# The Utilization of the Polymerase Chain Reaction for the Detection of Genes Coding for Aminoglycoside Modifying Enzymes in Bacteria of Clinical Isolates from Jordan University Hospital.

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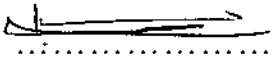
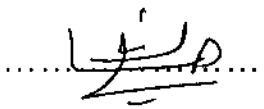
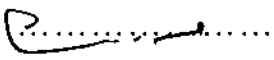
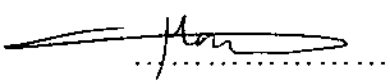
| Dr. Salwa Bdour |  |
| Dr Hala Khyami – Horani |  |
| Dr Hasan Abdel Ruhman |  |
| Dr Manar Atoum |  |

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# Abstract

**The Utilization of the Polymerase Chain Reaction for The Detection of Genes Coding for Aminoglycoside Modifying Enzymes in Bacteria of Clinical Isolates from Jordan University Hospital.**

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During the period of six months, a total of one hundred and sixteen bacterial isolates were collected from different clinical specimens to include: bile, blood, catheter, pus, seminal, sputum, urine, wound and urethral swabs. The susceptibility of some G (+) and G (-) bacteria to twenty-two different antibiotics were tested in this study. *Staphylococcus spp.*, *Enterococcus spp* and *Micrococcus spp.* were resistant by 16.7, 42.9 and 50%, respectively, toward the commonly used antibiotic, gentamicin. *Micrococcus spp.* is 100% resistant to ampicillin, cefoxitin and ceftazidime. In addition *Micrococcus spp.* is 75% resistant toward

imipenem and 50% resistant toward ceftriaxone and pipericillin. *Staphylococcus spp.* has shown the highest resistance toward erythromycin (20.8%). *Enterococcus spp.* showed the highest resistance toward clindamycin, erythromycin and nalidixic acid (28.5%). All the G(-) bacteria used in this study expressed resistance towards ampicillin, one of the most commonly prescribed antibiotics. The resistance profile ranged from 40-100% as expressed by *Klebsiella spp.* and *Proteus spp.*, respectively. *Enterobacter spp.* have shown resistance to 15 out of the 22 used antibiotics, ranging from 5.9 to 47%. In an attempt to determine the pattern of antibiotic resistance in clinical isolates obtained from Jordanian patients, and to design effective antibiotic policies , systemic analysis of resistance mechanisms in clinical isolates is indispensable.Detection of genes coding aminoglycoside modifying enzymes in the hospital isolates was carried out using Polymerase Chain Reaction (PCR). The focus of this work was on G(-) bacteria. Three species of bacteria were chosen for this study : *Enterobcter spp.*, *Pseudomonas spp.* and *Escherichia spp.*. PCR was performed using plasmid DNA prepared from the isolates. Colony PCR was followed , and the isolates showed to carry the following genes :*rrs, aacC2, aacC3, aacA-aphD* and *aphA3* .The restriction enzyme digestion confirmed primarly that the isolated genes had similar restriction sites as the published results.

## 1.Introduction

### Antibiotic Resistance

The bacteria causing diseases that are now becoming serious public health threats are neither strange nor exotic, but rather shockingly familiar. Tuberculosis, typhoid fever, meningitis, pneumonia, and septicemia are emerging global threats. The infectious agents causing these serious threats are the same bacteria identified many decades age. The only difference is that these and other microorganisms are no longer killed by the "miracle drugs" that have kept them at bay for the past six decades. The indiscriminate use of antibiotics has led to a fast-approaching crisis in which human dominance of the planet is threatened by a single, elementary cell of the microbial world (Harrison and Svec, 1998). Although a number of causes can be identified which contribute to the problem, clearly the antibiotic as a selective agent and the resistance gene as the vehicle of resistance are the two most important factors, making up a "drug resistance equation". Given sufficient time and quantity of antibiotic, drug resistance will eventually appear but a public health problem is not inevitable if the two components of the drug resistance equation are kept in check (Levy, 1997).

Because of the rapid multiplication rates and large sizes of bacterial populations, the ability of these populations to respond to environmental changes is remarkable. The adaptation of bacteria to the toxicity of antibiotics is

due, therefore, to the flexibility of bacterial populations. When a new antibiotics is introduced into clinical practice for the treatment of infections caused by bacteria that are not inherently resistant to the drug, the majority of bacteria respond to the new drug but after months and/or years of continuous use, reports often appear describing treatment failures due to infections by strains of bacteria that are resistant to the drug (Hugo and Russel, 1992).

**The origin and molecular basis of antibiotic resistance**

Researchers refer to insensitive bacteria in the clinic as being resistant to antibiotics, but rarely they consider what that means. Even the most resistant bacterium can be inhibited or killed by a sufficiently high concentration of antibiotic; patients, however, would not be able to tolerate the high concentration required in some cases. Bacterial species vary tremendously in their susceptibility to an antibiotic. Most strains of *Streptococcus pneumonia* in Britain are inhibited by 0.01 mg/l of benzylpencillin, whereas for *Escherichia coli* 32-64 mg/l are required to inhibit growth, a level which cannot be achieved in the human body (Hawkey, 1998). This introduces the concept of clinical resistance, which is dependent on outcome and is all too often ignored. Clinical resistance is a complex concept in which the type of infecting bacterium, its location in the body, the distribution of the antibiotic in the body and its concentration at the site of infection, and the immune status of the patient all interact (Hawkey, 1998).

**Mechanisms of antibiotic resistance in bacteria**

The mechanisms that bacteria exhibit to protect themselves from antibiotics can be classified into four basic types. Antibiotic modification is the best known where the resistant bacteria retain the same sensitive target as antibiotic sensitive strains, but the antibiotic is prevented from reaching it. This happens, for example with β lactamases which enzymatically cleave the four membered β- lactam ring, rendering the antibiotic inactive. Over 200 types of β-lactamase have been described (Livermor,1995). Most β- lactamases act to some degree against both penicillin and cephalosprins; others are more specific namely, cephalosporinases ( for example, AmpC enzyme found in *Enterobacter* spp) or penicillinases ( for example, *Staphylococcus aureus* penicillinase). β-Lactamases are widespread among many G(+) and G(-) bacterial species and exhibit varying degrees of inhibition by β-lactamase inhibitiors, such as clavulanic acid (Livermor, 1995)

Some antibiotic resistant bacteria protect the target of antibiotic by preventing the antibiotic from entering the cell or pumping it out faster than it can flow in (Chopra *et. al.*, 1992).

β-lactam antibiotics in G(-) bacteria gain access to the cell , through a water filled hollow membrane protein known as a porin. Lack of the specific D2

porin confers resistance of *Pseudomonas aeruginosa* to imipenem (Chopra *et. al.*, 1992).

This mechanism is also seen with low level resistance to fluoroquinolones and aminoglycosides. Increased efflux via an energy-requiring transport pump is a well recognised mechanism for resistance to tetracyclines and is encoded by a wide range of related genes, such as *tet (A)*, that have become distributed in the *Enterobacteriaceae* (Chopra *et. al.*, 1992).

Alteration in the primary site of action may mean that the antibiotic penetrates the cell and reaches the target site but is unable to inhibit the activity of the target because of structural changes in the target. *Enterococci* are regarded as being inherently resistant to cephalosporins because the enzymes responsible for the synthesis of the peptidoglycan in the bacterial cell wall have a low affinity for cephalosporins . Most strains of *Streptococcus pneumoniae* are highly susceptible to both penicillins and cephalosprins but can acquire DNA from other bacteria, which codes for structurally altered enzyme(s) (Tomasz and Munoz, 1995). The altered enzyme still synthesises peptidoglycan but has a low affinity for penicillins and hence contributes to resistance of *S. pneumoniae* to this antibiotic.

The final mechanism by which bacteria may protect themselves from antibiotics is the production of an alternative target (usually an enzyme) that is resistant to inhibition by the antibiotic while continuing to produce the original sensitive target. This allows bacteria to survive in the face of selection: the alternative

enzyme "bypassess" the effect of the antibiotic. The best known example of this mechanism is probably the alternative penicillin binding proteins (PBP2a), which is produced in addition to the "normal" penicillin binding proteins by methicillin resistant *Staphylococcus aureus* (MRSA). The protein is encoded by the *mecA* gene, and because PBP2a is not inhibited by antibiotics such as flucloxacillin the cell continues to synthesise peptidoglycan and hence has a structurally intact cell wall (Michel and Gutmann, 1997).

**Molecular epidemiology of resistance genes**

Resistance in bacteria can be intrinsic or acquired. Intrinsic resistance is a naturally occurring trait arising from the biology of the organism, e.g. vancomycin resistance in *Escherichia coli*. Acquired resistance occurs when a bacterium that has been sensitive to antibiotics develops resistance this may happen by mutation or by acquisition of new DNA.

Mutation is a spontaneous event that occurs regardless of whether antibiotic is present. A bacterium carrying such a mutation is at a huge advantage as the susceptible cells are rapidly killed by the antibiotic, leaving a resistant subpopulation. Transferable resistance was recognised in 1959, when resistance genes found in *Shigella* transferred to *E. coli* via plasmids. (Grubb, 1998). Plasmids are self replicating circular double stranded DNA, smaller than the bacterial genome, which encode their transfer by replication into another bacterial strain or species (Grubb,1998). They can carry and transfer multiple

resistance genes, which may be located on a section of DNA capable of transfer from one plasmid to another or to the genome as transposons (or "jumping gene"). Because the range of bacteria to which plasmids can spread is often limited, transposons are important in spreading resistance genes across such boundaries. The *mecA* gene in MRSA *S.areus* may well have been acquired by transposition. (Grubb, 1998).

Simjee *et al* (2000) described the nature of transposon mediated high-level gentamicin resistance in *Enterococcus faecalis* isolated in the United Kingdom. The resistance was attributed to the synthesis of the aminoglycoside modifying enzyme *aac6'-aph2"*. which was present on a 70 Kb plasmid in all the 42 isolates studied. This gene could be transferred by conjugation in association with the 70 Kb plasmid in 39 of the isolates studied. In three *Enterococcus faecalis* isolates, however, the resistance was transferable independent of the 70 Kb plasmid, suggesting the presence of a conjugative transposon. Long PCR studies showed that all 42 clinical isolates harboured a transposon similar to the originally identified transposon in *Enterococcus faecalis* isolated in the USA.

Transduction which is carried by bacteriophages can also transfer resistance, and this is frequently seen in *Staphylococci*. When bacteria die they release DNA, which can be taken up by competent bacteria in a process known as transformation. This process is increasingly recognised as important in the environment and is probably the main route for the spread of penicillin

resistance in *Streptococcus pneumoniae*, by creation of "mosaic penicillin binding protein genes." (Tomasz and Munoz, 1995).

**Origin of resistance genes**

In considering the evolution and dissemination of antibiotic resistance genes it is important to appreciate the rapidity of bacterial multiplication and the continual exchange of bacteria among animal, human, and agricultural hosts throughout the world. There is support for the notion that determinants of antibiotic resistance were not derived from the currently observed bacterial host in which the resistance plasmid is seen. DNA sequencing studies of lactamases and aminoglycoside inactivating enzymes show that despite similarities within the protein studies of the two families, there are substantial sequence differences (Craig, 1993). As the evolutionary time frame has to be less than 50 years it is not possible to derive a model in which evolution could have occurred by mutation alone form common ancestral genes. They must have been derived from a large and diverse gene pool presumably already occurring in environmental bacteria. Many bacteria and fungi that produce antibiotics possess resistance determinants that are similar to those found in clinical bacteria. Gene exchange might occur in soil or, more likely, in the gut of humans or animals. It has been discovered that commercial antibiotic preparations contain DNA from the producing organism, and antibiotic resistance gene sequences can be identified by the polymerase chain reaction (Craig, 1993).

Genes either exist in nature already or can emerge by mutation rapidly. Rapid mutation has been seen with (*a*) the *TEM* lactamase, resulting in an extension of the substrate profile to include third generation cephalosporins (first reported in Athens in 1963, one year after the introduction of ampicillin) and (*b*) the *IMI-1B* lactamase (reported from a Californian hospital before imipenem was approved for use in the United States) (Daikos *et al.*,1991). The selection pressure is heavy, and injudicious use of antibiotics, largely in medical practice, is probably responsible, although agricultural and veterinary use contributes to resistance in human pathogens. The addition of antibiotics to animal feed or water, either for growth promotion or, more significantly, for mass treatment or prophylaxis (or both treatment and prophylaxis) in factory farmed animals, is having an unquantified effect on resistance levels (Daikos *et al.*, 1991).

**Antimicrobial Agents Used in the Treatment of Infectious Disease**

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties (CLAS, 1995).

**Kinds of Antimicrobial Agents and their Primary Modes of Action**

**Cell wall synthesis inhibitors**

Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan . Generally they exert their selective toxicity against bacteria because human cells lack cell walls.

**ß-lactam antibiotics.**

Chemically, these antibiotics contain a 4-membered beta lactam ring. They are the products of two groups of fungi, Penicillium and Cephalosporium molds, and are correspondingly represented by the penicillins and cephalosporins.

The ß-lactam antibiotics are stereochemically related to D-alanyl-D-alanine which is a substrate for the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains. Penicillins bind to and inhibit the carboxypeptidase and transpeptidase enzymes that are required for this step in peptidoglycan biosynthesis. ß - lactam antibiotics are normally bactericidal and require that cells be actively growing in order to exert their toxicity.

Two other classes of ß- lactams are the carbapenems and monobactams. The latter are particularly useful for the treatment of allergic individuals. A person who becomes allergic to penicillin usually becomes allergic to the cephalosporins and the carbapenems as well. Such individuals can still be treated with the monobactams, which are structurally different so as not to induce allergy (CLAS, 1995)

Bacitracin is a polypeptide antibiotic produced by *Bacillus species*. It prevents cell wall growth by inhibiting the release of the muropeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane. Teichoic acid synthesis, which requires the same carrier, is also inhibited.

Glycopeptides, such as the antibiotic vancomycin, appear to inhibit both transglycosylation and transpeptidation reactions during peptidoglycan assembly. They bind to the muropeptide subunit as it is transferred out of the cell cytoplasm and inhibit subsequent polymerization reactions. Vancomycin is not effective against G(-)bacteria because it cannot penetrate their outer membrane. However, it has become important in clinical usage for treatment of infections by strains of *Staphylococcus aureus* that are resistant to virtually all other antibiotics (CLAS, 1995)

**Cell membrane inhibitors**

They disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital to bacteria, and compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in bacterial and eukaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically. The only antibacterial antibiotic of clinical importance that acts by this mechanism is Polymyxin produced by *Bacillus polymyxis*. Polymyxin is

effective mainly against G(-)bacteria and is usually limited to topical usage. Polymyxins bind to membrane phospholipids and thereby interfere with membrane function.

**Protein synthesis inhibitors**

Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of protein synthesis. Their attack is always at one of the events occurring on the ribosome and never at the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 70S (as opposed to 80S) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotics with this mode of action are the tetracyclines, chloramphenicol, the macrolides (e.g. erythromycin) and the aminoglycosides (e.g. streptomycin).

The tetracyclines consist of eight related antibiotics, which are all natural products of Streptomyces, although some can now be produced semisynthetically. Tetracycline, chlortetracycline and doxycycline are the best known. The tetracyclines act by blocking the binding of aminoacyl tRNA to the A site on the ribosome. Tetracyclines inhibit protein synthesis on isolated 70S or 80S (eukaryotic) ribosomes, and in both cases, their effect is on the small ribosomal subunit. However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an

effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline which is harmless to animal tissues can halt protein synthesis in invading bacteria (CLAS, 1995).

Chloramphenicol has a broad spectrum of activity but it exerts a bacteriostatic effect. Chloramphenicol inhibits the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis.

Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit. Binding inhibits elongation of the protein by peptidyl transferase or prevents translocation of the ribosome or both. Macrolides are bacteriostatic for most bacteria but are cidal for a few G(+)bacteria.

