DEVELOPMENT OF RESISTANCE BY REPEATED EXPOSURE OF BACTERIA TOANTIBIOTICS AND ULTRAVIOLET LIGHT

BY

SULEIMAN MUSTAPHA

SPS/14/MMB/00020

DISSERTATION

TO

DEPARTMENT OF MICROBIOLOGY, BAYERO UNIVERSITY,

KANO

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE MSc IN MICROBIOLOGY WITH SPECIALIZATION IN MEDICAL MICROBIOLOGY

SUPERVISED BY

Dr. SHAMSUDDEEN UMAR

FEBRUARY, 2017

DECLARATION

I the under signed, declare that this research work titled"Development of Resistance by Repeated Exposure of Bacteria to Antibiotics and Ultraviolet Light" was carried out by me in partial fulfilment of the requirement for the award of M.S.c in Medical Microbiology.

…… …….

Suleiman Mustapha Date

SPS/14/MMB/00020

CERTIFICATION

This is to certify that the research work for thisdissertation and subsequent write-up"SULEIMAN MUSTAPHA (SPS/14/MMB/00020)" was carried out under our supervision.

…….. …….

Dr. Shamsuddeen Umar Date

(Supervisor)

……. ……..

Dr. AminuBukar Date

(PG Coordinator)

……... ……

Dr.Abdulkadir.M. Magashi Date

(HOD)

APPROVAL

This Dissertation has been examined and approved for the award of Masters Degree in Microbiology (Medical).

……. …….

External ExaminerDate

…….. ……..

Internal Examiner Date

……. …….

(Supervisor)Date

DrShamsuddeen Umar

…….. ……..

(P.G Coordinator) Date

DrAminuBukar

…… ……

S.P.S Representative Date

DrNafiuHussaini

…… …….

H.O.D/Chief Exarminer Date

Dr A.M Magashi

ACKNOWLEDGMENT

The most beautiful praises be to Allah, we thank Him and seek for both guidance and protection from Him. I thank Him for his uncountable mercies bestowed upon me throughout my life time and for making it easy for me to complete this project work. May He enable us to continue to thank Him in both deeds and words.

I would also like to use this opportunity to sincerely thank my dear research supervisorDrShamsuddeen Umar. No words beautiful enough exist to show my gratitude and appreciations. It has been a great experience to be your project student. May Allah reward you abundantly.

I also want to extend my gratitude to my beloved Parents. May Allah shower his forgiveness and mercy upon you. Only Allah can reward you for the training and discipline you inculcated in us. May paradise be our final destination all.

I would also use this opportunity to thank all my friends and family members for their support and encouragements, more grease to your elbows. One love keeps us together.

DEDICATION

This work is dedicated to my beloved parents whose tireless efforts and patience contributed immensely to my success in all spares of life.

TABLE OF CONTENTS

CONTENT **Page**

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

ABSTRACT

The study was carried out on the development of resistance due to repeated exposure of some bacterial isolates to antibiotics and UV light.*Salmonella typhi* and *Shigelladysentriae* were isolated from stools using SS Agar. *Proteus mirabilis* and *Staphylococcus aureus* were isolated from urine using CLED Agar. After the isolation, standard inoculum of *Salmonella typhi,Shigelladysentriae, Proteus mirabilis* and *Staphylococcus aureus*were prepared, each and was streaked onto Mueller-Hinton Agar plates. Prepared Amoxicillin, Ciprofloxacin, and Gentamicin paper discs were placed at the center of the plates and incubated for 24 hours, at 35-37^{o}C. And zones of inhibition were formed. The zones of inhibition were measured and recorded, and then bacteria from the edges of the inhibition zones were picked up with a swab stick, and inoculated on to fresh Mueller-Hinton Agar plates. This process was repeated for each of 10 times. The same bacteria (*Salmonella typhi,Shigelladisenteriae, Proteus mirabilis* and *Staphylococcus aureus*) isolated were exposed to ultraviolet light (UV light), by placing the petri dishes containing the bacteria under the UV light at distance of 15cm, and wave length of 254nm for 1, 3, 5, 7 and 10 minutes. After the exposure, the bacteria were sub cultured in other Mueller-Hinton Agar plates. Amoxicillin, Ciprofloxacin, and Gentamicin paper discs were placed at the center of the plates of each bacteria. Zones of inhibition formed. The zones of inhibition were measured and recorded. Over the course of 10 exposures to test antibiotics, all the test organisms developed resistance to the antibiotics gradually as seen through decrease in diameter of their zones of inhibitions. Out of five different periods of exposure to UV light, all the test organisms developed resistance at 1 minute exposure and became more susceptible for the subsequent minutes exposures. The results indicated that the short time exposure of the test organisms to ultraviolet light during the sensitivity testing, increased their resistance as seen by decrease in the diameter of their zones of inhibition. It is also observable that the longer the organisms were exposed to UV light, the bigger the zone of inhibition.This means prolonged exposure to UV light makes the bacteria to be more susceptible to the test antibiotics.This may be due to the effect of the UV light on the genetic material of the organisms, that is,the excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines (specifically thymine) in DNA.

CHAPTER ONE

1.0 INTRODUCTION

Antibiotic resistance is a serious and growing global problem. It occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections. The bacteria survive and continue to multiply causing more harm. Bacteria can do this through several mechanisms. Some bacteria develop the ability to neutralize the antibiotic before it can do harm, others can rapidly pump the antibiotic out, and still others can change the antibiotic attack site so it cannot affect the function of the bacteria (WHO, 2014). Some organisms are naturally resistant but the term most often refers to acquired resistance, which can be a result of either new mutations or transfer of resistance genes between organisms (Woodford and Ellington, 2007). The increasing rates of antibiotic resistant infections are caused by antibiotic use from human and veterinary medicine. Any use of antibiotics can increase selective pressure in a population of bacteria, promoting resistant bacteria and causing vulnerable bacteria to die(Leekha*et al.*, 2011).

Antibiotics kill or inhibit the growth of susceptible bacteria. Sometimes one of the bacteria survives because it has the ability to neutralize or escape the effect of the antibiotic; that one bacterium can then multiply and replace all the bacteria that were killed off (Cassir*et al.*, 2014). Exposure to antibiotics therefore provides selective pressure, which makes the surviving bacteria more likely to be resistant. In addition, bacteria that were at one time susceptible to an antibiotic can acquire resistance through mutation of their genetic material or by acquiring pieces of DNA that code for the resistance properties from other bacteria. The DNA that codes for resistance can be grouped in a single easily transferable package. This means that bacteria can become resistant

to many antimicrobial agents because of the transfer of one piece of DNA (Hoffman *et al.,* 2015).

