(3)-I* (16.3%).

5. MDR *Pseudomonas aeruginosa* was detected in the study and demonstrated resistance to Carbapenems. The occurrence of numerous AME genes in MDR *P. aeruginosa* was of great interest indicating the possibility of more than one mechanism of aminoglycosides resistance to be at play at the same given time.

In respect to these findings, the alternative hypotheses is accepted that the rate of Aminoglycoside resistance in the genera *Escherichia*, *Klebsiella*, *Pseudomonas* and *Acinetobacter* implicated in invasive infections in Kenya is on the increase and these clinical isolates do posses mobile genetic determinants of antibiotics responsible for Aminoglycoside resistance.

5.2 RECOMMENDATIONS

1. Regulations should be set up by Public health officials to monitor the administration and effectiveness of Aminoglycosides antibiotics, and consequently manage the evolution against Aminoglycosides resistance. At the same time researchers should develop more potent antibiotics for treatment of more severe nosocomial infections.
2. Aga Khan University Hospital should develop an aggressive system where appropriate antimicrobial agents can be selected hence avoid unnecessary treatment with ineffective antibiotics, prognosis of the patients can be

improved, acquisition of resistance in pathogens could be decelerated, and Expenditure on antimicrobials and overall hospital costs can be reduced.

3. Further studies by researchers into the membrane structure of *P. aeruginosa* would open avenues for managing their resistance to antibiotics and offer hope to patients infected. Likewise, development of antibiotics that cripple the efflux mechanism of these bacteria would be a step in the right direction.

4. ESBL detection is not commonly carried out in many microbiology units in developing countries including Kenya and this could be attributed to lack of awareness and/or lack of resources and facilities to conduct ESBL identification. The present study can be used to advocate the necessity for ESBL detection in order to raise awareness and reduce the challenges of antibiotics resistance.

5. Determination of the 3D atomic structure of AMEs by X-crystallography would enable better understanding of the molecular basis of aminoglycosides resistance modification. This information could lead to the development of effective and potent inhibitors that will reverse aminoglycosides resistance. Monitoring of the effectiveness of Aminoglycoside antibiotics should be instituted at various levels of healthcare systems in Kenya.

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APPENDIX A
MEDIA PREPARATION

MacConkey Agar

Formula

Gelatin peptone 17.0
Bile salts No: 3 1.5g
Lactose 10.0g
Neutral red 0.03g
Sodium chloride 5.0g
Peptone mixture 3.0g
Bacteriological agar 13.5g

Preparation

Suspend 50 g in 1 liter of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 mins. Cool to 45-50°C and pour in 15 – 20 ml amounts into Petri dishes.

Use: For selection of Gram negative bacteria.

Mueller Hinton Agar.

Formula

Beef infusion 300.0g
Acid hydrolysate of casein 17.5g
Starch 1.5g
Agar 17.0g

Preparation

Dissolve 42 g in 1 liter of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 mins. Cool to 45-50°C and pour in 15 – 20 ml amounts into Petri dishes.

***Use*:** For sensitivity testing.

Tryptic Soy Broth

Formula

Pancreatic digest of casein 17.0g
Papaic digest of soy meal 3.0g
Sodium chloride 5.0g
Di-potassium phosphate 2.5g
Dextrose 2.5g

Preparation

Dissolve 30g in 1 liter of distilled water. Heat (if necessary) to dissolve medium completely. Mix and distribute into final containers. Autoclave at 121°C for 15 mins.

Use: Stocking media

Mueller Hinton broth

Formula

Beef, infusion form 300g
Casein acid hydrolysate 17.50g
Starch 1.50g

Preparation

Dissolve 21g in 1 liter of distilled water. Heat (if necessary) to dissolve medium completely. Mix and distribute into final containers. Autoclave at 121°C for 15 mins.

Use: For plasmid extractions instead of LB broth.

Tryptic Soy Both

Formula

Caesin peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5

Preparation

Dissolve 30g in 1000ml distilled water. Dispense into test tubes. Sterilize by autoclaving at 121^0C for 15mins.