Lincomycin and Clindamycin are a miscellaneous group of protein synthesis inhibitors with activity similar to the macrolides.

**Effects on Nucleic Acids**

Nalidixic acid is a synthetic chemotherapeutic agent that belongs to a group of compounds called quinolones. Nalidixic acid is a bactericidal agent that binds to the DNA gyrase enzyme (topoisomerase) which is essential for DNA replication and allows supercoils to be relaxed and reformed. Binding of the drug inhibits DNA gyrase activity.

Rifampicin acts quite specifically on bacterial RNA polymerase and is inactive towards RNA polymerase from animal cells or towards DNA polymerase. The

antibiotic binds to the beta subunit of the polymerase and apparently blocks the entry of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis (CLAS, 1995).

**Competitive Inhibitors**

The sulfonamides (e.g. Gantrisin) and Trimethoprim are inhibitors of the bacterial enzymes required for the synthesis of tetrahydofolic acid (THF), the vitamin form of folic acid essential for 1-carbon transfer reactions. Sulfonamides are structurally similar to para aminobenzoic acid (PABA), the substrate for the first enzyme in the THF pathway, and they competitively inhibit that step . Trimethoprim is structurally similar to dihydrofolate (DHF) and competitively inhibits the second step in THF synthesis mediated by the DHF reductase (CLAS, 1995)

**Aminoglycosides: Activity and resistance**

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections (Gilbert, 1995). Their history begins in 1944 with streptomycin and was thereafter marked by the successive introduction of a series of milestone compounds (kanamycin, gentamincin and tobramycin) which definitively established the usefulness of this class of antibiotics for the treatment of G(-) bacillary infections. In the 1970s, the semisynthetic aminoglycosides dibekacin, amikacin

and netilmicin demonstrated the possibility of obtaining compounds which were active against strains that had developed resistance mechanisms towards earlier aminoglycosides as well as displaying distinct toxicological profiles (Mingeot-Leclercq and Tulkens, 1999). Since then, the pace of development of new aminoglycosides has markedly slowed down.

**Basis of Antimicrobial action**

Aminoglycosides act primarily by impairing bacterial protein synthesis through binding to the 30s subunit of the prokaryotic ribosomes. Passage of these highly polar molecules across the outer membrane of G(-) bacteria (Hancock., 1981) is a self-promoted uptake process involving the drug-induced disruption of $Mg^{2+}$ bridges between adjacent lipopolysaccharide molecules (Vaara ,1992). Penetration through porin channels is unlikely because of the large size of aminoglycosides [approximately 1.8 x 1.0 x 1.0 nm (Chung *et al.*, 1985)]. Subsequent transport of aminoglycosides across the cytoplasmic (inner) membrane is dependent upon electron transport and is termed energy-dependent phase 1 [EDP-1 (Bryan and Kwan, 1983)]. It is rate limiting and is blocked or inhibited by divalent cations, hyperosmolarity, low pH (Xiong, *et.al.*,1996) and anaerobiosis. In the cytosol, aminoglycosides bind to the 30S subunit of ribosomes, again through an energy-dependent process [(energy-dependent phase II [EDP-II] (Bryan and Kwan, 1983)]. While this binding does not prevent formation of the initiation complex of peptide synthesis, it perturbs the

elongation of the nascent chain by impairing the proofreading process controlling translational accuracy (misreading and/or premature termination (Melancon *et al.*, 1992). The aberrant proteins may be inserted into the cell membrane, leading to altered permeability and further stimulation of aminoglycoside transport (Busse *et al.*, 1992).

**Resistance Mechanisms**

The emergence of resistant strains has somewhat reduced the potential of aminoglycosides in empiric therapies. The other main mechanisms which may affect all aminoglycosides are (i) a decreased uptake and/or accumulation of the drug in bacteria and (ii) the bacterial expression of enzymes which modify the antibiotic and thereby inactivate it (Davies and Wright, 1997; Shaw *et al.*, 1993).

**Decrease in drug uptake and accumulation.**

Reduced drug uptake mostly seen in *Pseudomonas spp.* and other non-fermenting G (-) bacilli, is likely to be due to membrane impermeabilization, but the underlying molecular mechanisms are largely unknown (Chambers and Sande, 1995). It is highly significant in the clinic, since it affects all aminoglycosides, is a stable characteristic, and results in a moderate level of resistance (intermediate susceptibility). Aerobic G (-) bacilli in general also show a phenomenon of adaptive resistance [transiently reduced antimicrobial killing in originally susceptible bacterial (Karlowsky *et al.*, 1994; Xiong *et al.*,

1997)]. Membrane protein changes and alteration in the regulation of genes of the anaerobic respiratory pathway in bacteria exposed to aminoglycosides are probably responsible for this phenomenon. This gives a pharmacodynamic rational for high dosages associated with long intervals between successive administrations (Daikos *et al.*, 1991). Active efflux has been evidenced for neomycin, kanamycin, and hygromycin A in *Escherichia coli* (Edgar and Bibi, 1997)

**Aminoglycoside-modifying enzymes**

Aminoglycoside-modifying enzymes catalyze the covalent modification of specific amino or hydroxyl functions, leading to a chemically modified drug. This drug binds poorly to ribosomes and for which the EDP-II of accelerated drug uptake also fails to occur, thereby resulting most often in high-level resistance. The enzymes modifying aminoglycosides are *N*-acetyltransferases *(AAC)*, which use acetylcoenzyme A as donor and affect amino functions, and *O*-nucleotidyltransferases *(ANT)* and *O*-phosphotransferases *(APH)*, which both use ATP as donor and affect hydroxyl functions (Shaw *et al.*, 1993).

Aminoglycoside-modifying enzymes are often plasmid encoded but are also associated with transposable elements. Plasmid exchange and dissemination of transposons facilitate the rapid acquisition of a drug resistance phenotype not only within a given species but also among a large variety of bacterial species. In epidemiological surveys aminoglycoside resistance mechanisms have first been ascertained by examining the susceptibility of the isolates to a panel of

clinically used and experimental aminoglycosides with specific susceptibilities to aminoglycoside modifying enzymes (Shaw *et al.*, 1993). Such studies quickly led to the recognition of a large diversity of phenotypes with almost every susceptible position in each drug being modified by several distinct enzymes. With the development of molecular biology techniques a considerably large number of genes have been characterized so that each phenotype has now been associated with the expression of several distinct proteins with the same aminoglycoside-modifying activity (Shaw *et al.*, 1993). Large variations in substrate specificity may develop from a few and sometimes a single amino acid change in the protein (Rather *et al.*, 1997; WU *et al.*, 1997). Moreover, several genes could derive from one or a few single common ancestors, suggesting a large plasticity in the type of activities a bacterium may express. It is therefore anticipated that bacteria will quickly catch up to or defeat the efforts at making a given aminoglycoside resistant to inactivation by a specific enzyme. This insight also places the existence of aminoglycoside-modifying enzymes in a new perspective. It is indeed likely that these enzyme have physiological functions in bacteria (acetylation, nucleotidylation or phosphorylation of natural substrates) and that their activities against aminoglycosides in the wild-type strains are simply too weak to confer a phenotype of resistance. Yet, these proteins may be overexpressed under the pressure of aminoglycosides (Wright and Ladak, 1997; Rather *et al*, 1997). In some cases, aminoglycosides may still remain poor substrates compared to the natural ones, but a moderate overexpression will

nevertheless be enough to cause low-level resistance and further selection of the so-called intermediate susceptibility strains. These strains often fail to be clearly recognized in routine microbiological testing unless their minimum inhibitory concentrations (MICs) are determined. Their presence may explain clinical failures seen with patients with low peak levels (Moore *et al.*, 1987) of aminoglycosides in serum.

**Nomenclature of genes encoding aminoglycoside-modifying enzymes**

The availability of molecular techniques has enabled researchers to distinguish more accurately the various genes involved in aminoglycoside resistance and to study more in depth the heterogeneity of these genes. Genetic analysis has revealed a high degree of heterogeneity in the genes encoding these modifying enzymes. Inevitably, this has led to a very complex situation that has generated some confusion and controversy particularly when it concerns the nomenclature of these genes. Moreover, the picture has become even more intricate by the use of two systems of nomenclature. In the standard nomenclature for bacterial plasmids proposed by Novick *et al.* (1976) the genes encoding the aminoglycoside acetyltransferases, nucleotidyltransferases and phosphotransferases are designated *aac*, *aad* and *aph*, respectively. In the family of the acetylating enzymes *aacA* stands for a 6`-N- acetyltransferase, while *aacB* and *aacC* stand for a 2`-N-acetyltransferase and a `3-N-acetlytransferase respectively. This designation can be followed by a number to indicate a subdivision. In this nomenclature, *aacA7* is supposed to be the seventh

discovered encoding a 6`-N-acetyl transferase enzyme. In the second widely used nomenclature (Shaw *et al.*,1993) the gene designation is written in the same way as in the enzyme designation, but in italic and lowercase characters. In this nomenclature the *aacA* gene would be described as an *aac(6`)* gene. This designation can be followed by a roman numeral to indicate a subdivision and a lowercase character to distinguish the various genes encoding a particular enzyme. Hence, the *aac(6`)-Im* gene is the 13$^{th}$ gene encoding a 6-N-acetyltransferase type I enzyme. It is clear that the simultaneous use of two systems of nomenclaure can be the cause of misunderstandings and confusion. Indeed, a given designation can refer to various genes and vice versa.

**PCR detection of genes coding for amino glycoside-modifying enzymes**

The polymerase chain reaction (PCR) is an *in vitro* technique, which allows the amplification of a specific Deoxy Ribonucleic Acid (DNA) region that lies between two known DNA sequences.

PCR amplification of DNA is achieved by using oligonucleotide primers, also known as amplimers. These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA. The primers are extended on single stranded denatured DNA (template) by a DNA polymerase, in the presence of deoxynucleoside triphophates (dNTPs) under suitable reaction conditions.This results in the synthesis of new DNA strands complementary to the template . Strand synthesis can be repeated by heat denaturation of double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at the optimal temperature of the enzyme reaction. Each repetition of strand synthesis comprises a cycle of amplification.

Potentially, after 20 cycles of PCR there will be $20^{20}$ fold amplification, assuming 100% efficiency during each cycle (Newton and Graham, 1994)

PCR products are generally less than 10 kbp in length. Many techniques can be used to detect and confirm the identity of the amplified species. Normally the simplest and most commonly used method is electrophoresis of an aliquot of the PCR product on an agarose or polyacrylamide gel and visualization by staining with ethidium bromide, which is a fluorescent dye that intercalates into

the DNA. After staining, ultraviolet transillumination allow visualization of the DNA in the gel (Newton and Graham , 1994)

The gel may be photographed to provide a permanent record of the experimental result. Size markers and previously amplified control PCR products can be electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons.

PCR fragments can be detected and / or confirmed using various methods:

1. Southern blot hybridization or dot blot hybridization and detection with radioactive or non-radioactive specific probes (Newton and Graham, 1994).
2. PCR products can be labeled during the amplification reaction, for example by the addition of labeled dNTPs, or by addition of $^{32}P$- labeled, fluorescent or biotinylated primers to the reaction (Newton and Graham, 1994).

Restriction fragment length polymorphisms (RFLPs) are used routinely in diagnostic laboratories for following the inheritance of genes or linked gene markers. Normally total genomic DNA is used, however with the advent of PCR it is possible to amplify from genomic DNA a short region of DNA surrounding the restriction site of interest followed by restriction digestion of the product. Restriction enzyme digestion of a PCR product can also confirm the identity of the amplified region. (Geha *et al*, 1994; Weller *et al* 1997; Wallet *et al*,1996).

**Potential advantages of genetic susceptibility testing methods:**

For several reasons, genetic methods, compared to conventional susceptibility methods, have the potential to provide a more rapid and reliable assessment of antimicrobial resistance. (i) Genetic susceptibility testing methods can be performed directly with clinical specimens obviating the need for isolation of the organism by culture.(ii) These methods assess the genotype of the organism, whereas conventional susceptibility techniques asses the phenotype or expression of the genotype under artificial or laboratory conditions. Although debate exists among authorities as to which of these assessment is more clinically relevant, it seems reasonable that the lowest-risk approach for the patient is to determine the genotype. This may be especially true if one is dealing with serious life-threatening infections such as meningitis or bacteremia or infections such as endocarditis or osteomyelitis, which require prolonged courses of antimicrobial therapy. (iii) In some cases, genotypes may be discerned long before phenotypes can be determined due to the slow growth of the organism. (iv) Some organisms cannot be cultured or are not easily cultured and so only genotypes can be determined in these cases. (v) Genetic methods may lessen the biohazard risk which may occur with the propagation by culture of a microorganism, a requirement for conventional test methods (Franklin, 1999).

Accordingly we have set the aims of our work as follows:

1. Collection of clinical isolates from the Jordanian patient.
2. Identification of the bacterial isolates using the biochemical classification kit.
3. Determination of the antibiotic sensitivity, using the disk diffusion method for each of the bacterial isolates.
4. Determination of the minimum inhibitory concentration of the antibiotic to which the bacterial isolate is believed to be resistant to according to the antibiotic disk diffusion result (McCartney, 1996)
5. PCR detection of the resistance gene, using a set of primers, that detect genes coding for different *aacA* isoenzymes that are present in G(-) bacteria.
6. Determination of the specificity of the amplification reaction enzyme digestion of the PCR products.
7. To determine whether the *aacA* enzyme is chromosomal or extrachromosomal(if possible) by:
    a- Isolation of plasmid DNA for each of the resistant bacterial strains.
    b- Transformation of the bacterial plasmids into *E.coli* DH5 competent bacterial cells which lack the antibiotic resistant plasmid.

# 2.Materials and Methods

## 2.1 Materials

### 2.1.1 Bacterial strains

All bacterial isolates from patients admitted to the Jordan University Hospital (teaching hospital with in-and-out patient facilities). The samples were either urine, blood, pus catheter, wound, bile, sputum, seminal or urethra swab.

Bacterial strain *E.coli* DH5 ATCC 53865 [F⁻ *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*1 *hsd*R17 *sup*E44λ⁻ (Hanahan, 1983)] was purchased from American Type Culture Collection (U.S.A)

### 2.1.2 Antibiotic discs

Disc diffusion method was adopted to provide a simple and reliable test specially applicable in routine clinical bacteriology (22 antibiotics were used)(Table 1). Antibiotic discs used were purchased from Medical Wire & Equipment (UK)

### 2.1.3 Antibiotics and media

Kanamycin was purchased from Medix (UK) . Nutrient broth agar, Nutrient agar, MacConkey agar and Mueller Hinton agar were purchased from Hi Media (India).

**2.1.4** API 20 E (Biochemical Identification System for Enterobacteriacae and other G(-) rods) was purchased from bioMerieux laboratories (France)

### 2.1.5 Electrophoresis Chemicals

Agarose, ethidium bromide (10mg/ml) and 1Kb DNA molecular size marker were purchased from Bio-Rad Laboratories (USA), Tris borate-EDTA buffer solution was purchased from Fluka Chemika Co., (Switzerland) and 100 bp DNA molecular size marker was purchased from Promega (USA).