Several molecular mechanisms of antibacterial resistance exist. Intrinsic antibacterial resistance may be part of the genetic makeup of bacterial strains (Alekshun, 2007). For example, an antibiotic target may be absent from the bacterial genome. Acquired resistance results from a mutation in the bacterial chromosome or the acquisition of extra-chromosomal DNA (Alekshun, 2007). Antibacterial-producing bacteria have evolved resistance mechanisms that have been shown to be similar to, and may have been transferred to, antibacterial-resistant strains (Nikaido, 2009). The spread of antibacterial resistance often occurs through vertical transmission of mutations during growth and by genetic recombination of DNA by horizontal genetic exchange. For instance, antibacterial resistance genes can be exchanged between different bacterial strains or species via plasmids that carry these resistance genes (Witte, 2004). Plasmids that carry several different resistance genes can confer resistance to multiple antibacterial. Cross-resistance to several antibacterial may also occur when a resistance mechanism encoded by a single gene conveys resistance to more than one antibacterial compound (Baker-Austin*et al.*, 2006).

UV radiation has become a global concern due to the fact that it is a major environmental mutagen and carcinogen, leading to conditions such as skin cancer (Kevin and Alice, 2001).Coupled with the fact that ozone layer (the atmospheric layer that filters out solar UV radiation from the sun) is continuously depleted by pollution, there is pressure to better understand the mechanics in how mutations arise so that possible remedies in the future can be devised. UV radiation may be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria. Such environmental stress caused by UV may in some way induce different evolutionary changes on bacteria that would have otherwise not been

selected for. This area thus provides avenues of physiological, ecological, and genetic investigation because mutations play a key role in biological processes such as evolution, carcinogenesis, aging, and generation of somatic genetic diversity (Livneh*et al.*, 1993).

(UV) light is non-ionizing radiation, such as ultraviolet (UV) light, exerts its mutagenic effect by exciting electrons in molecules. The excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines (specifically thymine) in DNA. When two pyrimidines are bound together in this way, it is called a pyrimidine dimer. These dimers often change the shape of the DNA in the cell and can cause problems during replication. The cell often tries to repair pyrimidine dimers before replication, but the repair mechanism can also lead to mutations as well (Michelle, 2015).

There are 3 general types of UV light; UVa, UVb and UVc. Each of these has a different wavelength. UVa and UVb have a longer wavelength than UVc; therefore, they have less energy and cannot penetrate cells as well. While they are still dangerous, they are not considered germicidal because they produce only a small effect on most microbes. If cells are exposed to UVc long enough then it can penetrate and kill them (Michelle, 2015). Ultraviolet light (opt 250 nm) causes bonds to form between adjacent pyrimidine residues (commonly referred to as thymine dimers, although the effect can also occur with cytosine) in the same polynucleotide strand. These are called pyrimidine dimers. UV irradiation can result in the formation of covalent links between pyrimidine dimers. These bonds distort the DNA conformation and inhibit DNA replication and transcription (Muhammad and Najafi, 2013).

1.1 Statement of the Research Problems and Justification

The development of resistance by bacteria as a result of continuous exposure or in appropriate use of antibiotic has resulted into difficulties in the management of various infection. Antibiotic resistance is a serious and growing global problem: a World Health Organization (WHO) report released April 2014 stated, "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country (WHO, 2014).

UV light has become a global concern due to the fact that it is a major environmental mutagen and carcinogen, leading to conditions such as skin cancer (Kevin and Alice, 2001). It can be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria.

Therefore it has become increasingly important for such study in order to give health practitioners more ideas on management of bacterial infections and development of resistance.

1.2 Aim of the Study

The aim of this study is to investigate the development of antibiotic resistance in*Salmonellatyphi, Shigelladysenteriae*isolated from stooland *Proteus mirabilis, Staphylococcus aureus*isolated from urinecaused by repeated exposure to antibiotics and ultraviolet light.

1.3 Objectives of the research

i. To isolate and identify *Salmonellatyphi, Shigelladysenteriae, Proteus mirabilis and Staphylococcus aureus* and subject them to antibiotics sensitivity testing.

ii. To study the resistance of the bacteria isolated to antibiotics as well as the susceptibility pattern of such bacteria.

iii. To determine the level of bacterial exposureto antibiotics that may induce resistance.

iv. To determine the effect of exposure of the bacterial isolates to ultraviolet light, on response of the test organisms to the antibiotics.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1History ofAntibiotics

Since the introduction in 1937 of the first effective antimicrobials, namely, the sulfonamides, the development of specific mechanisms of resistance has plagued their therapeutic use. Sulfonamide resistance was originally reported in the late 1930s, and the same mechanisms operate some 70 years later. Penicillin was discovered by Alexander Fleming in 1928, and in 1940, several years before the introduction of penicillin as a therapeutic agent, bacterial penicillinase was identified by two members of the penicillin discovery team (Abraham, 1940). Once the antibiotic was used widely, resistant strains capable of inactivating the drug became prevalent, and synthetic studies were undertaken to modify penicillin chemically to prevent cleavage by penicillinases (β-lactamases). Interestingly, the identification of a bacterial penicillinase before the use of the antibiotic can now be appreciated in the light of recent findings that a large number of antibiotic genes are components of natural microbial populations (D'Costa*et al.*, 2006).

2.2 How Bacteria Develop Resistance to Antibiotics

Antibiotic resistance occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections. The bacteria survive and continue to multiply causing more harm. Bacteria can do this through several mechanisms. Some bacteria develop the ability to neutralize the antibiotic before it can

do harm, others can rapidly pump the antibiotic out, and still others can change the antibiotic attack site so it cannot affect the function of the bacteria (*www.rxlist*, 2016).