Use: For stocking purposes.

Triple Sugar Iron Medium

Formula

Peptic digest of animal tissue	10.0
Casein enzymic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.2
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Final pH (at 25^0C) 7.4 ±0.2

Preparation
Suspend 65g in 1000ml of distilled water. Heat to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 121^0C for 15mins. Allow medium to set in a sloped form with a 1 inch long butt.

Use: For identification of Gram negative enteric bacilli on basis of dextrose, lactose and sucrose fermentation and hydrogen sulphide production.

Sulphur Indole Motility Medium
Formula

Peptic digest of animal tissue	30.0
Beef extract	3.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0

Preparation
Suspend 36.23g in 1000ml of distilled water. Heat to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 121^0C for 15mins. Allow medium to set in an upright position.

Use:
For determination of hydrogen sulphide production, indole formation and motility of enteric bacilli.

Simmons Citrate
Formula

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

Final pH 7.0±0.2

Preparation
Suspend 23g in 1000ml of distilled water. Boil to dissolve completely. Dispense into test tubes. Sterilize by autoclaving at 121^0C for 15mins.

Use: For identification of Gram negative bacteria on basis of citrate utilization

Urea medium
Formula

Peptone	1.0
Glucose	1.0

Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0

Final pH 6.8±0.2

Preparation

Suspend 2.4g in 95ml of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 115^0C for 20mins. Cool to 50^0C and aseptically add 1 ampoule of sterile Urea solution (SR20). Mix well, distribute 10ml amounts into sterile containers.

Use: For identification of Gram negative bacteria on basis of urease production.

MR-VP Medium (Glucose Phosphate Broth)

Formula:

Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0

Final pH 6.9±0.2

Preparation

Suspend 17g in 1000ml distilled water. Heat (if necessary) to dissolve medium completely. Distribute in test tubes in 10ml amounts and sterilize by autoclaving at 121^0C for 15mins.

Use:

For performance of Methyl red and Voges-Proskauer tests in differentiation of coli-areogenes group.

APPENDIX B

PLASMID EXTRACTION REAGENTS

Solution I

50 mMol glucose
25 mMol Tris Cl (pH 8.0)
10 mMol EDTA (pH 8.0)
Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 min at 10 lb/sq. in. on liquid cycle and stored at 4°C.

Solution II

0.2 N NaOH (freshly diluted from a 2 N stock).
1% SDS

Solution III

5 M potassium acetate 60 ml
Glacial acetic acid 11.5 ml
H_2O 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetic acid.

Electrophoresis buffer

Buffer	**Working Solution**	**Concentrated stock solution (per litre).**
Tris-borate (TBE) (pH 8.0)	0.5 X: 0.045 M Tris-borate 0.001 M EDTA	5 X: 54g Tris base 27.5g boric acid 20ml 0.5 M EDTA

APPENDIX C

KEMRI ETHICAL REVIEW LETTER

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: kemri-hq@nairobi.mimcom.net: director@kemri.org; Website: www.kemri.org

KEMRI/RES/7/3/1 **2ND MARCH 2009**

TO: **Ms. DORIS W. NDEGWA (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. N. WAMAE,**
THE DIRECTOR, CMR,
NAIROBI

Forwarded 03/03/2009

RE: **SSC PROTOCOL No. 1500 (2ND *REVISION*): MSc PROPOSAL ON CHARACTERIZATION OF AMINOGLYCOSIDE RESISTANT STRAINS OF *ESCHERICHIA, KLEBSIELLA, PSEUDOMONAS AND ACINETOBACTER* IMPLICATED IN INVASIVE INFECTIONS IN NAIROBI, KENYA**

Dear Ms. Ndegwa,

Reference is made to your letter dated 25th February 2009.

We acknowledge receipt of the revised proposal and note that data obtained from this investigation will be instrumental in improved drug administration and treatment regimens at the Aga Khan University Hospital.