### 2.1.6 PCR primers and chemicals

- Nuclease free water purchased from Promega (USA)
- PCR core system II purchased from Promega (USA); it includes :

1. Taq DNA polymerase 10X Buffer, W/15 m M $MgCl_2$
2. PCR Nucleotide Mix, (10mM) 40m M (10mM each of dATP, dCTP, dGTP, dTTP ) 200 µl.
3. Upstream control primer, 15µM (100µl).
4. Down stream control primer, 15µM (100µl).
5. Positive control plasmid DNA 1ng/µl in TE Buffer (100 µl)

- Taq DNA polymerase. Storage Buffer B, 5 units/ µl, 20mM Tris HCl (pH8 at 25°C) 100mM KCl, 0.1 mM EDTA, 1mM DTT, 50%. Glycerol, 0.5%. Tween ®20, and 0.5% Nonidet P40, (250 unit), purchased from Promega (USA).
- pBK - CMV plasmid (Invitrogen, UK)

**PCR Primers listed below were purchased from Operon (USA)**

All primers were dissolved in 1 ml deionized nuclease free water (Van De klundert and Vleigenthart, 1993)

| | Length | OD | pmol | µg |
|---|---|---|---|---|
| **Name rrs1** | | | | |
| Seq (5`-3`)<br>GGATTAGATACCCTGGTAGTCC | 22 | 10 | 47257.55 | 318 |
| **Name rrs2** | | | | |
| Seq (5`-3`)<br>TCGTTGCGGGACTTAACCCAAC | 22 | 11.5 | 55736.31 | 372 |
| **Name aacC1-1** | | | | |
| Seq (5`-3`)<br>ACCTACTCCCAACATCAGCC | 20 | 11.3 | 62058.89 | 368 |
| **Name aacC1-2** | | | | |
| Seq (5`-3`)<br>ATATAGATCTCACTACGCGC | 20 | 11.5 | 59696.69 | 360 |
| **Name aacC2-1** | | | | |
| Seq (5`-3`)<br>ACTGTGATGGGATACGCGTC | 20 | 11.5 | 59313.05 | 365 |

| | Length | OD | pmol | µg |
|---|---|---|---|---|
| **Name aacC2-2** | | | | |
| Seq (5`-3`)<br>CTCCGTCAGCGTTTCAGCTA | 20 | 10.4 | 59029.07 | 355 |
| **Name aacC3-1** | | | | |
| Seq (5`-3`)<br>CACAAGAACGTGGTCCGCTA | 20 | 10.9 | 55100.51 | 335 |
| **Name aacC3-2** | | | | |
| Seq (5`-3`)<br>AACAGGTAAGCATCCGCATC | 20 | 12.3 | 61154.14 | 371 |
| **Name aacC4-1** | | | | |
| Seq (5`-3`)<br>CTTCAGGATGGCAAGTTGGT | 20 | 9.2 | 47123.81 | 290 |
| **Name aacC4-2** | | | | |
| Seq (5`-3`)<br>TCATCTCGTTCTCCGCTCAT | 20 | 9.6 | 58622.38 | 348 |
| **Name aadC-1** | | | | |
| Seq (5`-3`)<br>GCAAGGACCGACAACATTTC | 20 | 11.9 | 59165.39 | 359 |

| | Length | OD | pmol | µg |
|---|---|---|---|---|
| **Name aadC-2** | | | | |
| Seq (5'-3)<br>TGGCACAGATGGTCATAACC | 20 | 10.6 | 53222.41 | 325 |
| **Name aacA-aphD-1** | | | | |
| Seq (5'-3)<br>CCAAGAGCAATAAGGGCATA | 20 | 11.7 | 53618.02 | 329 |
| **Name aacA-aphD-2** | | | | |
| Seq (5`-3`)<br>CACTATCATAACCACTACCC | 20 | 11.8 | 61885.87 | 369 |
| **Name aphA3-1** | | | | |
| Seq (5`-3`)<br>GCCGATGTGGATTGCGAAAA | 20 | 11.7 | 56513.06 | 349 |
| **Name aphA3-2** | | | | |
| Seq (5`-3`)<br>GCTTGATCCCCAGTAAGTCA | 20 | 10.1 | 53346.09 | 323 |

**2.1.7.** Restriction enzymes p urchased from Promega (U.S.A)

*Cla* I restriction enzymes, 500units. Concentration 10units/µl Buffer C, supplied with this enzyme (100mM Tris HCl (pH7.9), 500mM NaCl, 100m M $MgCl_2$, and 10mM DTT at 37°C.

*Eco* RV Restriction enzyme, 2000 units. Concentration 10 units /µl Buffer D supplied with this enzyme (60 mM Tris-HCl (pH 7.9), 1.5 M N aCl, 60mM $MgCl_2$, and 10mM DTT at 37°C.

*Bst* EII Restriction Enzyme, 2000 units. Concentration 10 units /µl Buffer D supplied with this enzyme.

*Ssp* I Restriction Enzyme, 500units. Concentration 10 units /µl Buffer C, supplied with this enzyme (100mM Tris HCl (pH7.9), 500mM NaCl, 100m M $MgCl_2$, and 10mM DTT at 37°C.

Bovin albumin purchased from Promega (U.S.A)

**2.1.8.**Miscellaneous chemicals

The following chemicals were of analytical grade and used directly without any further pruification.

Calcium chloride dihydrate ($CaC1_2.2H_2O$) 99%, glacial acetic acid 99%, ether and chloroform 99%, all were purchased from Gainland Chemical Company (U.K).Tris base ($C_4H_{11}NO_3$) molecular biology grade was purchased from Promega, (USA).Sodium Hydroxide (NaOH), Sodium dodacyl sulphate ($CH_3(CH_2)_{11}OSO_2ON_a$) (SDS) were purchased from

Merck-Schuchardt, (Germany).Phenol solution approx, 90% aqueous solution was purchased from Schalau (European Union).Ethylene diamino tetra-acetic disodium salt dihydrate ($C_{10}H_{14}N_2NaO_8.2\ H_2O$) and methanol ($CH_3OH$) HPLC grade were purchased from Fluka Chemika Co., (Switzerland).Glucose monohydrate and PEG 6000 were purchased from Riedel-de Haen, (Hannover).Ethanol absolute ($CH_3CH_2OH$) was purchased from Panreac (Spain).

## 2.2. Apparatus

The following equipment were used throughout the work:-

1. GeneAmp PCR system 9600, Perkin Elmer (USA.)
2. Biometra electrophoresis power pack p25, made by Biotron, (Germany)
3. Gel Documentation System, UVP, (U.S.A)
4. Portable Express Equipment autoclave, made by Dixons Surgical Instrument, LTD (UK)
5. Incubator WTB Binder (Germay).
6. Orbital incubator SI 50 (shaking incubator) Stauart Scientific (U.K).
7. pH Meter, WPA Cambridge (U.K)
8. GFL water bath (Germany)
9. Laboratory centrifuge R&C Remi Equimpment (India).
10. Refregerated (Hettich) centrifuge, EBA 12R

11.Laminar flow, Holten Laminar Air ( Denemark )

12.Sartorius Analytical Balance (Germany).

13.Carry U.V-visible spectrophotometer, Varian, pty. Ltd, (Australia)

## 2.3. Experimental methods

### 2.3.1 Bacterial methodology

All samples were collected from patient admitted to Jordan University Hospital (teaching hospital with in and outpatient facilities). The samples were either urine, blood, pus, catheter, wound, bile, sputum, seminal or urethra swab. The samples were streaked onto nutrient or MacConkey agar at 37°C for 24 hr. *Enterobacteriaceae* and other G(-) bacteria were identified (see page 55 ). The antimicrobial susceptibility tests were carried out with Mueller-Hinton agar using the standard antibiotics for the disc diffusion method.

#### 2.3.1.1 Antimicrobial susceptibility test:

**Disk diffusion method**

Each plate of nutrient agar received 0.5ml of one of the assigned cultures. The inoculum was spread evenly over the agar surface using a sterile glass spreader. The agar surface was allowed to dry for 3-5 minutes before the antibiotic discs (A,B,C or D) were applied.

Using a sterile forceps and under aseptic condition, one ring of antibiotics was plated on each inoculated agar surface, and pressed gently with the tip of the forceps to ensure intimate contact. The plates were incubated at 37°C for 24 hours in the up-right position. The inhibition zones surrounding the antibiotic discs were measured with a

metric roller to the nearest millimeter. The resistance (R) or susceptibility (S) of each bactrium to the antibiotics were recorded.

**Table 1: Antibiotic disks used in the Disk Diffusion Method**

| A | | B | |
|---|---|---|---|
| Ampicillin | 10μg | Ampicillin | 1μg |
| Cephalothin | 30μg | Cephalothin | 30μg |
| Chloramphenicol | 30μg | Cotrimoxazde | 25μg |
| Clindamycin | 2μg | Gentamicin | 10μg |
| Erythromycin | 15μg | Nalidixic acid | 30μg |
| Gentamicin | 10μg | Nitrofurantoin | 300μg |
| Oxacillin | 1μg | Norfloxacin | 10μg |
| Vancomycin | 30μg | Tetracycline | 25μg |
| **C** | | **D** | |
| Amikacin | 30μg | Amikacin | 30μg |
| Ampicillin | 10μg | Pipericillin | 100μg |
| Cefoxitin | 30μg | Imipenem | 10μg |
| Ceftazidime | 30μg | Carbenicillin | 30μg |
| Ceftriaxone | 30μg | Ceftazidime | 30μg |
| Chloramphenicol | 30μg | Cefotaxime | 30μg |
| Gentamicin | 10μg | Gentamicin | 30μg |
| Pipericillin | 100μg | Netilimicin | 30μg |

### 2.3.1.2 Minimum inhibitory Concentration (MIC)

*In vitro* susceptibility of the 17 G (-) isolates (see page 55) *(Pseudomonus spp., Escherichia spp., and Enterobacter spp.)* to kanamycin (see page 56; Appendix 1) was tested using agar dilution method described by (McCartney, 1996). Nutrient broth (5ml) was inoculated with organisms and adjusted to the turbidity of Mcfarland 0.5 turbidity standard. This turbidity was equivalent to $10^7$ CFU/ml.

Cultures were then used for inoculation of plates containing different concentrations of antibiotic. An inoculator delivering (1-2µl) bacterial suspension was used for inoculation. Inoculated plates were incubated at 37°C for 24 hr. Minimum inhibitory concentration (MIC) was read as the lowest concentration of an antibiotic at which complete inhibition occurs or allows growth of less than ten colonies of the bacterial strains/plate .

### 2.3.1.3. Identification of the G (-) bacteria, using the API 20E biochemical Kit.

The 17 G(-) isolates were identified using the API 20E (bioMerieux) which consists of standardized and miniaturized biochemical tests and a data base as an identification system. The API 20E biochemical tests contain dehydrated substrates in separate wells (ortho-nitro-phenyl-β-galactopyranoside, arginine,

lysine, ornithine, sodium citrate, sodium thiosulfate, urea, tryptophane, sodium pyruvate, kohn's gelatin, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose). These wells were inoculated with a bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of the kit provided reagents. The reactions are read according to the specified tables (negative or positive).Then the identification was obtained by referring to the API 20E Analytical Profile index.

#### 2.3.1.4 Rapid, small scale isolation of plasmid DNA

The method described By Sambrook *et al.*, (1991) was used for isolation of plasmid DNA:

1. A single colony of 2mm diameter was picked from each kanamycin containing plate of the different subcultured bacteria. The picked colony was inoculated to 5ml nutrient broth containing 50µg/ml of the corresponding antibiotic and grown over 16 hrs in a shaker incubator at 37°C .
2. The cells were harvested at 2500 rpm at 4°C for 10 minutes and the pellets resuspended in 120µl solution (I) Appendix (1), using eppindorf tubes. After vortexing, the mix was incubated at RT. for 5 minutes.
3. 400µl solution (II) Appendix (1), was added and mixed by inverting the eppindorf tube, then the tubes were incubated on ice for 10 minutes

4. 300µl solution (III) Appendix (1) was added, mixed, and the tubes incubated on ice for a further 10 minutes.
5. The samples were centrifuged for 5 minutes.
6. To 900µl supernatant, 500µl [phenol: chloroform (1:1) ] was added. The tubes were then centrifuged for 3 minutes.
7. To 720µl aqueous phase, 0.43ml isopropanol was added and the tubes centrifuged at room temperature.
8. The pellets were washed with 70% ethanol, and repelleted for 3 minutes.
9. The pellets were dried, and resuspended in (1:10) TE Buffer.
10. The samples were run on 0.7% agarose gel, and the gel photographed at the end of electrophoresis.

### 2.3.1.5. Agarose gel electrophoresis

In order to prepare an agarose gel, the following had to be followed: (Sambrook *et al.* ,1991)

1. The gel was 0.7 –2 % (w/v) agarose prepared in 150 ml borate-tris buffer. The agarose was heated in a boiling water bath until it dissolved.
2. The solution was cooled to 50°C and one drop of ethidium bromide (10mg/ml) was added.
3. The edges of a clean, dry, plastic plate was sealed with special ends, the slurry was pored, the comb was clamped into a position near one end of the

gel leaving 0.5mm of agarose between the bottom of teeth and the base of the gel to ensure that the wells are completely sealed.

4. After 45 minutes, the tray was placed in the electrophoresis tank that contained two liters of the borate-tris buffer after which the comb was removed.

5. In microfuges, 20µl of each plasmid was mixed with 6x loading buffer. Appendix (1).

6. Samples (10-20 µl) were slowly applied to the gel, 1kb size marker loaded in the first and last lane using disposable micropipette tip and automatic 10 µl micropipettor.

7. The gel was run at 20 mV for 20 hr. and photographed.

### 2.3.1.6 Bacterial Transformation

The method described by Sambrook *et. al.* ,1991 was used for transformation of *E.coli* DH5 strain with two different plasmids, obtained from the following strains:

A. *E.coli* #1, hospitals isolate.

B.*E-coli* #2, hospitals isolate.

1- The initiation of the DH5 culture was started directly from the stock of the bacterial strains, on nutrient agar. A single colony of the bacterial strain was cultured in 5ml of nutrient broth for 24 hours at 37°C. Each culture was scaled up to 100ml of nutrient broth for further 3 hours at 37°C. O.D. was

measured at 600 nm every 30 minutes until it reached 0.6 at which the following process started.

2- Aseptically the cells were transferred to a sterile 50ml glass centrifuge tube, cooled to 0°C by storing them in ice for 10 minutes.

3- The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C in R&C laboratory centrifuge.

4- The media was decanted from the cell pellets, and the tubes were inverted for 1 minute to allow the last traces of media to drain.

5- Pellets were resuspended in 10 ml of ice-cold 0.1M $CaCl_2$ and stored in ice for 15 minutes.

6- The cells were recovered by centrifugation at 4000rpm for 10 minutes at 4°C in the refrigerated centrifuge (Hettich).

7- The fluid from the cell pellet was decanted and the tube kept in an inverted position for 1 minute to allow the traces of fluid to drain away.

8- Pellets of DH5 were resuspended in 4ml of ice-cold 0.1M $CaC1_2$ (2 ml for each 50ml of original culture) resulting in competent cells.

9- Two tubes were labeled, each received 200µl competent DH5. One tube DH5 received 1 µg *E.coli* plasmid DNA #1 .The second DH5 received 1 µg *E.coli.* plasmid DNA #2. The 200 µl of each competent cell suspension were transferred by a chilled sterile pipette tip to a sterile microfuge tube.

10- The tubes were transferred to a water bath set at 42 °C for exactly 90 seconds, with out shaking.

11- The tubes were rapidly transferred to an ice bath to allow the cells to chill for 1-2 minutes. In order to be subjected to heat-cold shock.

12- 800µl of liquid broth were added to each tube, and the cultures were then incubated for 45 minutes in a water-bath adjusted to 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmids.

13- Each tube was plated on two nutrient agar plates one with 50µg/ml kanamycin and the other with no antibiotic as control.

14- The plates were incubated at 37°C for 24 hrs.

### 2.3.1.7. Optical density (O.D.) measurement of Double-Stranded DNA Plasmid (spectrophotmetric determination):

For quantitating the amount of DNA, OD readings should be taken at wavelengths of 260 and 280nm. The reading at 260 nm allows the calculation of the concentration of nucleic acid in the sample. As 1 $OD_{260}$ corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA and 20 µg/ml for oligonucleotides. The ratio between the readings at 260 nm and 280 nm ($OD_{260}$ / $OD_{280}$) provides and estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have $OD_{260}/OD_{280}$ of 1.8 and 2.0 respectively. If there is contamination with protein or phenol, the $OD_{260}/OD_{280}$ will be significantly less than the

values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

10 µl of each prepared plasmid was diluted to 1000 µl of TE-buffer pH8 in an autoclaved eppendorf tube in order to get 1 ml of each plasmid. The 1ml quartz cells were used, and the readings were taken at both wave length for each plasmid (Sambrook *et al.* ,1991).