Antibiotics kill or inhibit the growth of susceptible bacteria. Sometimes one of the bacteria survives because it has the ability to neutralize or escape the effect of the antibiotic; that one bacterium can then multiply and replace all the bacteria that were killed off. Exposure to antibiotics therefore provides selective pressure, which makes the surviving bacteria more likely to be resistant. In addition, bacteria that were at one time susceptible to an antibiotic can acquire resistance through mutation of their genetic material or by acquiring pieces of DNA that code for the resistance properties from other bacteria. The DNA that codes for resistance can be grouped in a single easily transferable package. This means that bacteria can become resistant to many antimicrobial agents because of the transfer of one piece of DNA (*www.rxlist*,2016).

2.3 Factors Driving Resistance Development

The rate and extent of resistance development depends on a number of factors, some of which are outside our direct control, but some of which we can influence through measures to alter human behaviour, such as improving medical procedures, rationalising prescription practices, influencing consumers'perceptions and expectations, and enforcing restrictions on antibiotic use in animal farming (Livermore, 2004). One major controllable factor is the volume of use of antibiotics as this will set the overall selection pressure for resistance development.As a rule it is observed that the frequency of resistance both at the community and the individual level is correlated to antibiotic use. Determining the relative risk of resistance development in the case of any specific antibiotic or dosage regimen is complicated. Influential factors are the antibacterial

spectrum of the drug and its pharmacokinetics, such as the building up of concentrations in the gastrointestinal tract, skin and saliva(Livermore, 2003).

Another uncontrollable factor that has a major influence on resistance development is the fitness cost of resistance combined with the ability of the resistant bacteria to compensate for these costs. Thus, it is often observed that antibiotic resistance exacts a fitness cost of the bacteria, causing them to grow more slowly, become less virulent (for example, in their ability to cause infection) and less transmissible (Andersson, 2003). Furthermore, these fitness costs can be reduced or completely eliminated as a result of additional genetic changes, and therefore not lead to loss of resistance. Such compensatory evolution is potentially of great concern since it will cause a stabilization of the resistant bacteria in the population.

Bacteria behave differently when they are continuously exposed to antibiotics, and the rate and extent to which resistance develops are strongly dependent on the particular combination of bacterial species and type of drug. A few years after the introduction of penicillin, reports from British hospitals indicated an almost 50% prevalence of resistant *Staphylococcus aureus*. In contrast, despite of an extensive use of penicillins for the treatment of infections caused by *Streptococcus pyogenes*there has still not been a single case of a penicillin-resistant strain found in a clinical setting. These cases are two extremes of fast and slow resistance development. Importantly, it is generally seen for most combinations of drugs and bacteria that the frequency of resistance at the population level increases both with the length and volume of use and the absence of resistance development. The example of *S. pyogenes*mentioned above is certainly unusual and is not a typical case. It is obviously of great interest to understand why rates of resistance development differ so much (Levin, 2001).

2.4 Mechanisms and Origins of Antibiotic Resistance

The molecular mechanisms of resistance to antibiotics have been studied extensively and have involved investigations of the genetics and biochemistry of many different facets of bacterial cell function (Alekshun*et al.*, 2007). In fact, the study of antibiotic action and resistance has contributed significantly to our knowledge of cell structure and function. Resistance processes are widely distributed in the microbial kingdom and have been well described for a variety of commensals (Marshall *et al*, 2009)and pathogens; most can be disseminated by one or more distinct gene transfer mechanisms. A few of the resistance types that illustrate the difficulties in maintaining effective antibiotic activity in the face of the genetic and biochemical flexibility of bacteria deserve special mention.

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

i. Drug inactivation or modification: for example, enzymatic deactivation of penicillin G in some penicillin-resistant bacteria through the production of β-lactamases. Most commonly, the protective enzymes produced by the bacterial cell will add an acetyl or phosphate group to a specific site on the antibiotic, which will reduce its ability to bind to the bacterial ribosomes and disrupt protein synthesis (Criswell, 2004).
ii. Alteration of target site: for example, alteration of PBP—the binding target site of penicillins—in MRSA and other penicillin-resistant bacteria. Another protective mechanism found among bacterial species is ribosomal protection proteins. These proteins protect the bacterial cell from antibiotics that target the cell's ribosomes to inhibit protein synthesis. The mechanism involves the binding of the ribosomal protection proteins to the ribosomes of the bacterial cell, which in turn changes its conformational

shape. This allows the ribosomes to continue synthesizing proteins essential to the cell while preventing antibiotics from binding to the ribosome to inhibit protein synthesis.

iii. Alteration of metabolic pathway: for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid.

iv. Reduced drug accumulation: by decreasing drug permeability or increasing active efflux (pumping out) of the drugs across the cell surface (Li *et al*., 2009) These specialized pumps can be found within the cellular membrane of certain bacterial species and are used to pump antibiotics out of the cell before they are able to do any damage. These efflux pumps are often activated by a specific substrate associated with an antibiotic (Aminov, 2007).

Antibiotic resistance can be a result of horizontal gene transfer (Ochiai, 1959) and also of unlinked point mutations in the pathogengenome at a rate of about 1 in 10^8 per chromosomal replication. Mutations are rare but the fact that bacteria reproduce at such a high rate allows for the effect to be significant. A mutation may produce a change in the binding site of the antibiotic, which may allow the site to continue proper functioning in the presence of the antibiotic or prevent the binding of the antibiotic to the site altogether. Research has shown the bacterial protein LexA may play a key role in the acquisition of bacterial mutations giving resistance to quinolones and rifampicin. DNA damage induces the SOS gene repressor LexA to undergo autoproteolytic activity. This includes the transcription of genes encoding Pol II, Pol IV, and Pol V, which are three nonessential DNA polymerases that are required for mutation in response to

DNA damage (Cirz*et al*., 2005).The antibiotic action against the pathogen can be seen as an environmental pressure. Those bacteria with a mutation that allows them to survive live to reproduce. They then pass this trait to their offspring, which leads to the evolution of a fully resistant colony. Although these chromosomal mutations may seem to benefit the bacteria by providing antibiotic resistance, they also confer a cost of fitness. For example, a ribosomal mutation may protect a bacterial cell by changing the binding site of an antibiotic but it will also slow the process of protein synthesis (Criswell, 2004). Additionally, a particular study specifically compared the overall fitness of antibiotic resistant strains of Escherichia coli and Salmonella typhimurium to their drug-sensitive revertants. They observed a reduced overall fitness in the antibiotic resistant strains, especially in growth rate (Levin, 2000).