It is also observed that the proposed study shall inform strategies for addressing emerging aminoglycoside resistance among the *Escherichia*, *Klebsiella*, *Pseudomonas* and *Acinetobacter* strains.

The Committee is satisfied that the issue raised at the 163rd meeting of 19th February 2009 has been adequately addressed. The study is hereby granted approval for implementation effective this **2nd day of March 2009** to **1st March 2010**.

Please note that you are responsible for reporting any changes to the approved study to the Scientific Steering Committee and to the Ethical Review Committee prior to execution. This includes changes to research design, personnel and funding. You may embark on the study.

Sincerely,

C. WASUNNA,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

In Search of Better Health

APPENDIX D
AMINOGLYCOSIDE SENSITIVITY RESULTS
Aga Khan University Hospital New Isolates (2007-2008).

Micro-organisms	Lab No.	Year	Specimen source	AK	K	CN	S	TOB	NEO	SPCT	AK	K	CN	S	SPCT	TOB	NEO
AKUH																	
Klebsiella	1	2007	Pus swab	18	16	17	12	6	13	17	S	I	S	I	I	R	I
	2	2007	blood	20	6	6	6	6	14	14	S	R	R	R	R	R	I
	3	2007	urine	17	6	16	6	17	11	34	S	R	S	R	S	S	R
	6	2007	urine	20	19	18	19	19	18	40	S	S	S	S	S	S	S
	7	2007	urine	19	6	6	15	6	14	23	S	R	R	S	S	R	I
	8	2008	blood	6	6	6	6	6	20	14	R	R	R	R	R	R	S
	9	2007	urine	20	19	19	6	19	18	30	S	S	S	R	S	S	S
	11	2008	blood	18	11	11	6	6	18	34	S	R	R	R	S	R	I
	14	2007	tracheal aspirate	16	6	6	6	6	16	26	I	R	R	R	S	R	I
	33	2008	HVS	18	6	16	6	6	10	30	S	R	R	R	S	S	R
	34	2008	sputum	20	17	6	6	6	15	20	S	I	I	R	S	R	I
	36	2008	urine	16	16	14	6	18	14	20	I	I	I	R	S	R	I
E.coli	17	2007	urine	19	17	6	12	14	16	21	S	I	R	I	S	I	I
	18	2007	urine	16	6	19	12	6	18	26	I	R	S	I	S	R	S
	19	2008	urine	17	6	18	7	6	17	22	S	R		R	S	R	S
	21	2008	urine	20	13	20	11	9	17	23	S	R	S	R	S	R	S
	22	2007	urine	16	16	21	10	10	17	28	S	I	S	R	S	R	S
	23	2007	urine	22	6	6	10	6	17	30	I	R	R	R	S	R	S
	25	2007	urine	23	22	20	10	21	20	26	S	S	S	R	S	S	S
	26	2007	urine	23	21	21	10	21	20	33	S	S	S	R	S	S	S
	55	2008	urine	19	13	6	17	8	8	32	S	R	R	S	S	R	I