### 2.3.2. Polymerase chain reaction (PCR)

1. PCR master mixture was prepared according to the recipe listed below:

- 0.2 µl(1 unit)Taq DNA polymerase .
- 0.2 µl PCR Nucleotide Mix, 10 mM
- 6 µl Taq DNA polymerase 10 x buffer with 15mM $MgCl_2$
- 4 µl of plasmid DNA prepared by the miniprep method was added followed by 12µl (500- 700 pmol ) from each primer (down stream, upstream).
- Volume was made up to 60µl with nuclease free water.

2. PCR negative control contained the PCR reagent mix and nuclease free water instead of DNA.

3. PCR positive control contained PCR reagent and the plasmid carrying antibiotic marker (neo gene).

4. The DNA was denatured for 3min at 94°C in a thermal cycler, and the fragments were amplified for 32 cycles of 30s at 94°C, 45s at 60°C, and 2 min at 72°C. The mixture was cooled and stored at 4°C

5. Colony PCR (Van De klundert & Vliegenthart , 1993) was also carried out , in which the DNA template was prepared directly as listed in the following procedure:

   a. 10 colonies were suspended in 500µl of sterile phosphate buffer saline.

   b. The suspension was centrifuged for 2 min at 4500 rpm

   c. The supernatant was removed with a drawn-out Pasteur pipette.

   d. The pellet was suspended in 120µl of distilled water.

   e. The suspension was heated for 10 min at 94°C. (GeneAmp PCR system 9600, Perkin Elmer).

   f. 20 µl of cell lysate containing chromosomal DNA and plasmid was used as a template in 60 µl of PCR mixture

6. Each of the PCR amplification reactions were slowly applied to a previously prepared 2% agarose gel, the samples were run at 100 mV for 2 hrs. using 100bp DNA marker, and photographed.

7. Double-stranded DNA plasmid digestion and Gel electrophoresis. Restriction enzymes analysis of the amplified fragments was done to test for the specificity of the amplification reaction.

### 2.3.3 Restriction enzyme digestion

Restriction digestion were carried out as follows:

10µl of the amplified PCR fragment

2 µl Enzymes (*Cla* I, *Eco* RV, *Bst* EII *or Ssp* I)

2 µl 10x Buffer

0.2 µl Bovine Serum albumin

5.8 µl autoclaved water

The above mixture in microfuge tubes were placed in water bath set at 37°C over 18hr after which 3µl of 6x loading buffer was added to each tube then loaded into 2% agarose gel. Samples were slowly applied to the gel, 100bp size marker was loaded in the first and last lanes using disposable micropipette. The gel was run at 20 mV for 20 hr and photgraphed. (The undigested amplicons were run along side the digested ones).

## 3. Results and Discussion

During a period of six months, a total of 116 bacterial isolates were collected and 22 different antibiotics were used to test the susceptibility of these isolates to them using the disk diffusion technique. Table 2 lists the antibiotics used and their abbreviations. The bacterial isolates were collected from different clinical specimens to include: bile, blood, catheter, pus, seminal, sputum, urine, wound and urethra swabs. Clinical isolates were identified by routine standard biochemical tests. Table 3 shows the bacteria distribution with reference to the source of the samples. *Escherichia spp.* as G(-) and *Staphylococcus spp.* as G(+) were the most common type of bacterial infection in the isolates collected.

Table 4 represents the resistance profile of the 3 G(+) and 6 G(-) bacteria used in the study. G(+) bacteria used in this study; *Staphylococcus spp.*, *Enterococcus spp.* and *Micrococcus spp.* are 16.7, 42.9 and 50%, respectively, resistant toward the commonly used antibiotic, Gentamycin. *Micrococcus spp.* was 100% resistant to Amp, Cxt, and Caz. In addition *Micrococcus spp.* is resistant toward Imp (75%) and 50% resistant toward Ctr and Pip. *Staphylococcus spp* have shown resistance toward Ery (20.8%), Oxc (16.7%), Cln (12.5%), Clt (8.3%) and 4.2% resistance toward Amp, Imp, Nal, and Nor. *Enterococcus spp.* showed 28.6% resistance toward Cln, Ery, Nal and 14.3% resistance toward Cot, Oxc and Tet (Figure 1, Table 4). The most resistant G(+) bacterium toward 7 of the used antibiotics was found to be *Micrococcus*

*spp.* Figure 2,3 and Table 4 summarizes G(-) bacteria growth profile in the presence of the different antibiotics. All the G(-) bacteria used in the study have expressed resistance towards Amp, one of the most commonly prescribed antibiotics. *Klebsiella spp.* and *Proteus spp.* expressed the resistance profile which ranged from 40 to 100%, respectively. *Ecsherichia spp.* showed the highest resistant profile toward Amp (58.6%), Tet (34.4%), Cot (27.5%) and Nal (24.1%). In addition it showed resistance of 6.9% toward Clt and 3.4% toward Amk, Chl, Nor, and Pip. *Enterobacter spp.* showed resistance toward most of the antibiotics used: Gen (47.1%), Amp (41.2%), Car (29.4%), Cxt (23.5%), Caz (23.5%), Cot (23.5%), Ctx (17.7%), Net (17. 7%), Amk (11.8%), Clt (11.8%), Imp (11.8%), Nal (5.9%), Nor (5.9%), Tet (5.9%), Ctr (5.1%) and Chl (3.4%), (Figure 2, Table 3). *Pseudomonus spp.* showed resistance toward Amp (37.5%), Net (25.0%), and 18.8% toward Amk, Cxt, Gen and 12.5% toward Imp and Nit. In addition *Pseudomonus spp.* showed 6.3% resistance toward Ctr, Clt, Cot and Chl (6.3%). *Proteus spp.* showed 100.0% resistance toward Amp only (Fig 3). *Klebsiella spp.* showed its highest resistance toward Amp (90.0%) followed by similar level of resistant toward Amk and Gen (36.4%). In addition *Klebsiella spp.* showed resistance toward Ctr (18.2%), Pip (27.3%) and showed similar resistant toward Car, Cot, Net and Tet (9.1%) (Fig. 3). *Acinetobacter spp.* showed highest resistance toward Amk, Amp and Gen (40.0%) and 20.0% resistance toward Ctx, Cxt, Caz, Ctr and Chl.

Table 2: Antibiotics used in the study and their abbreviations.

| Antibiotic | Abbreviation | Antibiotic | Abbreviation |
|---|---|---|---|
| **Amikacin** | Amk | **Erythromycin** | Ery |
| **Ampicillin** | Amp | **Gentamicin** | Gen |
| **Carbenicillin** | Car | **Imipenem** | Imp |
| **Cefotaxime** | Ctx | **Nalidixic Acid** | Nal |
| **Cefoxitin** | Cxt | **Netilimicin** | Net |
| **Ceftazidime** | Caz | **Nitrofurantoin** | Nit |
| **Ceftriaxone** | Ctr | **Norfloxacin** | Nor |
| **Cephalothin** | Clt | **Oxacillin** | Oxc |
| **Chloramphenicol** | Chl | **Pipericillin** | Pip |
| **Clindamycin** | Cln | **Tetracyclin** | Tet |
| **Cotrimoxazole** | Cot | **Vancomycin** | Van |

Table 3: The Distribution of bacterial strains verses the source of the samples.

| Bacteria | Bile | Blood | Catheter | Pus | Swab | Seminal | Sputum | Urine | Wound | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| *Enterococcus* | 1 | | 1 | | 3 | | | 2 | | 7 |
| *Micrococcus* | | 0 | 1 | | 1 | | 1 | | | 4 |
| *Staphylococcus* | 2 | 2 | 2 | 2 | 10 | 1 | 2 | 3 | | 24 |
| *Acinetobacter* | | | 2 | | | | 2 | 1 | | 5 |
| *Escherichia* | | | | 2 | 5 | | | 22 | | 29 |
| *Enterobacter* | 1 | 3 | 1 | 2 | 2 | | 2 | 6 | | 17 |
| *Klebsiella* | | | | 3 | 2 | | 2 | 4 | | 11 |
| *Proteus* | | | | | | | 2 | | 1 | 3 |
| *Pseudomonas* | | 1 | 3 | 2 | 4 | | 1 | 4 | 1 | 16 |
| Total | 4 | 7 | 10 | 11 | 27 | 1 | 12 | 42 | 2 | 116 |

Table 4: Resistance of the bacterial isolates expressed as a percent versus the antibiotic used in the study.

| *Antibiotics* | *Staphylococcus* | *Enterobacter* | *Pseudomonus* | *Escherichia* | *Klebsiella* | *Acinetobacter* | *Enterococus* | *Micrococus* | *Proteus* |
|---|---|---|---|---|---|---|---|---|---|
| **Amikacin** | – | 11.8 | 18.8 | 3.4 | – | 40.0 | – | – | – |
| **Ampicillin** | 4.2 | 41.2 | 37.5 | 58.6 | 90.9 | 40.0 | – | 100.0 | 100.0 |
| **Carbenicillin** | – | 29.4 | – | – | 9.1 | – | – | – | – |
| **Cefoxitin** | – | 23.5 | 18.8 | – | – | 20.0 | – | 100.0 | – |
| **Cefotaxime** | – | 17.6 | 6.3 | – | – | 20.0 | – | – | – |
| **Ceftazidime** | – | 23.5 | - | – | 36.4 | 20.0 | – | 100.0 | – |
| **Ceftriaxone** | – | 5.1 | 6.3 | – | 18.2 | 20.0 | – | 50.0 | – |
| **Cephalothin** | 8.3 | 11.8 | 6.3 | 6.9 | – | – | – | – | – |
| **Chloramphenicol** | – | – | 6.3 | 3.4 | – | 20.0 | – | – | – |
| **Clindamycin** | 12.5 | – | – | – | – | – | 28.6 | – | – |
| **Cotrimoxazole** | – | 23.5 | 6.3 | 27.5 | 9.1 | – | 14.3 | – | – |
| **Erythromycin** | 20.8 | – | – | – | – | – | 28.6 | – | – |
| **Gentamicin** | 16.7 | 47.1 | 18.8 | – | 36.4 | 40.0 | 42.9 | 50.0 | – |
| **Imipenem** | 4.2 | 11.8 | 12.5 | – | – | – | – | 75.0 | – |
| **Nalidixic Acid** | 4.2 | 5.9 | – | 24.1 | – | – | 28.8 | – | – |
| **Netilimicin** | – | 17.6 | 25.0 | – | 9.1 | – | – | – | – |
| **Nitrofurantoin** | – | – | 12.5 | – | – | – | – | – | – |
| **Norfloxacin** | 4.2 | 5.9 | – | 3.4 | – | – | – | – | – |
| **Oxacillin** | 16.7 | – | – | – | – | – | 14.3 | – | – |
| **Pipericillin** | – | – | – | 3.4 | 27.3 | – | – | 50.0 | – |
| **Tetracyclin** | – | 5.9 | – | 34.4 | 9.1 | – | 14.3 | – | – |
| **Vancomycin** | – | – | – | – | – | – | – | – | – |