There are three known mechanisms of fluoroquinolone resistance. Some types of efflux pumps can act to decrease intracellular quinolone concentration (Morita, 1998) In Gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Robicsek, 2006).

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. A recent study demonstrated that the extent of horizontal gene transfer among *Staphylococcus* is much greater than previously expected—and encompasses genes with functions beyond antibiotic resistance and virulence, and beyond genes residing within the mobile genetic elements.

For a long time, it has been thought that, for a microorganism to become resistant to an antibiotic, it must be in a large population. However, recent findings show that there is no necessity of large populations of bacteria for the appearance of antibiotic resistance. We know now that small populations of E.coli in an antibiotic gradient can become resistant. Any heterogeneous environment with respect to nutrient and antibiotic gradients may facilitate the development of antibiotic resistance in small bacterial populations and this is also true for the human body. Researchers hypothesize that the mechanism of resistance development is based on four SNP mutations in the genome of E.coli produced by the gradient of antibiotic. These mutations confer the bacteria emergence of antibiotic resistance (Chan, 2001).

2.4.1Genetic Jugglery

The genes for β-lactamase enzymes are probably the most international in distribution; random mutations of the genes encoding the enzymes have given rise to modified catalysts with increasingly extended spectra of resistance (Guiadkowski, 2008). The archetypical plasmid-encoded β-lactamase, TEM, has spawned a huge tribe of related enzyme families, providing ample proof of this adaptability. The β-lactamase genes are ancient and have been found in remote and desolate environments, which implies that novel β-lactamases with altered substrate ranges occur in the environment (Allen *et al.*, 2009).

Macrolide antibiotics, such as erythromycin and its successors, were introduced to contend with the problem of methicillin resistance and are widely used for the treatment of Gram-positive infections. Not surprisingly, strains resistant due to a number of different mechanisms are now widely disseminated (Roberts, 2008). The macrolides and related antibiotics act by binding at different sites in the peptide exit tunnel of the 50S ribosome subunit. Resistance can occur by

modification of the RNA or protein components of the tunnel. A specific rRNA modification that engenders resistance to all antibiotics acting at this site on the ribosome was described recently (Long *et al.,* 2006) and this modification is spreading.

Another example of bacterial genetic jugglery comes from the recent appearance of a novel FQ resistance mechanism. When the highly potent FQs were introduced in 1987, a few foolhardy experts predicted that resistance to this new class of gyrase inhibitors was unlikely, since at least two mutations would be required to generate a significant resistance phenotype. It was also suggested that horizontally transmitted FQ resistance was unlikely to occur. However, mutants of the target bacterial gyrase genes and efflux of the FQs from the cell have increasingly been encountered (Piddock, 2006). More unexpectedly, a transmissible mechanism of FQ inactivation has made its appearance. This mechanism comes about because one of the many aminoglycoside *N*-acetyltransferases has the capacity to modify a secondary amine on the FQs, leading to reduced activity (Depardieu *et al*, 2007). The latter does not result in high-level FQ resistance but may impart a low-level tolerance that favors the selection of resistance mutations. Another unpredicted FQ resistance mechanism is known as Qnr, a widespread family of DNA-binding proteins, and is responsible for low levels of quinolone resistance (Strahilevitz*etal.*, 2009).

2.4.2Intrinsic Resistance

Intrinsic resistance refers to the existence of genes in bacterial genomes that could generate a resistance phenotype, i.e., proto- or quasi-resistance. Different genera, species, strains, etc., exhibit ranges of antibiotic response phenotypes. Since the beginning of this millennium, the availability of genomewide mutagenesis techniques and rapid bacterial genome sequencing has revealed many potential/intrinsic gene functions in bacteria that may lead to resistance

phenotypes in clinical situations. For example, a common genetic route to enhanced antibiotic resistance is gene amplification, notably for resistance to the sulfonamides and trimethoprim (Kashmiri and Hotchkiss, 1975).

Phenotypic analyses of partial or "complete" gene knockout libraries by saturation mutagenesis of bacterial genomes permit the identification of specific mutants eliciting hypersensitivity responses to antibiotics. It is assumed that overexpression of the corresponding wild-type gene would generate a resistance phenotype. Such prognostic studies have been carried out with a number of organisms and have led to the prediction of novel resistance classes. This type of analysis was first done with a partial mutant library of *Acinetobacterbaylyi* (Gomez and Neyfakh, 2006). A more comprehensive survey of the Keio *E. coli* mutant gene library identified a total of 140 distinct isolates that were hypersensitive to a range of different antibiotic classes; related studies have been done with *Pseudomonas aeruginosa* (Fajardo*et al.*, 2008). Many of the putative "susceptibility" genes identified, such as genes that are genetically recessive, might not lead to a resistance phenotype. Nonetheless, such approaches identify potential r genes and provide information on the systems biology of resistance. RNA microarray analyses of the effects of antibiotics have provided similar predictive information (Brazas and Hancock, 2005 Simply put, increasing the number of copies of the target genes for an antibiotic can lead to reduction in the intracellular concentration of the inhibitor as a result of titration.

2.4.3The Resistome

It has been known for some time that bacterial strains resistant to antibiotics can be isolated by plating environmental bacteria on antibiotic-containing media in the laboratory. This is not surprising for antibiotic-producing actinomycetes, since most possess genes encoding resistance

to the compounds that they produce. In several cases, the resistance mechanisms have been identified and shown to be specific enzymatic modifications of the antibiotics. Streptomycetes have long been known to produce a variety of β-lactamases that may well be the source of some of the clinical forms of β-lactam resistance (Ogawara*et al.*, 1999). As mentioned earlier, environmental *Kluyvera* species have been found to be the origins of the CTX-M genes. In other cases, resistance of producing organisms to their products has been identified as due to efflux systems (Piddock, 2006). Multiple mechanisms of resistance, as found in the tetracycline producer *Streptomyces rimosus*, are frequent in producing bacteria. Based on biochemical and genetic similarities, such resistance mechanisms have presaged those found subsequently in antibiotic-resistant pathogens (Petkovic*et al.*, 2006).