	56	2008	urine	19	18	18	6	17	17	37	S	S	S	R	S	S	I
	57	2007	urine	21	11	6	12	6	6	30	S	R	R	I	S	R	I
	58	2008	urine	22	19	21	17	20	20	35	S	S	S	S	S	S	S
	59	2007	urine	21	21	21	10	20	20	10	S	S	S	R	R	S	S
	60	2008	urine	19	15	6	6	13	13	37	S	I	R	R	S	I	I
	61	2007	urine	19	13	10	14	10	10	33	S	R	R	I	S	R	S
	62	2008	urine	18	11	9	6	9	9	18	S	R	R	R	S	R	S
Acinetobacter	27	2007	tracheal aspirate	6	6	6	6	18	6	15	R	R	R	R	R	S	R
	28	2007	pus swab	6	6	6	6	19	6	21	R	R	R	R	S	S	R
	29	2007	urine	21	21	20	6	21	21	10	S	S	S	R	R	S	S
	30	2007	urine	14	17	6	6	15	15	18	R	I	R	R	I	S	I
	31	2007	urine	15	16	6	6	15	16	19	I	I	R	R	I	S	I
	35	2008	tracheal aspirate	6	6	6	6	20	10	18	R	R	R	S	I	S	R
	64	2007	tracheal aspirate	23	23	22	20	21	21	26	S	S	S	S	S	S	S
Pseudomonas	40	2008	urine	19	6	6	6	6	14	10	S	R	R	I	R	R	I
	43	2008	tracheal aspirate	17	6	6	6	6	13	10	S	R	R	I	R	R	I
	51	2007	CSF	6	6	6	12	6	11	30	R	R	R	R	S	R	R
	54	2008	urine	6	6	6	6	6	9	36	R	R	R	R	S	R	R
	63	2007	urine	6	6	6	6	6	15	10	R	R	R	R	R	R	R
	65	2007	tracheal aspirate	10	6	6	6	6	19	10	R	R	R	R	S	R	S
	70	2007	tracheal tube tip	14	6	6	6	10	15	10	R	R	R	R	S	R	S
	74	2007	urine	6	6	6	6	23	22	10	R	R	R	R	R	S	S
	77	2007	urine subdural tissue	8	6	6	6	10	21	10	R	R	R	R	S	R	R
	78	2007	urine	7	6	6	6	10	10	30	R	R	R	R	R	R	R
	79	2007	urine	6	6	6	6	12	13	10	R	R	R	R	S	R	R
	80	2007	tracheal aspirate	6	6	6	6	10	14	10	R	R	R	R	R	R	R
	81	2007	pus swab	10	6	6	6	10	19	10	R	R	R	R	R	R	R
	84	2007	tracheal aspirate	6	6	6	6	14	16	10	R	R	R	R	R	I	R

86	2007	urine	6	6	6	6	12	14	10	R	R	R	R	R	R	R
88	2007	blood	8	6	11	6	6	17	30	R	R	R	R	S	R	R
		Sum	**754**	**553**	**542**	**428**	**599**	**763**	**1116**							
		Mean	**14.8**	**11**	**10.6**	**8.4**	**11.7**	**15**	**21.9**							

Stored KEMRI isolates (2001-2006)

Micro-organisms Stored	**Lab No.**	**Year**	**Specimen Source**	**AK**	**K**	**CN**	**S**	**TOB**	**NEO**	**SPCT**	**K**	**AK**	**CN**	**S**	**TOB**	**SPCT**	**NEO**
E.coli	6308	2004	urine	23	11	19	13	6	10	18	R	S	S	I	R	S	I
	6476	2004	urine	20	16	6	11	6	19	16	I	S	R	R	R	I	S
	6679	2004	urine	20	11	6	6	6	13	24	R	S	R	R	R	S	I
	6680	2004	urine	26	24	26	23	20	19	25	S	S	S	S	S	S	S
	6681	2004	urine	19	14	6	14	11	13	22	I	S	R	I	R	S	I
	5875	2003	urine	18	14	6	15	20	21	30	I	S	R	S	S	S	R
	6701	2004	urine	21	16	6	17	10	15	31	I	S	R	S	R	S	I
	6741	2004	urine	20	6	6	10	6	9	30	R	S	R	R	R	S	I
	6742	2004	urine	19	11	6	6	6	15	30	R	S	R	R	R	S	R
	6743	2004	urine	18	9	8	9	6	14	28	R	S	R	R	I	S	R
	6744	2004	urine	20	8	19	6	13	16	26	R	S	S	R	R	S	R
	6686	2004	urine	20	10	6	14	6	20	26	R	S	R	I	R	S	R
	6687	2004	urine	19	16	9	15	10	9	28	I	S	R	S	R	S	R
	6688	2004	urine	20	20	20	16	13	18	25	S	S	S	S	R	S	I
	6689	2004	urine	18	13	6	9	11	20	27	R	S	R	R	R	S	I
	6698	2004	urine	19	14	6	10	11	14	25	I	S	R	R	R	S	S
	6699	2004	urine	21	18	6	16	10	14	24	S	S	R	S	R	S	R
	6702	2004	urine	19	22	19	18	6	17	39	S	S	S	S	R	S	R