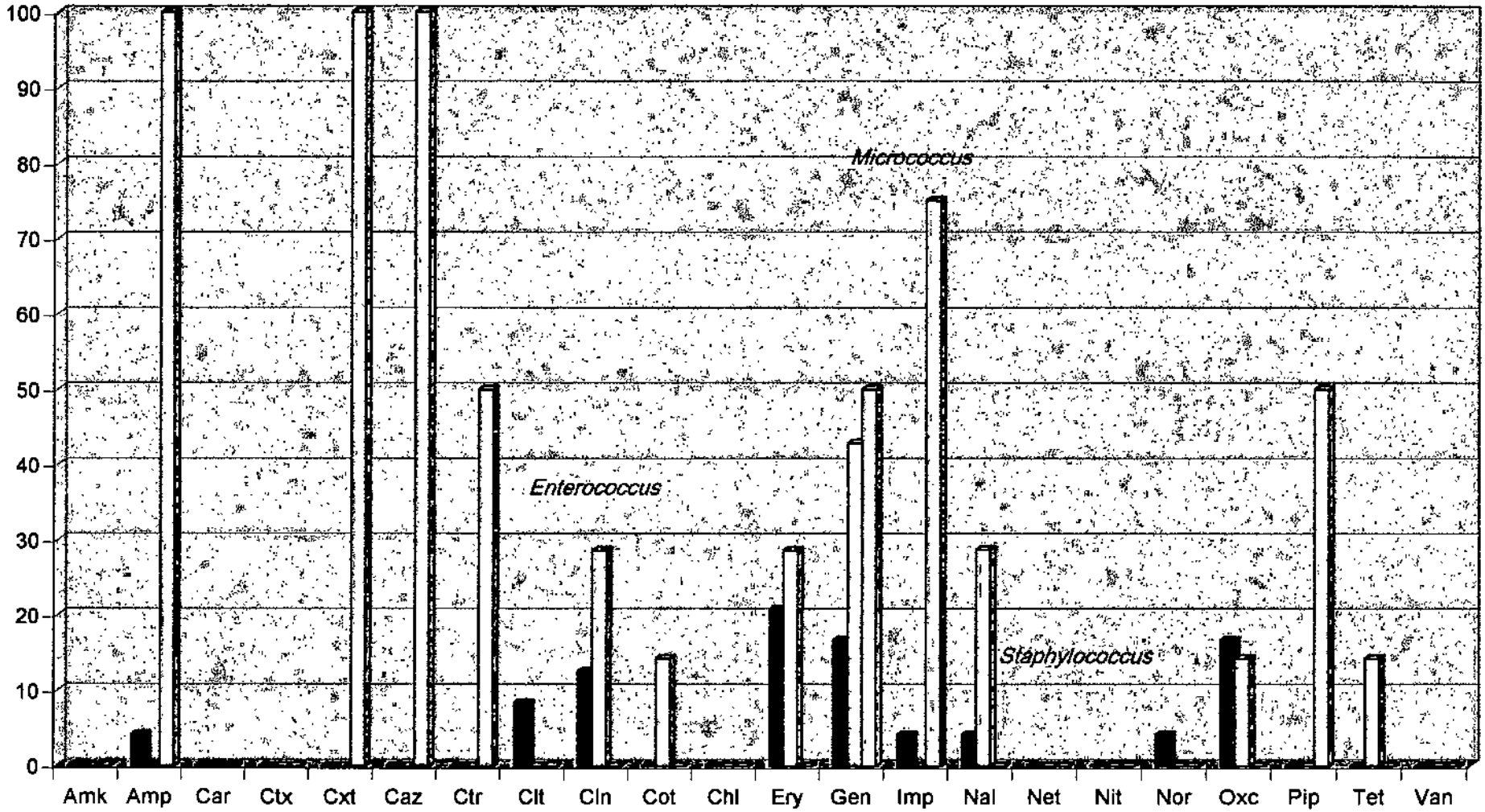


**Figure 1: G(+) Bacterial Growth Profile in the Presence of the Listed Antibiotics**

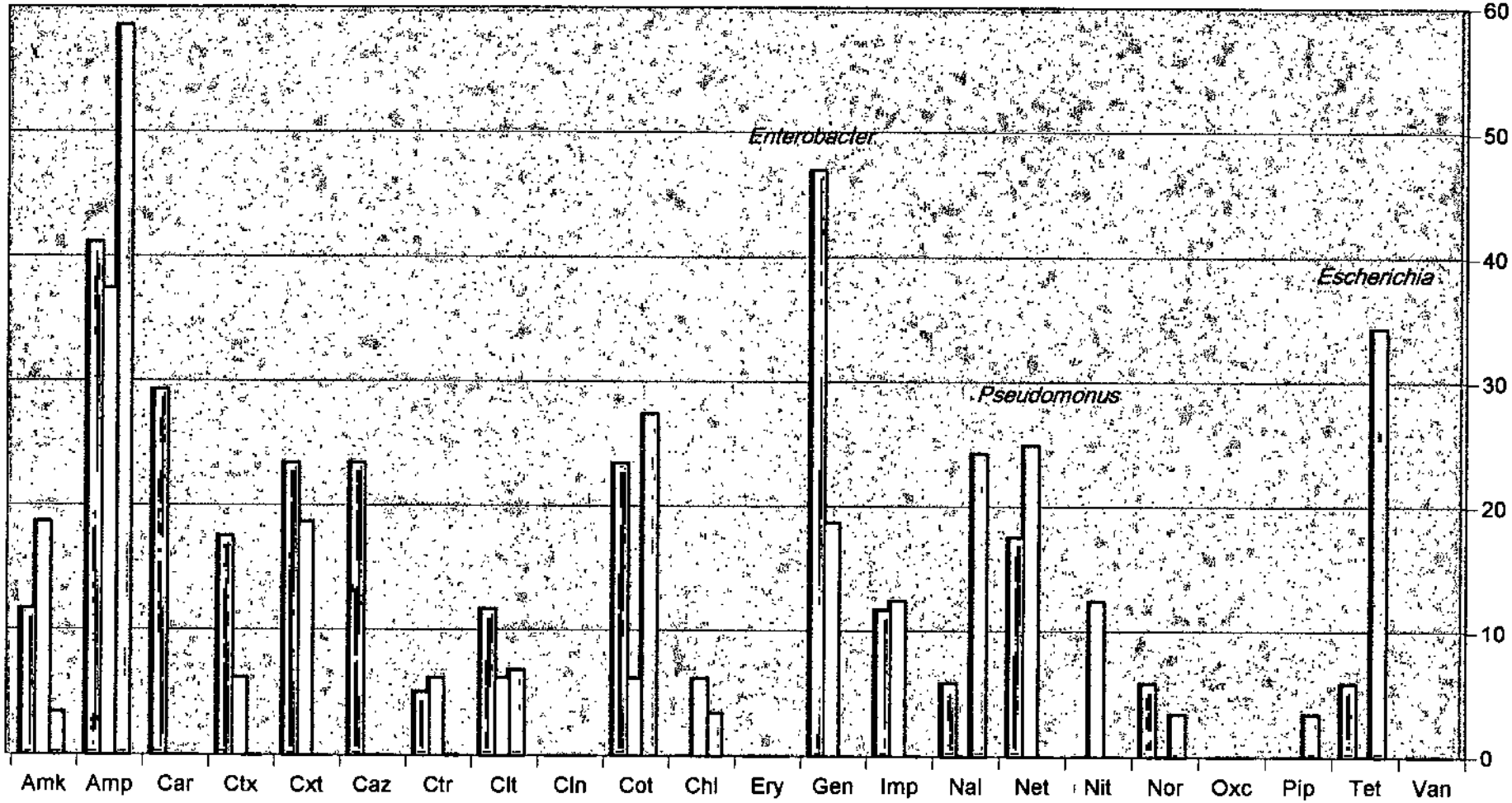


**Figure 2: G(-) Bacterial Growth Profile in the Presence of the Listed Antibiotics**

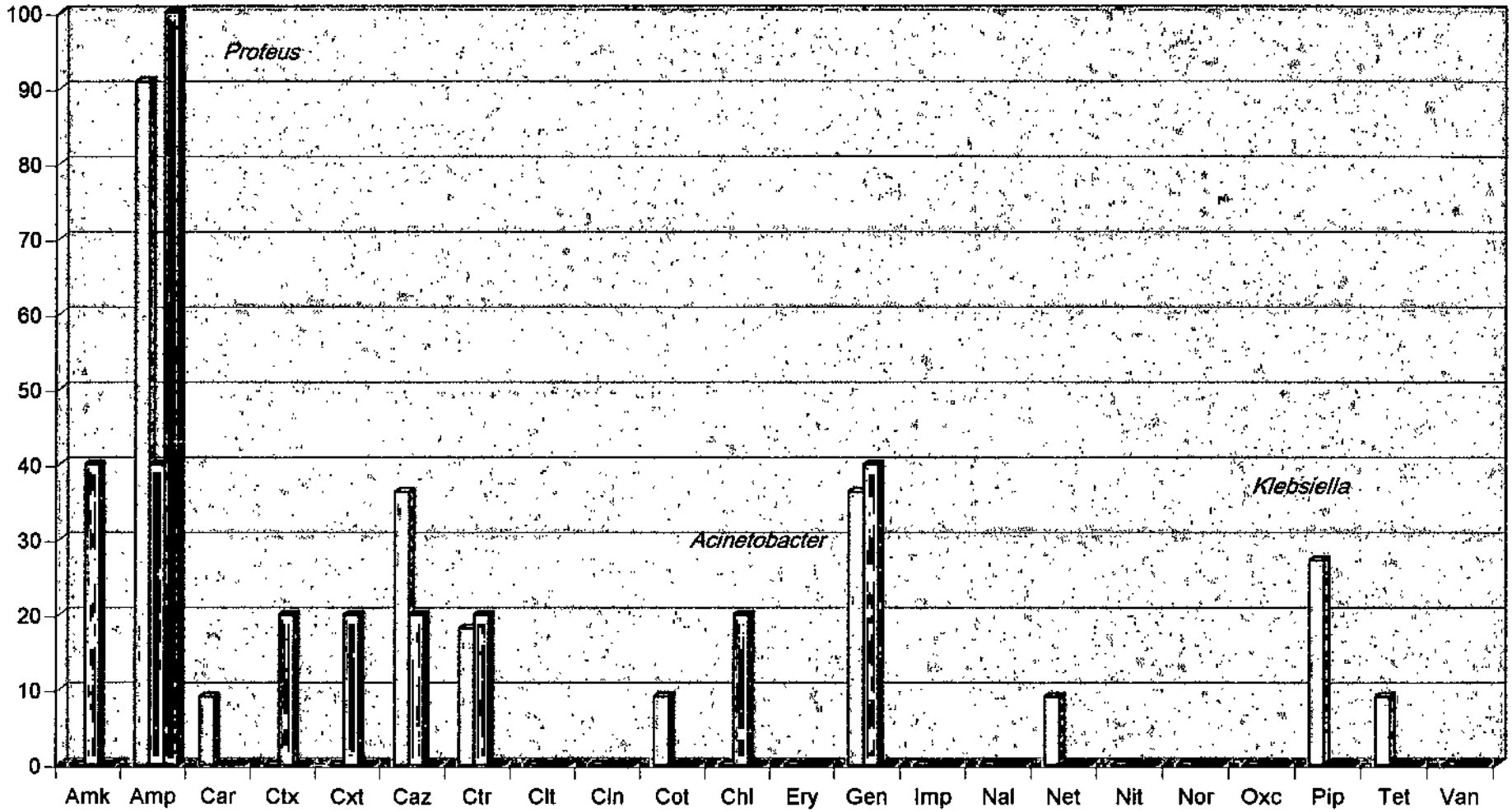


**Figure 3: G(-) Bacterial Growth profile in the Presence of the listed Antibiotics**

Excessive and inappropriate use of antibiotics contributes to the development of antibiotic resistance (Bdour *et al.*, 1985). Aminoglycoside antibiotics often form the cornerstone of effective therapy of sever bacterial infections. They are usually combined with beta – lactam antibiotic, and the agents act synergistically (Van de Klundert and Vleigenthart, 1993) Bacterial resistance against aminoglycosides thus constitutes a major threat to a successful antimicrobial treatment of patients. In most cases resistance is due to the production by bacteria of aminoglycoside- modifying enzyme (AME). To date, there are some 20 different (AME) known (Van de Klundert and Vleigenthart, 1993). In an attempt to determine the pattern of antibiotic resistance in clinical isolates obtained from Jordanian patients, and to design effective antibiotic policies, systematic analysis of resistance mechanisms in clinical isolates is indispensable. Furthermore, such an analysis can be useful for early signaling of an impending epidemic of antibiotic resistance. Detection of genes coding aminoglycoside modifying enzyme in the hospital clinical isolates was carried out using Polymerase Chain Reaction (PCR).

In an attempt to scale down our work we focused on G(-) bacteria since aminoglycoside modifying enzymes were found predominantly in G(-) bacteria. Three species of bacteria were chosen for this study: *Escherishia spp.* (29 islates), *Pseudomonus spp.* (16 isolates) and *Enterobacter spp.* (17 isolates). All the 62 isolates were purified and cultured in 5 ml nutrient broth media

supplemented with 50 µg/ml kanamycin. Since the *neo* gene coding aminoglycoside resistance express resistance to Kanamycin selectively in prokaryotes and gentamicin in eukaryotes. Kanamycin was used for the selection of resistant strains.

Only 17 isolates were grown on the antibiotic containing media. These 17 isolates were identified using API 20E a standardized and miniaturized Biochemical test specially designed for G(-) bacteria. Table 5 summarizes the results. *In vitro* susceptibility of the 17 G (-) isolates to kanamycin was tested using the MIC method. Table 6 shows the lethal concentration of kanamycin to the different isolates.

Plasmid DNA was obtained form the 17 different isolated by mini-preparation (Fig 4) .


**Figure (4) Plasmids mini preparation**

**Table 5: Identification of G(-) bacteria**

| *Isolates* | *Microorganism* |
|---|---|
| *1.* | *Pseudomonas aeruginosa* |
| *2.* | *Pseudomonas aeruginosa* |
| *3.* | *Pseudomonas aeruginosa* |
| *4.* | *Escherichia coli #1* |
| *5.* | *Escherichia hermonii* |
| *6.* | *Enterobacter gergoviae* |
| *7.* | *Escherichia fergusonii* |
| *8.* | *Escherichia coli #2* |
| *9.* | *Escherichia fergusonii* |
| *10.* | *Escherichia coli #3* |
| *11.* | *Proteus penneri* |
| *12.* | *Enterobacter cloacae* |
| *13.* | *Enterobacter aerogenes* |
| *14.* | *Klebsiella oxytoca* |
| *15.* | *Enterobacter amnigenus* |
| *16.* | *Escherichia coli #4* |
| *17.* | *Enterobacter sakazakii* |

**Table 6: *In Vitro* susceptibility of the 17 G (-) isolates to kanamycin expressed as mg / l**

| # | Microorganism | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | *Pseudomonas aeruginosa* | + | + | + | + | + | + | - | - | - |
| 2. | *Pseudomonas aeruginosa* | + | + | + | + | + | + | - | - | - |
| 3. | *Pseudomonas aeruginosa* | + | + | + | + | + | + | + | - | - |
| 4. | *Escherichia coli #1* | + | + | + | + | + | + | + | - | - |
| 5. | *Escherichia hermonii* | + | + | + | + | + | + | + | - | - |
| 6. | *Enterobacter gergoviae* | + | + | + | + | + | + | - | - | - |
| 7. | *Escherichia fergusonii* | + | + | + | + | + | + | + | - | - |
| 8. | *Escherichia coli #2* | + | + | + | + | + | + | - | - | - |
| 9. | *Escherichia fergusonii* | + | + | + | + | + | + | + | - | - |
| 10. | *Escherichia coli #3* | + | + | + | + | + | + | - | - | - |
| 11. | *Proteus penneri* | + | + | + | + | + | + | - | - | - |
| 12. | *Enterobacter cloacae* | + | + | + | + | + | + | + | - | - |
| 13. | *Enterobacter aerogenes* | + | + | + | + | + | + | - | - | - |
| 14. | *Klebsiella oxytoca* | + | + | + | + | + | + | - | - | - |
| 15 | *Enterobacter amnigenus* | + | + | + | + | + | + | - | - | - |
| 16. | *Escherichia coli #4* | + | + | + | + | + | + | - | - | - |
| 17. | *Enterobacter sakazakii* | + | + | + | + | + | + | - | - | - |

An attempt was carried out to determine if the antibiotic resistance gene is extrachromosomal or chromosomal. DNA plasmids prepared from two randomly chosen *E.coli* isolates were introduced into the host *E.coli* DH5. The resulting transformants were grown on nutrient agar supplemented with kanamycin (50 µg /ml). The two transformants grew on nutrient media supplemented with kanamycin indicating that the resistance gene was carried on the plasmid and not on the bacterial genome.

This experiment demonstrated the presence of the antibiotic resistance gene(s) on the plasmid of the two *E. coli* isolates .

It was an indicative experiment, that the antibiotic resistance gene could be carried on an extrachromosomal plasmid , but not exclusively.

Further in the research, colony PCR was adopted to cover the possibility that the antibiotic resistance gene could be chromosomal and not extrachromosomal.

The utilization of the polymerase chain reaction to detect genes coding for aminoglycoside modifying enzymes was carried out on the plasmids DNA prepared from the 17 isolates that previously showed high-level resistance towards Kanamycin.

Van De klundert, and Vleigenthart, (1993) describes the Polymerase Chain Reaction (PCR) detection method for four genes coding for four different aminoglycoside modifying enzymes in a single strain using colony PCR. Genes encoding aminoglycoside ´6-N-acetyltransferases *(accA)*, were

identified. Four sets of primers delineating DNA fragments of 209 bp, 250 bp, 260 bp and 347 bp, specific for the four known *aacA* genes, and probes within these fragments, were constructed based on the nucleotide sequences of the *aacA* genes (Vanhoof *et al.*, 1993). The specificity of the primers was evaluated using reference strains encoding various aminoglycoside-modifying enzymes. The primers reacted with their corresponding *aacA* genes and did not cross-react with genes coding for other aminoglycoside-modifying enzymes.

Figure 5 shows the plasmid pBK-CMV carrying the neo gene under the control of the viral SV40 promoter. The pBK-CMV was used as a positive control in the PCR amplification of the resistance genes . The amplification of the neo gene gave fragment of approximately 300bp (Fig. 6).

PCR amplification procedure was followed using plasmid DNA prepared form the isolates. Negative and positive controls were used .