In a recent, all-inclusive approach to quantifying the r genes/phenotype density in the environment, Wright and colleagues screened a collection of morphologically distinct spore-forming actinomycetes (including many known antibiotic-producing strains) for resistance to 21 different antibiotics (D'Costa*et al.*, 2006). A significant number of strains were resistant to an average of 7 or 8 antibiotics; they were naturally multidrug resistant. The population of r genes in nature is referred to as the environmental antibiotic resistome (Wright, 2007). Clearly, different environments would be expected to vary in the number and type of resistances. Novel resistance mechanisms, as well as many mechanisms related to those found in pathogens, were identified in the collection. This is the best evidence available for the presence of a vast environmental pool of genes with the potential to be captured and expressed as resistance determinants for any overused inhibitor. However, more studies are necessary to establish a strong environment-clinic connection (Canton, 2009).

Similar surveys of other antibiotic-producing bacteria, such as the *Bacillaceae*, pseudomonads, cyanobacteria, and the extensive family of *Actinobacteria*, a phylogenetic group known to produce many low-molecular-weight molecules, will be valuable in extending our understanding of the nature of r genes existing in the wild (Ventura *et al.*, 2007).

2.5 Superbugs and Super Resistance

Many of the bacterial pathogens associated with epidemics of human disease have evolved into multidrug-resistant (MDR) forms subsequent to antibiotic use. For example, MDR *M. tuberculosis* is a major pathogen found in both developing and industrialized nations and became the 20th-century version of an old pathogen. Other serious infections include nosocomial (hospital-linked) infections with *Acinetobacterbaumannii*, *Burkholderiacepacia*, *Campylobacter jejuni*, *Citrobacterfreundii*, *Clostridium difficile*, *Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Haemophilusinfluenzae*, *Klebsiellapneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia* spp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Stenotrophomonasmaltophilia*, and *Streptococcus pneumoniae*. The term "superbugs" refers to microbes with enhanced morbidity and mortality due to multiple mutations endowing high levels of resistance to the antibiotic classes specifically recommended for their treatment; the therapeutic options for these microbes are reduced, and periods of hospital care are extended and more costly. In some cases, super resistant strains have also acquired increased virulence and enhanced transmissibility. Realistically, antibiotic resistance can be considered a virulence factor(Ryan, 1992).

Tuberculosis is the archetypical human pathogen; it evolved with the human race and currently infects as much as one-third of the world population. While the ground-breaking discoveries of streptomycin and isoniazid provided vital treatments, resistance development was rapid. George

Orwell, who suffered from TB while writing the Novel *1984*, was apparently infected by an antibiotic-resistant strain of *M. tuberculosis*. The introduction of cocktails of anti-TB drugs has become an essential treatment regimen, with considerable success; however, for a variety of reasons, multidrug resistance continues to compromise TB therapy throughout the world. *M. tuberculosis* strains resistant to four or more of the front-line treatments (i.e., extremely drug-resistant [XDR] strains) have appeared and spread rapidly in the last decade or so (Shah*et al*., 2007). And now there are TDR strains, which are totally drug resistant (Velayati*et al*., 2009)! There have been no validated reports of a role for HGT in the development of resistance in *M. tuberculosis*. Antibiotic resistance in *M. tuberculosis* occurs exclusively by spontaneous mutation.

The most prevalent Gram-negative pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Klebsiellapneumoniae*, cause a variety of diseases in humans and animals, and a strong correlation between antibiotic use in the treatment of these diseases and antibiotic resistance development has been observed over the past half-century. This is especially apparent with the β-lactam class of antibiotics and their related inactivating enzymes, the β-lactamases. At this time, several groups and classes have been identified, comprising up to 1,000 resistance-related β-lactamases.

Concerning hospital-acquired diseases, *Pseudomonas aeruginosa* has evolved from being a burn wound infection into a major nosocomial threat. In this case, again, antibiotic resistance mechanisms evolved coincidentally with the introduction of new antibiotic derivatives, compromising the most effective treatments (such as the β-lactams and aminoglycosides). *P. aeruginosa* is of considerable concern for patients with cystic fibrosis (Horrevorts*et al*., 1990);

the pathogen is highly persistent and can avoid human immune defenses. Resistance development is associated with the lengthy antibiotic treatment of cystic fibrosis patients.

Acinetobacterbaumannii is a more recent Gram-negative pathogen and is also primarily nosocomial. As with the pseudomonads, it comes equipped with a suite of r genes and pathogenicity determinants that results in enhanced rates of mortality and morbidity (Peleg*et al*., 2008). It is thought that the infectious properties of *Acinetobacter* organisms derive from their robust survival and biodegradation capabilities in the environment; in addition, many strains are naturally competent for DNA uptake and have high rates of natural transformation. *A. baumannii* is evolving rapidly; recent genome sequence studies showed that some derivatives have at least 28 genomic islands encoding antibiotic resistance determinants; more than half of these inserts also encode virulence functions in the form of type IV secretion systems (Barbe*et al*., 2004).

Currently, the most notorious superbug is the Gram-positive organism *Staphylococcus aureus.* Whether it is the most serious superbug can be debated, since one wonders to what extent its bad reputation is due to its extensive press coverage. *S. aureus* has a close association with humankind: it is carried as a nasal commensal in 30% of the population, and its presence has long been linked to common skin infections such as boils. It does not have the historical reputation of *M. tuberculosis*, but in recent years, this multidrug-resistant pathogen has emerged as the major nosocomial infection (Enright*et al*., 2002). Following the discovery of penicillin, it seemed that *S. aureus* infections were controllable; however, the respite from resistance was short-lived. The landmark discovery and introduction of methicillin (the first designer antiresistance antibiotic) in 1959 were thought to be a sure defense against the penicillinases, but the appearance of methicillin-resistant *S. aureus* (MRSA) within just 3 years led inexorably to other multiantibiotic-resistant variants, and the acronym now denotes multidrug-resistant *S.*

aureus. Relatively recently, MRSA has moved outside the hospital and become a major community-acquired (CA) pathogen, with enhanced virulence and transmission characteristics. CA-MRSA has most of the properties of MRSA, albeit with different *mec* gene clusters, and has acquired new pathogenicity genes, such as the gene encoding the cytotoxic Panton-Valentine leukocidin (DeLeo*et al*., 2009). These are regulated by defined signaling systems (Novick and Gesinger, 2008).