	6703	2004	urine	22	6	14	6	20	19	28	R	S	I	R	S	S	I
	6763	2004	urine	23	12	6	19	6	20	25	R	S	R	S	R	S	R
	6301	2004	urine	20	17	19	7	13	21	17	S	S	S	R	I	I	S
	6303	2004	urine	20	18	19	16	6	20	30	S	S	S	S	R	S	R
	6305	2004	urine	22	11	6	19	8	19	26	R	S	R	S	R	S	R
	6309	2004	urine	20	14	8	9	6	10	20	I	S	R	R	R	S	I
	6310	2004	urine	29	21	25	6	10	11	25	S	S	S	R	R	S	S
	6812	2006	urine	20	15	10	20	9	14	23	I	S	R	S	R	S	R
Klebsiella	5313	2003	urine	29	30	29	27	22	20	30	S	S	S	S	S	S	R
	5413	2003	catheter	19	6	6	15	7	8	25	R	S	R	S	R	S	I
	5414	2003	urine	21	18	20	18	22	20	26	S	S	S	S	S	S	R
	5415	2003	blood	17	11	18	6	19	21	24	R	S	S	R	S	S	R
	5419	2003	urine	21	6	6	6	15	13	20	R	S	R	R	S	R	R
	5420	2003	urine	22	19	10	6	18	18	30	S	S	R	R	S	I	R
	5422	2003	urine	21	9	6	8	12	20	26	R	S	R	R	S	R	R
	5423	2003	urine	22	21	22	19	22	21	26	S	S	S	S	R	S	R
	5424	2003	urine	21	21	21	17	21	20	29	S	S	S	S	S	S	R
	5425	2003	urine	22	11	21	10	20	17	27	R	S	S	R	S	S	S
	5874	2003	urine	20	18	20	16	21	20	27	S	S	S	S	S	S	R
	1662	2001	sputum	20	18	19	21	26	21	30	S	S	S	S	S	S	R
	1664	2001	blood	23	16	6	6	20	20	30	I	S	R	R	S	S	R
	1665	2001	pus	17	6	6	6	14	16	19	R	S	R	R	S	S	S
	1666	2001	pus	20	14	6	11	17	26	26	I	S	R	R	I	R	R
	1667	2001	pus	16	6	10	12	16	19	20	R	I	R	R	S	I	R
	1669	2001	pus	18	6	13	6	15	20	20	R	S	I	R	S	R	R
	1671	2001	sputum	24	6	6	13	19	16	26	R	S	R	R	S	R	R
	1690	2001	blood	18	6	6	6	16	16	28	R	S	R	R	S	I	R
	6685	2004	urine	21	21	20	6	18	19	18	S	S	S	R	S	R	R

	6764	2004	urine	19	6	6	6	14	15	28	R	S	R	R	S	I	R
Pseudomonas	5876	2003	urine	6	7	6	6	10	10	28	R	R	R	R	R	R	R
	6682	2004	nose swab	9	6	6	6	6	10	30	R	R	R	R	R	S	R
A. baumannii	5801	2003	urine	21	22	21	6	22	19	25	S	S	S	R	S	S	S
	6364	2004	urine	18	6	6	6	17	10	18	R	S	R	R	S	I	S
	6365	2004	urine	18	6	24	6	6	10	10	R	S	R	R	S	R	R
	6690	2004	urine	20	19	19	13	20	17	26	S	S	S	R	S	S	R
			Sum	**1059**	**708**	**652**	**614**	**710**	**872**	**1340**							
			Mean	**20**	**13**	**12.3**	**12**	**13.4**	**16.5**	**25.3**							