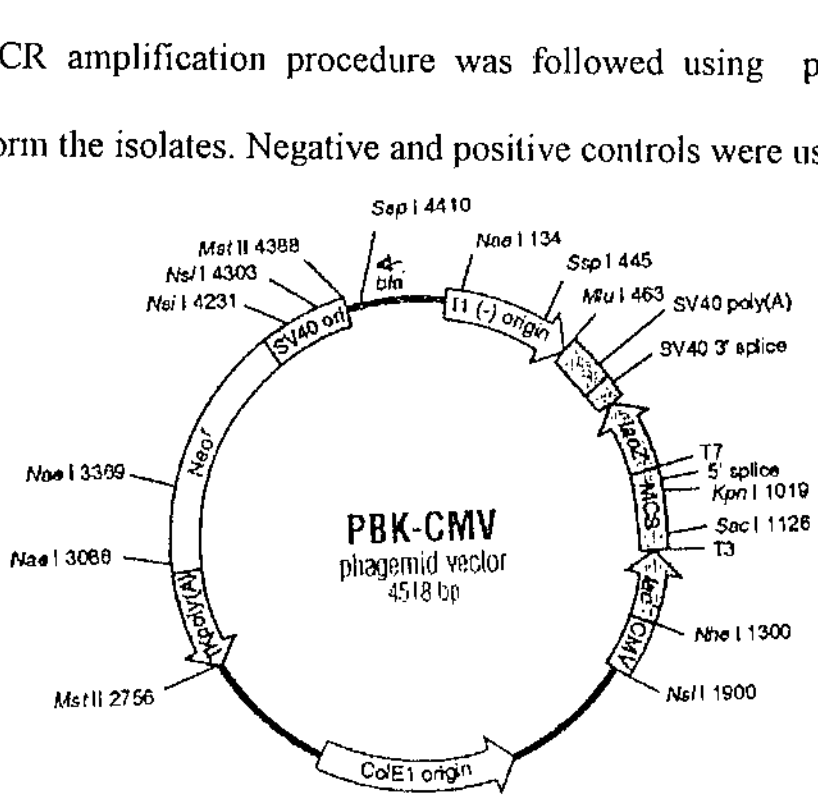


**Figure (5): pBK-CMV plasmid map**

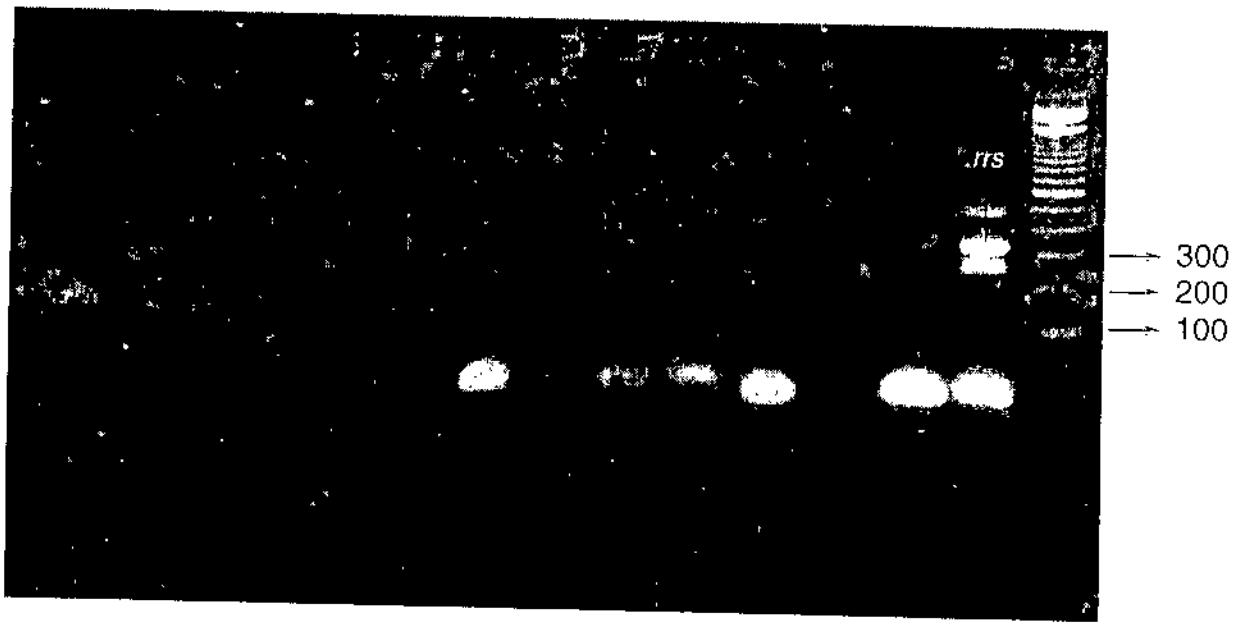


**Figure (6): PCR products using plasmid DNA**

Figures 6 and 7 shows unspecific amplification using *rrs* and *aacC3* primers. While Figure 8 shows the amplification of two different genes (*rrs* and *aacC2*) from the same isolates.

Only four strains of the 17 isolates whose plasmid DNA was prepared and used for PCR amplification, showed to carry genes encoding aminoglycoside resistance. Failure of gene detection indicates the possibility that resistance might be due to mechanisms other than chemical modification of the aminoglycoside or that the gene is carried on the chromosomal DNA and not on the plasmid DNA.

Accordingly further analysis of the former isolates were carried out following the colony PCR procedure. Colony PCR gives an extra advantage over

plasmid DNA PCR since it could amplify both chromosomal and extrachromosomal antibiotic resistance genes.

In an attempt to optimize the amount of bacterial cell lysate (thermal lysis under hypotonic conditions) used in colony PCR .2,5, 10 and 20 µl lysate correponding to 1,2,3 and 4 lanes (Fig. 9) were used in the PCR reaction mixture. A single isolate was used employing the *rrs* primer. All yielded 320 bp PCR product corresponding to the published results except the 2µl cell lysate (Van De klundert, and Vleigenthart, 1993).

The 17 isolates were subjected to colony PCR protocol using 20 µl cell lysate and the 8 sets of primers with each isolate.

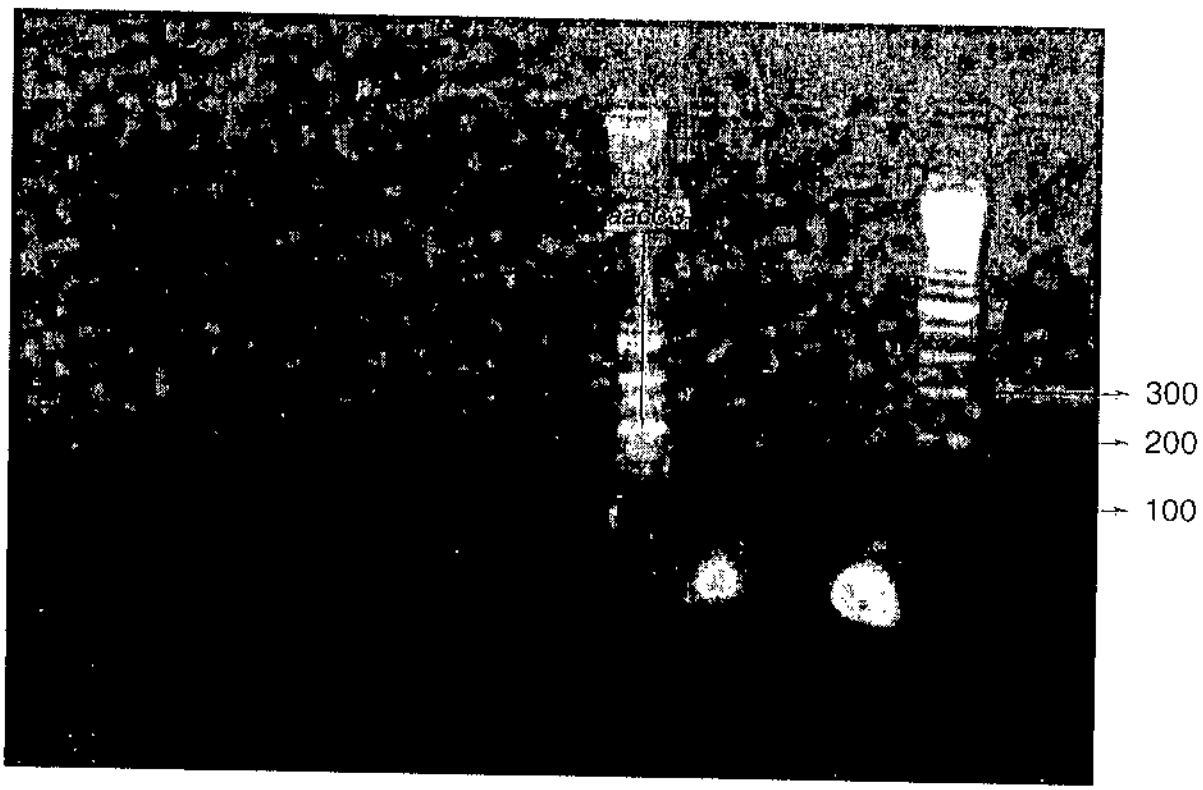


**Figure (7): PCR products using plasmid DNA**

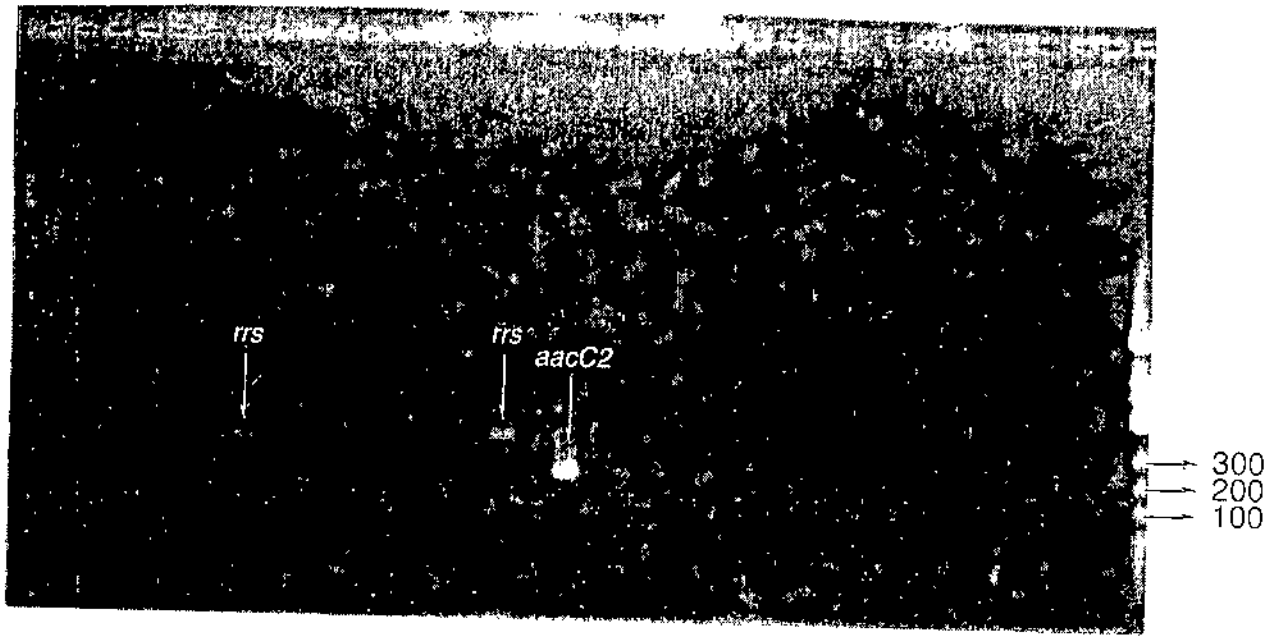


**Figure (8): PCR products using plasmid DNA showing *rrs* and *aacC2* genes**

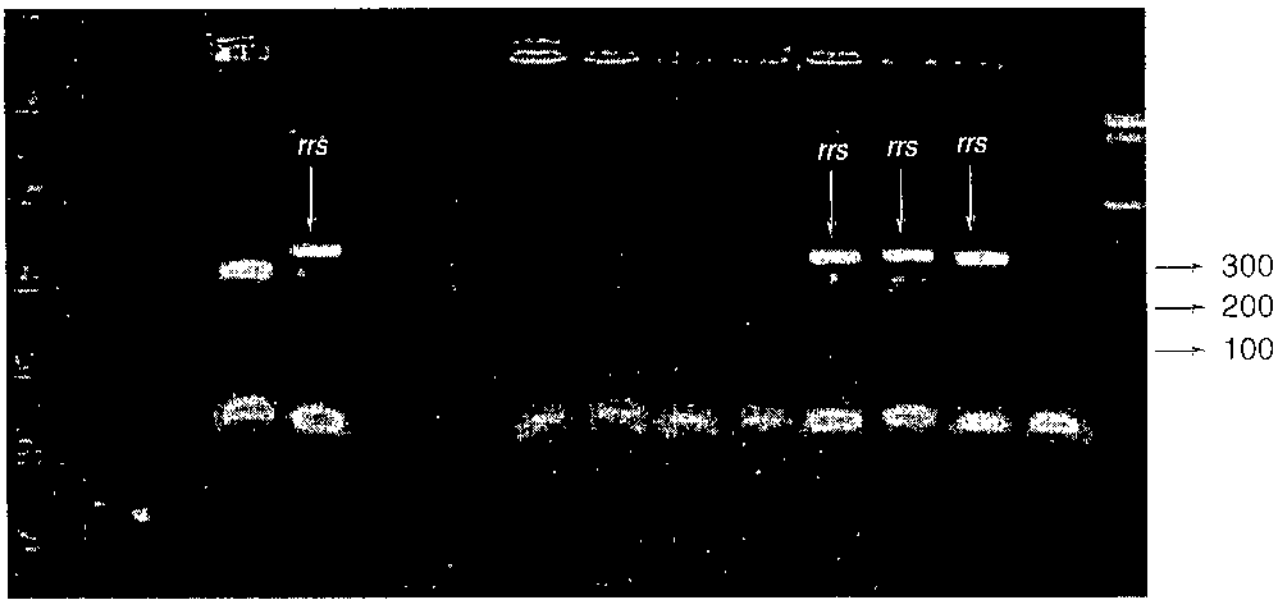


**Figure (9): Colony PCR using 2,5,10 and 20 µl Lysate and *rrs* primers**

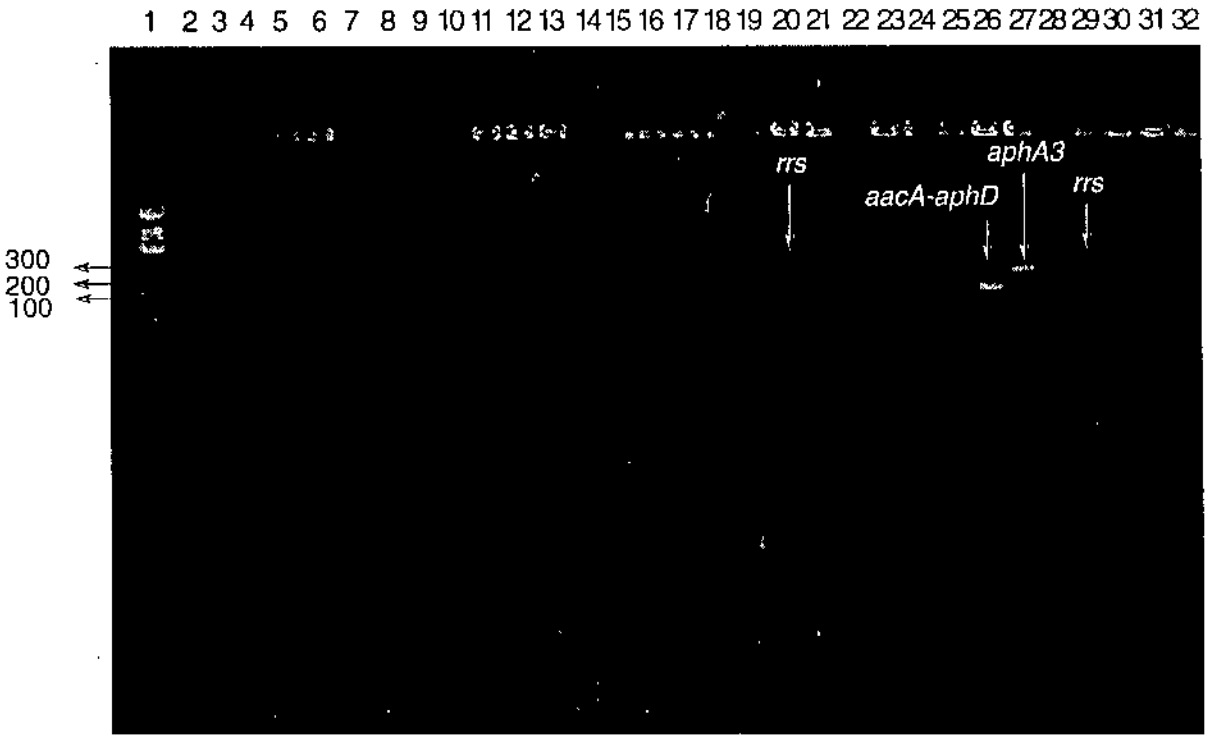


**Figure (10) Colony PCR showing the amplification of the *rrs, aac-aphD* and *aphA3* genes**

Figure 10 shows the amplification of part of the sequence in the *rrs* gene (320bp) from two different isolates (lanes 20 & 29) in addition one isolate gave the *aacA-aphD* (lane 26) and the *aphA3* (lane 27) genes corresponding to 220 and 292 bp respectively. Figure 11 shows the amplification of unspecific bands from one isolate in lane 5. Another isolate (lane 11) shows the amplification of a sequence in the *rrs* gene (320bp). Figure 12 shows the amplification of a sequence in the *rrs* gene from three different isolates (lanes 2,11&20). One isolate showed in addition to the *rrs* the *aacC2* gene (237 bp) (lane 22). Figure 13 shows the amplification of the *rrs* from three different isolates. Additionaly, isolates 2 and 4 shows the amplification of the *aacC2* gene (237 bp).