A long-recognized hospital denizen, the toxin-producing anaerobe *Clostridium difficile*, is increasingly found as the cause of severe intestinal infections; recently, hyper-virulent toxin-producing strains have been recognized (Vernaz*et al*., 2009). Being a Gram-positive spore former, it is a hardy organism and is readily transmitted by hospital personnel, on equipment, and as aerosols. Its renewed prominence is considered the result of extensive hospital use of antibiotics such as expanded-spectrum cephalosporins, the newer penicillins, and fluoroquinolones that cause significant depletion of the Gram-negative intestinal microflora, thus enhancing *C. difficile* colonization. In other words, these infections are the direct result of antibiotic use.

Superbugs are omnipresent in the biosphere; their consequences are aggravated enormously in volatile situations such as civil unrest, violence, famine, and natural disasters and, of course, by poor or nonexistent hospital practices. Superbugs are not the only microbial threats, but they are recognized as the most menacing with respect to morbidity and mortality worldwide. In terms of the number of infections and consequences, *Vibrio cholerae* should be at the head of the superbug list (Lipp*et al*., 2002). While fortunately it is not common in industrialized nations, *V. cholerae* is endemic in Asia and South America.

With respect to the global control of endemic and pandemic infectious diseases, a significant problem is the availability of reliable systems for tracking outbreaks of serious infections. Despite the heroic efforts of the World Health Organization, such reporting is nonexistent in many parts of the world. A lack of information concerning the early stages of an epidemic bacterial infection has retarded appropriate remedial action in many cases.

2.6 Antibiotics Resistance Due to Anthropogenic Activities

The predominant role of human activities in the generation of environmental reservoirs of antibiotic resistance cannot be disputed. Since the 1940s, ever-increasing amounts of antibiotics designated for human applications have been manufactured, used clinically, released into the environment, and widely disseminated, thus providing constant selection and maintenance pressure for populations of resistant strains in all environments. Obtaining accurate figures on the quantities of antimicrobials produced by the pharmaceutical industry is difficult (it is not in the best interest of pharmaceutical companies to provide this information), but it can be estimated that many millions of metric tons of antibiotic compounds have been released into the biosphere over the last half-century. Since the only available evidence indicates that little in the way of antibiotics is contributed by naturally occurring antibiotic-producing strains in their native environments (Gottlieb, 1976) we must assume that commercial production provides the vast bulk of the antibiotics found in the biosphere. Some alternative uses of antimicrobial agents are as follows: (i) growth promotion/prophylactic use in animals; (ii) therapeutic/prophylactic use in humans; (iii) therapeutic/prophylactic use in aquaculture; (iv) therapeutic/prophylactic use in household pets; (v) pest control/cloning for plants and agriculture; (vi) use as biocides in toiletries and in hand care and household cleaning products; and (vii) culture sterility, cloning,

and selection in research and industry. It should be noted that therapeutic use in humans accounts for less than half of all applications of antibiotics produced commercially.

Taking into consideration the large-scale disposal of toxic wastes, metals, disinfectants, biocides, and residues of manufacturing processes, the amounts of noxious xenobiotics released into the biosphere are inestimable. The fact that many of the chemicals disposed are recalcitrant to biodegradation only compounds the issue. The dumping of ciprofloxacin into rivers at levels in excess of 50 kg a day by pharmaceutical manufacturers in Hyderabad, in central India (Fick*et al*, 2009) is possibly the most extreme of the horror stories concerning irresponsible disposal; however, similar levels of pollution probably occur (unreported) elsewhere in the world. Quite apart from providing powerful selection for the formation of resistant strains in all bacterial genera (this information has not yet been published), physiological damage to local resident populations of insects, birds, animals, and humans cannot be overestimated (Carlsson*et al*., 2009).

2.7 Genetics of Antibiotics Resistance

The appearance and dissemination of antibiotic-resistant pathogens have stimulated countless studies of the genetic aspects of the different phenomena associated with resistance development, such as gene pickup, heterologous expression, HGT, and mutation (White *et al,* 2005). The genetics of plasmids is not discussed in any detail here, nor are the interactions between plasmid-encoded and chromosomal resistances, except to say that early preconceptions about the stability, ubiquity, and host ranges of r genes and their vectors have largely become fiction. For example, acquisition of resistance has long been assumed to incur a serious energy cost to the microorganism, and indeed, many resistant mutants may be growth limited under laboratory

conditions. As a result, it was considered that multidrug-resistant strains would be unstable and short-lived in the absence of selection (Andersson, 2006). However, as frequently demonstrated, laboratory conditions (especially culture media) do not duplicate real-life circumstances; available evidence suggests that pathogens with multiple mutations and combinations of r genes evolve and survive successfully *in vivo*. Two recent studies of the development of multimutant, multidrug-resistant *S. aureus* and *M. tuberculosis* provide examples that overturn earlier beliefs. In the first study, isolates from a hospitalized patient treated with vancomycin were sampled at frequent intervals after hospital admission and analyzed by genome sequencing. In the steps to the development of the final (mortal) isolate, 35 mutations could be identified over the course of 3 months! Similarly, it has been reported that genome sequencing of antibiotic-resistant strains of *M. tuberculosis* revealed 29 independent mutations in an MDR strain and 35 mutations in an XDR strain. The functions of these mutations are not understood; they could well be compensatory changes. Such studies emphasize the need for detailed systems biology analyses of resistance development *in situ* (Mwangi*et al.*, 2007).

2.7.1Transmissionof Resistance Gene

Essentially any of the accessory genetic elements found in bacteria are capable of acquiring r genes 2and promoting their transmission; the type of element involved varies with the genus of the pathogen. There are similarities but also clear differences between the Gram-positive and Gram-negative bacteria; nonetheless, plasmid-mediated transmission is far and away the most common mechanism of HGT (Norman *et al.*, 2009). Surprisingly, bacteriophages carrying antibiotic r genes have rarely been identified in the environment or in hospital isolates of resistant bacteria; however, there is no question about the association of phages with the

insertional mechanisms required for the formation of mobile resistance elements and with the functions of chromosomally associated r genes. They are frequently seen as phage "fingerprints" flanking genes encoding resistance or virulence on different vectors. It appears that such events are quite common in *S. aureus* (Skurray and Firth, 1997).