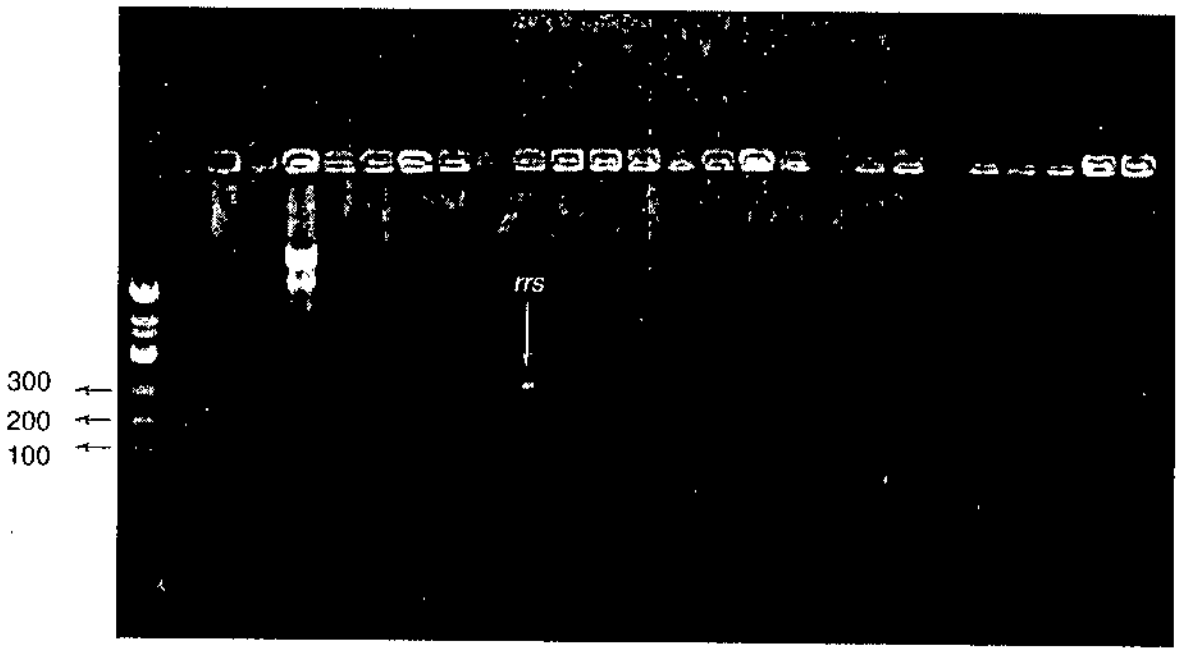


**Figure (11): PCR unspecific amplification**

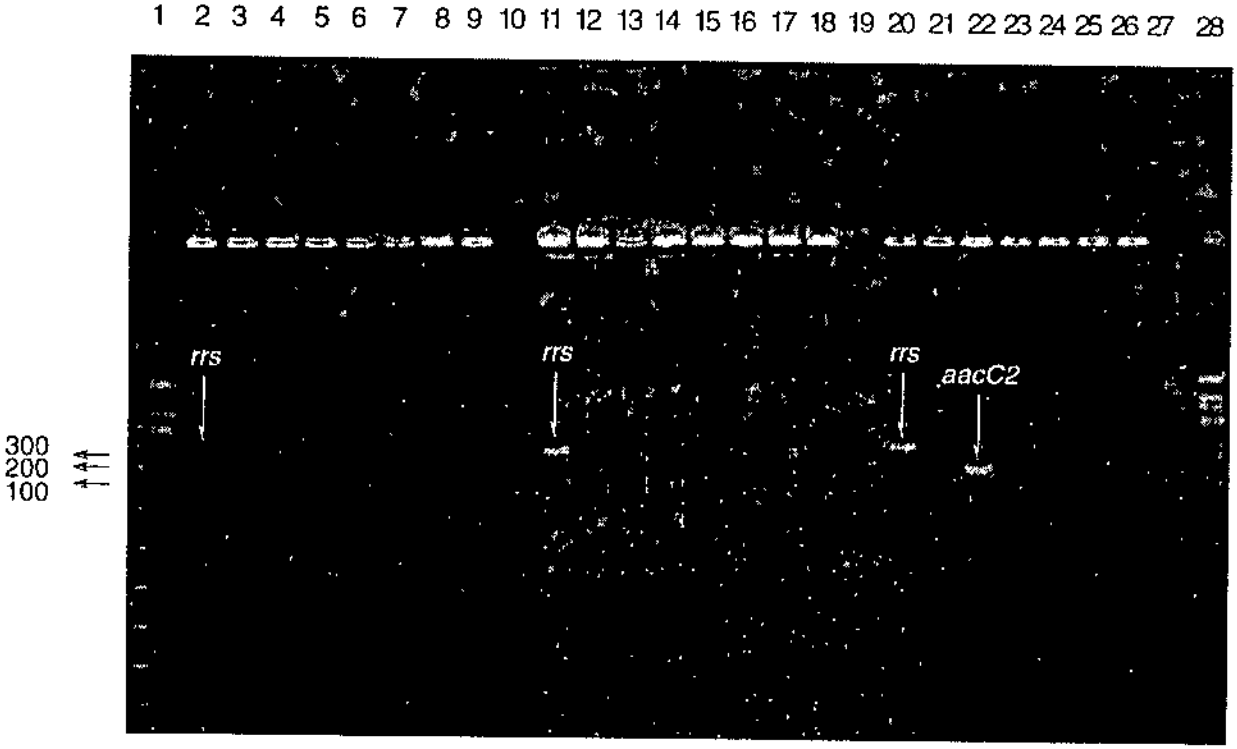


**Figure (12): Amplification of *rrs* gene from three different isolates using Colony PCR**

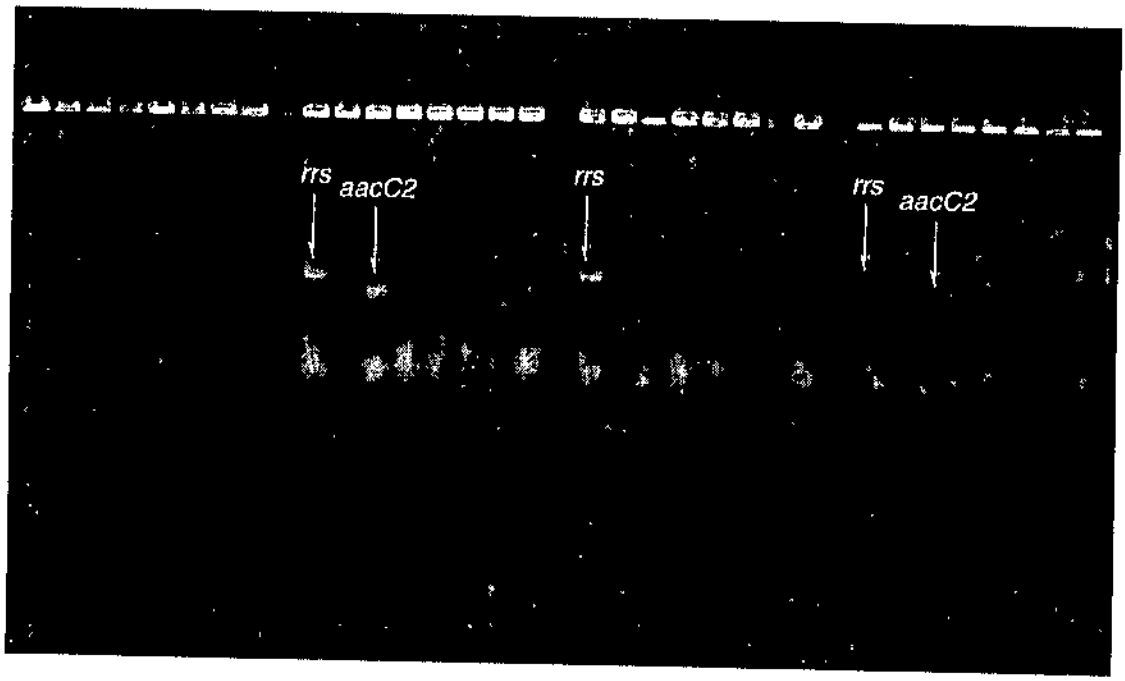


**Figure (13): Amplification of *rrs* and *aacC2* genes using colony PCR**

The 17 isolates showed to carry the following genes: *rrs, aacC2, aacC3, aacA-aphD* and *aphA3*. In order to determine the specifity of PCR and to compare the genes amplified and detected from the Jordanian isolates to the published results, restriction enzymes digestion were carried out. Figures 14 shows an outline of PCR for detection of *aacC* genes in G(-) bacteria. The *rss* gene has no restriction site while the *aacC2* has a site for *Cla* I giving two fragments of 39 and 198. There is a site for *Bst*EII in the *aacC3* gene giving two fragment of 61 and 124 bp. Figure 15 shows the outline of PCR for detection of aminoglycoside genes. The *aacA-aphD* has a site for *Ssp* I giving two fragment of 72 and 148 bp. There is a site for *Eco*RV in the *aphA3* gene giving two fragments of 52 and 240 bp.

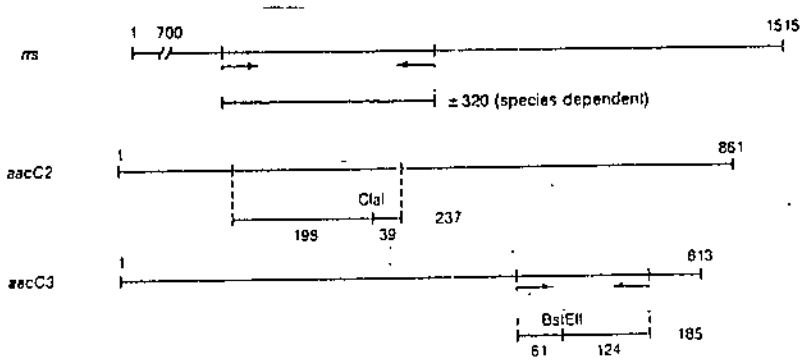


**Figure (14): Outline of PCR for detection of *aacC* genes is G (-) bacteria.** (Van De klundert, and Vleigenthart, 1993)

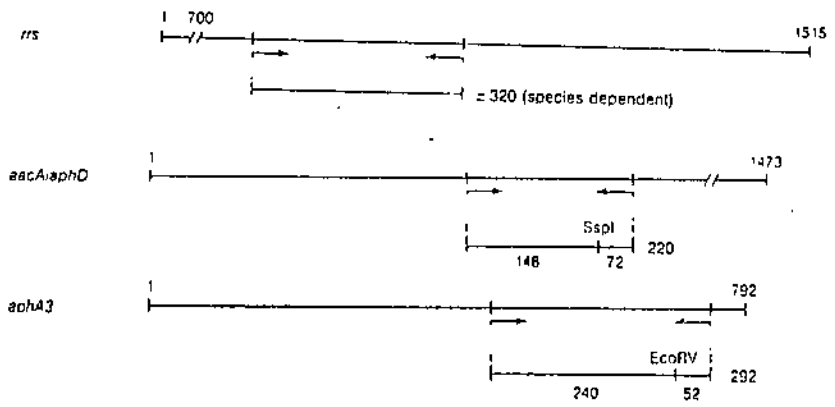


**Figure (15): Outline of PCR for detection of aminoglycoside genes.**

(Van De klundert, and Vleigenthart, 1993)

Figure 16 shows the *rrs* gene (320 bp) isolated from 12 different isolates run together with a size marker to confirm the size of the amplified fragment.

Figure 17 shows from left to right 100 bp size marker followed by the digested and undigested *aacC2* gene PCR product for four different isolates. Lane one shows size marker lanes 2,4,6 & 8 shows the undigested *aacC2* (237 bp) while lanes 3,5,7 & 9 shows the *ClaI* digested *aacC2* (39 and 198 bp). Due to the samll size of the fragment 39 bp it is hard to visulize it although the agarose gel is 2%.

Lane 12 shows the 61 bp fragment of the *BstEI* digested *aacC3* amplified gene. Lanes 13 and 14 shows non specific amplification. Lane 16 shows the undigested *aacA-aphD* while lane 17 shows 72 and 148 bp fragments of the Ssp Idigested *aacA-aphD*.

Lane 19 shows the undigested *aphA3* amplified gene and lane 20 shows the 52 and 240 bp fragments of the *Eco*RVdigested *aphA3*.

Figure 16 and 17 shows that five different genes *rrs*, *aacC2*, *aacC3*, *aacA-aphD* and *aphA3* coding for antibiotic resistance have been isolated and mapped. The analysis by restriction enzymes confirms primarily that the amplified genes have similar restriction sites as the published results (Van De klundert, and Vleigenthart, 1993)

Table 7 sumarizes the finding of this thesis by listing the different G (-) isolate verses the antibiotic resistance gene they carry.