Gene transmission by conjugation has been studied extensively in the laboratory and in approximating environmental conditions, and the frequencies of the transfer events often vary significantly. Experiments suggest that frequencies of conjugative transmission in nature are probably several orders of magnitude higher than those under laboratory conditions (Sorensen *et al.*, 2005). It has been shown that transfer in the intestinal tracts of animals and humans occurs *ad libitum* (Shoemaker *et al.*, 2001); it's a bordello down there! Recent studies have demonstrated diverse antibiotic r genes in the human gut microbiome (Sommer*et al.*, 2009).

In the streptococci, meningococci, and related genera, the exchange of both virulence and pathogenicity genes is highly promiscuous; the principal mechanism for DNA traffic appears to be transformation (Springman*et al.*, 2009). Finally, with respect to direct DNA acquisition in the environment, *Acinetobacter* spp. are naturally competent, and HGT is frequent; pathogenic strains typically carry large genomic islands (Perez *et al.*, 2007). Might *Acinetobacte*r and related environmental genera play roles in the capture and passage of r genes from environment to clinic? Such processes surely involve multiple steps and intermediate bacterial strains, but it has been suggested that heterogeneous gene exchange occurs readily in networks of multihost interactions (Dionisio*et al.*, 2002).

Horizontal gene transfer has occurred throughout evolutionary history, and one can consider two independent sets of events, largely differentiated by their time span and the strength of selection

pressure. What happened during the evolution of bacteria and other microbes and organisms over several billions of years cannot be compared to the phenomenon of antibiotic resistance development and transfer over the last century. Contemporary selection pressure of antibiotic use and disposal is much more intense; selection is largely for survival in hostile environments rather than for traits providing fitness in slowly evolving populations.

Consistent with the concept of the recent evolution of antibiotic resistance plasmids and multiresistant strains, studies with collections of bacterial pathogens isolated before the "antibiotic era" showed that plasmids were common but r genes were rare (Datta and Hughes, 1983). Genome sequence analyses of environmental microbes revealed that they are replete with plasmids—mostly large and often carrying multigene pathways responsible for the biodegradation of xenobiotic molecules, such as the polychlorinated phenolic compounds that have been used and distributed widely since the days of the industrial revolution. In summary, what is occurring in our lifetimes is an evolutionary process intensified by anthropogenic influences rather than the slower, random course of natural evolution. The existing processes of gene acquisition, transfer, modification, and expression that were in place are expanding and accelerating in the modern biosphere.

Laboratory studies have characterized numerous genetic mechanisms implicated in the evolution of antibiotic-resistant populations; the roles of plasmids, phages, and transformation are well established, but other processes may exist. For example, bacterial cell-cell fusion might be favored in complex mixed microbial communities, such as those found in biofilms (Gillings*et al.*, 2009). The efficiency of the processes is not critical; selection and the efficiency of heterologous gene expression are likely the most important constraints. However, low-level

expression of a potential r gene in a new host may provide partial protection from an antagonist; subsequent gene tailoring by mutation with selection would lead to improved expression. Promoter function under environmental conditions is not well understood (Cases and de Lorenzo, 2005); it appears that promoters of Gram-positive origin can function well in Gram-negative bacteria but that the converse is not often true. Does this imply a favored direction for bacterial gene transfer, as Brisson-Noel *et al.,*(1988) have suggested. During therapeutic use, the exposure of bacterial pathogens to high concentrations of antibiotics for extended periods creates severe selection pressure and leads to higher levels of resistance. The pathway from an environmental gene to a clinical r gene is not known, but it obviously occurs with some facility. Knowledge of the intermediate steps in this important process would be revealing—how many steps are there from source to clinic?

In the laboratory, HGT occurs under a variety of conditions and can be enhanced by physical means that facilitate DNA exchange, for example, physical proximity by immobilization on a filter or agar surface, and there are likely numerous other environmental factors that promote gene uptake. It is worth noting that antibiotics, especially at subinhibitory concentrations, may facilitate the process of antibiotic resistance development (Davies*et al*., 2006). For example, they have been shown to enhance gene transfer and recombination, in part through activating the SOS system (Guerin *et al.*, 2010); in addition, antimicrobials have been shown to induce phage production from lysogens. Such factors may play important roles in enhancing the frequency of gene exchange in environments such as farms, hospitals, and sewage systems, which provide ideal incubation conditions for r gene acquisition.

On the positive side, it should be noted that studies of antibiotic resistance mechanisms and their associated gene transfer mechanisms in pathogens have played seminal roles in the development of recombinant DNA methods, providing the experimental foundation for the modern biotechnology industry (Helinski, 2004). The use of restriction enzymes and plasmid cloning techniques completely transformed biology. The subsequent extension of bacterial recombinant DNA methods to plant, animal, and human genetic manipulations required only minor technical modifications, such as the construction of appropriate bifunctional antibiotics and cognate r genes in pro- and eukaryotes. The applications are truly universal, with increasingly evident benefits to all aspects of pure and applied biology.

2.8 Repeated Exposure of Bacteria to Antibiotics

Antibiotic resistance can be defined in several ways. The simplest definition is when a bacterium or a population of bacteria has a resistance mechanism that makes it less susceptible to treatment than other members of the same species. The mechanism can be caused by mutations in the bacteria or can be a result of horizontal gene transfer, which is the transfer of genes (resistance genes in this case) between bacteria in a manner other than reproduction. Horizontal gene transfer can occur through several different processes: transformation, transduction, and conjugation. Transformation is the genetic change that occurs when bacteria takes in naked DNA from its environment. Typically, a plasmid will contain a gene that will give antibiotic resistance to a portion of a population of bacteria. Transduction refers to the process of the transfer of genetic material from one bacterium to another by a virus (Antibiotic Resistance Q&A)(CDC, 2014). Researches use a clinical breakpoint, determined by a MIC value, to separate resistant bacteria from susceptible bacteria (Canton andMorosini, 2011). Several different strains of gram-

positive bacteria, such as the strain of Staphylococcus aureus known as VRSA, are already resistant to drugs of last resort. Mechanisms of resistance to natural or semi-synthetic antibiotics include glycosylation and phosphorylation (Levy and Marshall, 2004).