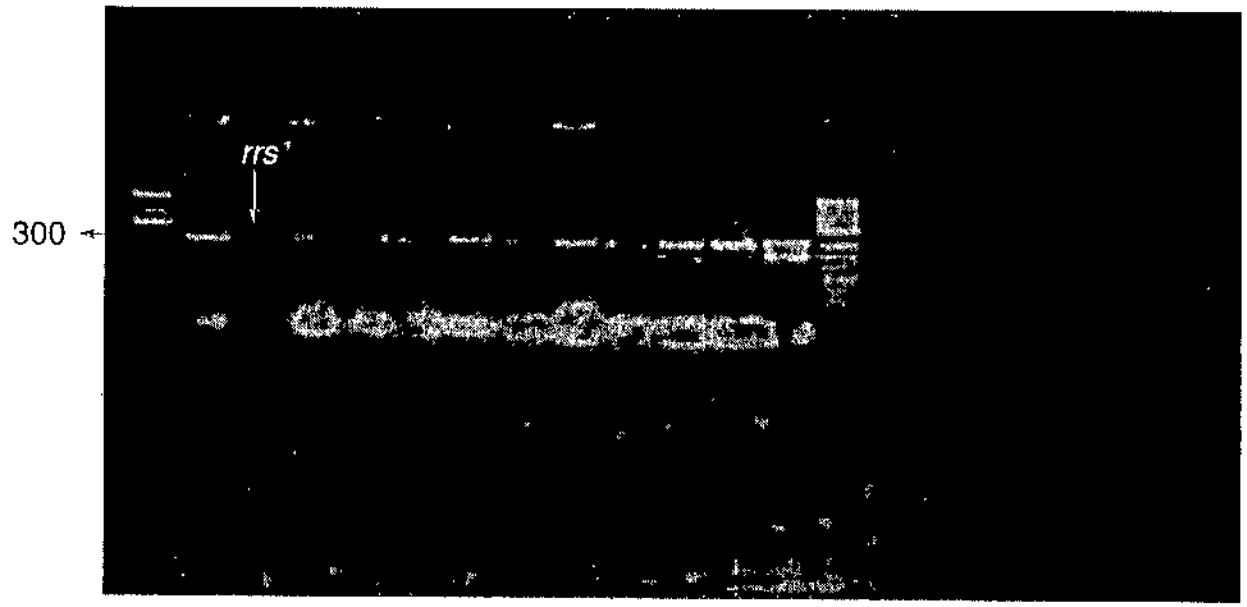


**Figure (16): Amplification of the *rrs* gene from 12 different isolates**

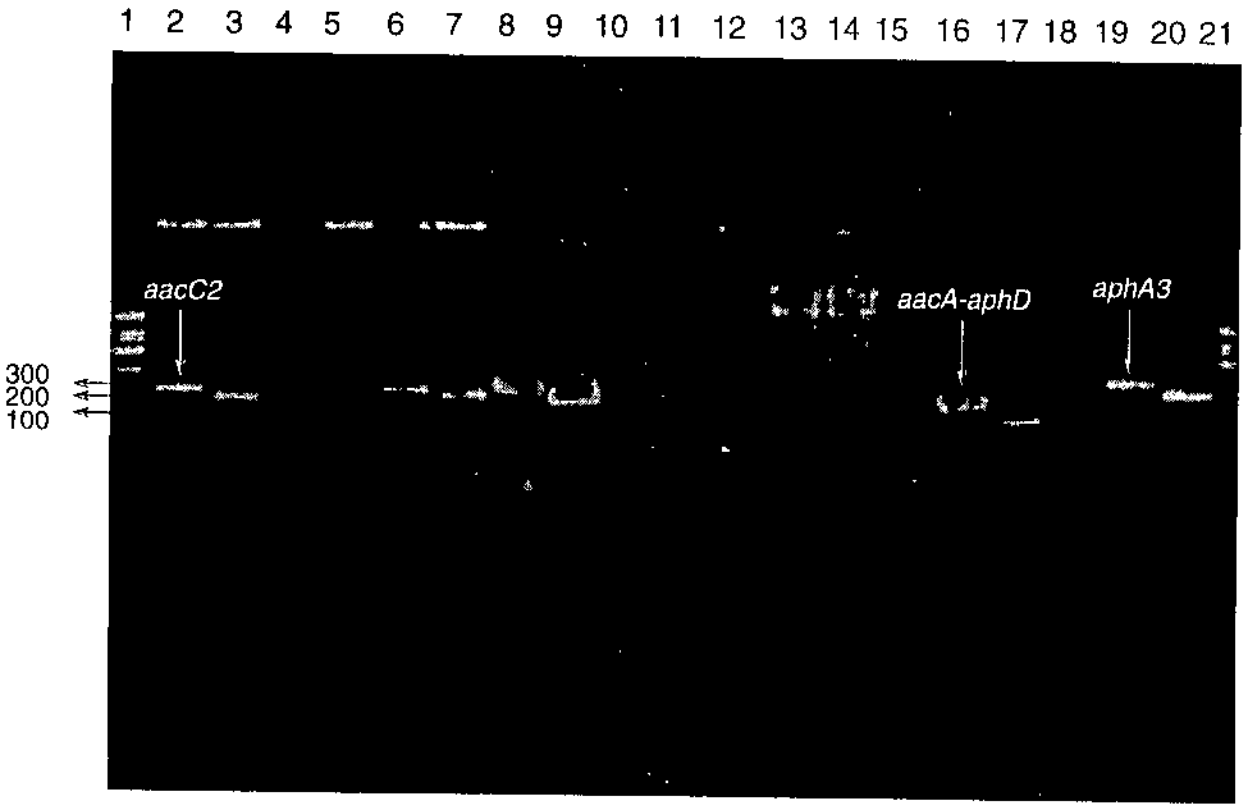


**Figure (17): Analysis of the amplified fragments of the *aacC2, aacC3, aacA-aphD* and *aphA3* genes using restriction enzymes**

**Table 7: G(-) bacteria carrying different antibiotic resistance genes**

| Bacteria | Gene | Isolates |
|---|---|---|
| ***Escherichia coli #1*** | *aacC3* | 4 |
| ***Enterobacter aerogenes*** | *rrs* | 13 |
| ***Klebsiella oxytoca*** | *rrs, aacC2* | 14 |
| ***Escherichia coli #4*** | *rrs* | 16 |
| ***Enterobacter amnigenus*** | *aacC3* | 15 |
| ***Escherichia hermanii*** | *rrs, aacC2* | 5 |
| ***Enterobacter gergoviae*** | *rrs* | 6 |
| ***Escherichia fergusonii*** | *aacc2* | 7 |
| ***Escherichia coli #2*** | *rrs* | 8 |
| ***Escherichia fergusonii*** | *rrs* | 9 |
| ***Escherichia coli #3*** | *rrs, aacC2* | 10 |
| ***Enterobacter cloacae*** | *aacA– aphD, aphA3* | 12 |

Kallova *et al.* (1995) included in there study seven *amikacin*-resistant strains of *Enterobacteriaceae* isolated in Slovakia and Germany . The strains were also resistant *in vitro* to high levels of gentamicin, tobramycin, netilmicin and isepamicin. All isolated plasmids from transconjugants encoded resistance to aminoglycosides by genes encoding the enzymes *aac(6')-I and aph(2")*. Aminoglycoside resistance was found to be transferable to *Escherichia coli* in all isolates, and R plasmids o were detected in donor and transconjugant strains.

Using a multiplex PCR procedure carried out on bacterial thermolysates (Noppe-Leclercq *et al.*,1999) analyzed the aminoglycoside resistance gene content of *Acinetobacter baumannii* strains responsible for nosocomial infections . In a single reaction were combined three primer pairs in order to amplify the genes coding for *aac(6')-Ih, aac(3)-I*, and *aac(3)-II*, three primer pairs for the genes coding for *ant(2")-I, aph(3')-VI*, and rRNA 16S as internal control, and finally two primer pairs for the genes coding for *aac(6')-Ib* and *aph(3')-I*.

Ploy *et al.* (1994) studied the distribution of *aac(6')-I* genes in 62 strains of *Acinetobacter spp.* resistant to amikacin, netilmicin, and tobramycin and susceptible to gentamicin, a phenotype compatible with synthesis of an *aac(6')-I* enzyme, by PCR and by DNA hybridization. Both methods gave similar results. Among the 51 *Acinetobacter baumannii* strains, *aac(6')-Ib* was found in 19 isolates and *aac(6')-Ih* was found in the remaining strains.

Seward *et al.* (1998) studied 24 multiresistant clinical isolates of *Acinetobacter spp.* from 15 hospitals in 11 countries worldwide. The full aminoglycoside resistance profile was determined for each isolate, with subsequent confirmation of enzyme content and genetic location by polymerase chain reaction (PCR) and hybridisation techniques. All produced at least one aminoglycoside-modifying enzyme, most commonly *aac(3)-I* and *ant(3")-I* in various combinations. Other enzymes found were *aac(3)-II, aac(6')-I, ant(2"), aph(3')-I* and *aph(3')-VI.* Nine isolates transferred resistance mediated by *aac(3)-I, ant(2")-I, aph(3')-I* or *aph(3)'-VI* by conjugation to a sensitive strain of *A. baumannii,* but most resistance was non-transferable.

Vanhoof *et al.* (1999) obtained A total of 1102 consecutive clinical blood isolates, including 897 *Enterobacteriaceae* and 205 non-fermenting *bacilli,* from 13 university and university hospitals. In total, 157 isolates were found not to be susceptible to aminoglycosides. By PCR, 179 aminoglycoside resistance mechanisms, i.e. 150 genes encoding modifying enzymes and 29 permeability mechanisms, were detected in 148 isolates.

Van De klundert, and Vleigenthart, (1993) , used the 8 set of primers we used in our research , and it enabled them to detect , in one PCR test, as many as four genes in a single strain. The specificity of amplification reaction was checked by restriction enzyme digestion of the generated amplimers. Our results confirmed the similarity between the genes amplified from bacterial isolates from the Jodanian patient and the published results.

- **Conclusions**

The clinical isolates from the Jordanian Patients in this study revealed a high level of resistance against antibiotics used. There is little doubt that the indiscriminate uses of antibiotics for the serious and more trivial illnesses have a major contributing factor relating to the problem of resistance. Our study showed a high resistance profile for *Micrococcus*, *Enterococcus*, *Eischerichia*, *Enterobacter*, *Pseudomonas*, *Acinetobacter* and *Klebsiella* towards the antibiotics used.

This study showed that multi-resistant strains occurred among the clinical isolates. This may suggest that R-plasmids or genes coding for antibiotic inactivating enzymes may be carried on transferable plasmid which could be responsible for this multi-resistance. More than one antibiotic could be a substrate for certain antibiotic-modifying enzymes. However, Roe, *et al*, (1971) reported that Carbenicillin-resistance in *P.aeruginosa* strains appeared through the transfer of the R-factor from *Proteus* and other members of *Enterobacteriaceae*.

The findings of this study provide evidence that antibiotic resistance of some G(+) and G(-) bacteria is a real problem in hospitalized patients. Such resistance would limit the choices in the number of antibiotics available to the clinicians to treat bacterial infections. In order to reduce the problem, indiscriminate use of antibiotics should be controlled. Prophylactic use should be limited to high risk patients only. This is the only way to discourage the

proliferation of antibiotic-resistant microbes and safeguard the effectiveness of existing antimicrobial drugs. In an attempt to determine the pattern of antibiotic resistance in clinical isolates obtained from Jordanian patients, and to design effective antibiotic policies, systemic analysis of resistance mechanisms in clinical isolates is indispensable. Detection of genes coding aminoglycoside modifying enzymes in the hospital isolates was carried out using polymerase chain reaction (PCR). The focus of this work was on G(-) bacteria. Three species of bacteria were chosen for this study : *Enterobcter spp.*, *Pseudomonas spp.* and *Escherichia spp.*. PCR was followed using plasmid DNA prepared from the isolates. To further analyze the isolates colony PCR procedure was followed. Colony PCR gives an extra advantage over direct PCR since it could amplify not only extrachromosomal DNA but chromosomal DNA as well, since antibiotic resistance could be either chromosomal or extrachromosomal. The isolates showed to carry the following genes :

*rrs*, *aacC2*, *aacC3*, *aacA-aphD* and *aphA3* . In order to compare the genes amplified and detected from the Jordanian isolates to the published results, restriction enzyme digestion were carried out. The restriction enzyme digestion confirmed primarily that the isolated genes have similar restriction sites as the published results.

## 5. References


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## Appendix 1

## Recipes for used solutions

### 1 . Kanamycin

25mg/ml solution of the kanamycin sulfate prepared in sterilized water, stored in aliquots at (-20 °C ).

### 2. 1M calcium chloride

Dissolve 14.75 g of $CaCl_2.2H_2O$ in pure sterilized water and make up to final volume of 100 ml, store them in 10-ml aliquots at (-20°C), when preparing competent cells the aliquots is diluted to 100 ml with pure sterilized water.

### 3. Lysis Buffer (Solution I)

50 mM Glucose

10 mM EDTA

25 mM Tris.HCl (pH 8.0)

0.301 gm of Tris.HCl were dissolved in 20 ml, the pH was adjusted to 8.0 then 0.372 g of EDTA and 0.99 g of glucose were added, then the volume was adjusted to 100 ml with water, and the solution was sterilized by autoclaving.

**4.Solution (II) ( 0.2 N NaOH & 1% SDS )**

**-0.2 N NaOH**

0.8 g of NaOH were dissolved in distilled water and made up to final volume of 100 ml and the solution was sterilized by autoclaving.

**- 1 %SDS**

1 g of SDS were dissolved in distilled water and made up to final volume of 100 ml and the solution was sterilized by autoclaving.

**5. 3M sodium acetate, pH4.6 ( solution III )**

29.45 g of potassium acetate and 12 ml g of acetic acid were dissolved in distilled water and made up to final volume of 100 ml and the solution was sterilized by autoclaving.

**6. TE pH 8.0**

10 mM Tris.HCl adjusted to pH 8.0

10 mM EDTA

0.121 g of Tris.HCl dissolved in 20 ml water, pH was adjusted to 8.0 by 0.1M NaOH, 0.372 g of EDTA were added and the solution was made up to final volume of 100 ml with distilled water, divided into 5 ml aliquots and were sterilized by autoclaving.

**7. Loading Buffer 6x**

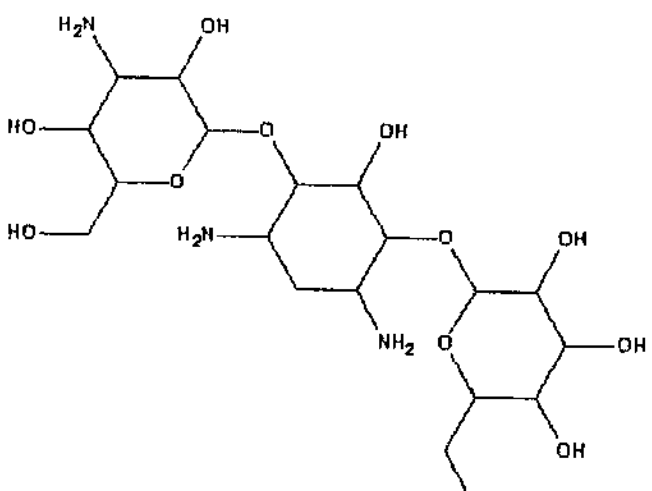
0.25% Bromophenol blue

0.25% Xylene cyanol

15% Ficoll type (400) in $H_2O$

0.25% g bromophenol blue, 0.25g xylene cyanol and 15 g (ficoll type 400) were dissolved in 100 ml water, divided into 5 ml aliquots and were sterilized by autoclaving and kept at 4 °C.

**8. Kanamycin chemical structure.**



## الملخص

استخدام طريقة البوليميريز متسلسل التفاعل لتحديد الجينات الرامزة للأنزيمات المسؤولة عن تعديل الأمينوكلايكوسايد في البكتيريا المعزولة من النماذج الطبية من مستشفى الجامعة الأردنية

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تم جمع مائة و ستة عشر عزلة بكتيرية خلال فترة ستة شهور، تشمل عينات من نماذج طبية مختلفة لكل من : عينة من الصفراء ، عينة من الدم ، مسحة من قثطار، عينة من قيح، عينة من سائل منوي ، عينة من قشع، عينة من بول، مسحة من جرح، مسحة من احليل. تم في هذا البحث دراسة استعدادية العزلات البكتيرية موجبة الغرام وسالبة الغرام لأثني و عشرين مضاد حيوي مختلف، حيث أظهرت العنقوديات و المكورات المعوية و المكورات الدقيقة مقاومة للمضاد الحيوي جنتاميسين بنسب ١٦,٧%و ٤٢,٩%و ٥٠% على التوالي، في حين أظهرت المكورات الدقيقة مقاومة لكل من الأمبسلين و السيفوكسيتين و السفتازيدّم بنسبة ١٠٠% و للأميبينيم بنسبة ٧٥% و لسفترايكسون و بيبريسلين بنسبة ٥٠%.

أظهرت العنقوديات مقاومة عالية للمضاد الحيوي ايرثرومايسين بأعلى نسبة و هي ٢٠,٨%.و أظهرت المكورات

المعوية أعلى مقاومة للمضادات الحيوية كليندااميسين و ايرثرومايسين و ناليديكسك اسيد بنسبة ٢٨%.

لقد أظهرت جميع البكتيريا سالبة الغرام المستخدمة في هذا البحث مقاومة للمضاد الحيوي الأكثر استعمالاً :الأمبسلين، كما أظهرت بكتيريا الكلبسيلا و المتقلبة مقاومة تراوحت بين ٤٠- ١٠٠% ، أما بكتيريا الأمعائيات فقد أظهرت مقاومة لخمسة عشر مضاد حيوي من الأثني و العشرين المستخدمة في هذا البحث ، و ذلك بنسب تتراوح بين ٥,٩% إلى ٤٧%.

ولتحديد نمط مقاومة البكتيريا المعزولة من المريض الأردني للمضادات الحيوية ولأجل وضع سياسات فعالة لاستخدام هذه المضادات فانه لا مناط من التحليل النظامي لآليات المقاومة في هذه العزلات . لذلك تم استخدام طريقة البوليميريز متسلسل التفاعل في البحث عن جينات رامزة للأنزيمات المسؤولة عن تعديل الأ مينوكلا يكوساید المعزولة من النماذج الطبية .و قد تم التركيز في هذا البحث على البكتيريا سالبة الغرام ( الأشريكيات و الزائفة و الأمعائيات ) باستعمال الحامض النووي الريبوزي اللاكروموسومي المستخرج من هذه العزلات لإستخدام طريقة البوليميريز متسلسل التفاعل . و استخدمت أيضا مستعمرة بكتيرية مباشرةً لإجراء التفاعل. و قد تبين ان العزلات البكتيرية تحمل الجينات التالية *rrs, aacC2, aacC3, aacA-aphD, aphA3* . و باستخدام عملية هضم الأنزيمات "restriction enzymes digestion" تبين ان الجينات التي تم تحديدها مشابهة لتلك المذكورة في نشرات علمية سابقة.