As the problem continues to grow and larger populations of bacteria become resistant to current antibiotics, new antibiotics and treatments must be developed. Despite the growing fear of "superbugs," many members of the public do not understand how antibiotic resistance can develop and what can be done in everyday life to prevent it. Making knowledge about resistance more available and understanding current research on the topic can slow the spread of resistant bacteria while new antibiotics are developed and tested. Much of current research focuses on the how antibiotic resistance develops in bacteria, such as E. coli, as well as how over-prescribing antibiotics and patient error can speed the spread (Canton andMorosini, 2011). (James, 2015) also confirmed that repeated exposure of E. coli to Ampicillin does lead to the antibiotics resistance. Researchers study how populations of bacteria survive exposure to antibiotics and acquire resistance genes through horizontal gene transfer, among other processes. Many patients ignore dosage guidelines and unknowingly contribute to antibiotic resistance in their own bodies and their immediate vicinities.

The appearance of drug-resistant bacteria quickly follows the introduction of a new antibiotic, which suggests an extremely fast rate of adaptation. Not long after penicillin was introduced, penicillin-resistant Staphylococcus aureus appeared in hospitals (Levy and Marshall, 2004). Furthermore, comparison of pre-antibiotic and post-antibiotic samples indicate an increase in antibiotic resistance genes for the ß-lactamase, tetracycline, and macrolide families of antibiotics (Bhullar*et al*., 2012). Repeated exposure to tetracycline antibiotics (which inhibits the process of

translation and therefore the creation of protein) causes the development of resistance in the form of ribosomal protection. Bacteria that can protect against the inhibition of protein synthesis will be largely resistant to this family of antibiotics.

Although it is obvious that the more dangerous, resistant strains have mutated and acquired stronger resistance genes over time, the presence of inherent resistance genes should not be overlooked. Bacteria can inherently possess the genes for antibiotic resistance and a vast amount of research explores how these genes express themselves and how bacteria populations develop resistance genes. Many organisms have an intrinsic resistome that contributes to the surprisingly quick development of resistance to a certain antibiotic. There are certain genes in bacteria, many of which are involved in "housekeeping" functions, which are widely considered pre-resistance genes (Canton andMorosini, 2011). Gene duplication contributes heavily to the evolution of housekeeping genes into antibiotic resistance genes. Furthermore, studies of the metabolic pathways of microbes shows that β -lactam antibiotics and β -lactamase enzymes were created by such microbes over two billion years ago (Spellberg*et al.*, 2008).

2.9 Reversing Resistance of Bacteria to Antibiotics

In the case of both of the major genetic processes, mutations or horizontal gene transfer, the resistantmicrobe is affected not only in its ability to withstand the antibiotic, but potentially also in its interactionwith the host and its ability to be transmitted between hosts. It is generally observed that most resistancemechanisms will confer a reduction in bacterial fitness, which might be expressed as reduced growthand survival inside and outside a host, and reduced virulence or transmission rate from environment tohost or between hosts. The observation that resistance is associated with a biological cost has led to thewidespread idea that by reducing the

volume of antibiotic use the frequency of resistant bacteria in a populationcan also be reduced. However, this picture is complicated by the fact that bacteria may reduce thecosts associated with the resistance through compensatory evolution (Andersson, 2003). The role of compensatorymutations that maintain the fitness of resistant strains is now well established and increasing levels ofbiologically competitive resistant bacteria are detected in the community, with no decrease in vitalitycompared to non-resistant strains (Levin, 2001). Thus, even in environments where antibiotic pressure is absent, thesebacteria may be difficult to remove.Clinical evidence supporting the reversibility idea is weak. Two epidemiological studies, of erythromycinresistance in *S. pyogenes*(Seppala *et al.*, 1997) and penicillin resistance in *Streptococcus pneumoniae*(Austin, 1999) havebeen suggested as providing support for the reversibility of resistance in community settings. In thesecases, the rate and extent of the decline in the frequency of resistance associated with reduced antibioticuse were small, which is in accord with predictions from modelling. In addition, the weak apparent correlationbetween reduced antibiotic consumption and decreased frequency of resistance could have beencaused by many other factors, for example, clonal shifts where a susceptible clone happened by chanceto increase in frequency coincidentally with the reduction in antibiotic use. Thus, the epidemiologicalstudies that are available at the moment provide no strong support for reversibility. In addition, severallaboratory and epidemiological studies indicate that various processes are predicted to cause long-termpersistence of resistant bacteria. One process is compensatory evolution, where the costs of resistanceare ameliorated by additional genetic changes, resulting in the stabilization of resistant bacteria in thepopulation. Even though most resistance is associated with fitness cost, some resistance mutations appearto be gratuitous. The

occurrence of such cost-free resistances will also cause irreversibility sincethe driving force for reversibility is absent.

Finally, genetic hitch-hiking between non-selected and selectedresistances will confer stabilization of the resistant bacteria. Thus, when two resistance genesare located near each other, on, for example, a plasmid, they tend to be inherited together. As a result,selection for one of the resistance genes tends to cause selection also for the nearby, genetically linkedgene. An interesting example of such hitch-hiking was provided by a recent study of sulphonamideresistant*E. coli*. Here, it was demonstrated that even after a drastic reduction in the use of sulphonamidein the United Kingdom from 1991 to 1999 the frequency of sulphonamide-resistant *E. coli* did not decrease,but actually increased slightly, from 40% to 46%. The explanation for this finding is most likelyhat the sulphonamide-resistant gene(s) is genetically linked on a plasmid to other resistance genes thatwere continuously selected during this time period (Enne*et al.*, 2001). In conclusion, if antibiotic resistant bacteria haveascended to a high frequency within the community they are likely to remain there for a long time.In hospital settings the rate and extent of reversibility are much higher than in communities, as shownby both actual experiments and clinical intervention studies as well as by theoretical models. Thereason for this difference is that the main driving force for reversibility in hospitals, in contrast to communities,is not the biological cost of resistance. Instead, in hospitals we observe a dilution effect asincoming patients, whether infected or not infected, are in most cases bringing susceptible bacteria intoclinical wards and therefore affect the levels of resistant bacteria. Thus, we predict that reversibility can occur in hospitals in response to reduced antibiotic use as long as the frequency of resistance is lower inthe community than it is in the hospital(Lipsitch*et al.,* 2000).

2.10Consequences of Antibiotic Resistance for the Individual

When a patient receives treatment with antibiotics, both the causative pathogen and the normal nonpathogenic microflora in the body will be affected. The indigenous microflora make up a complex ecological system of great importance for human health. Besides being essential for the digestion of food and to metabolise drugs, they also produce essential vitamins and are important for the activation and maintenance of the immune system in the gut. Ideally, antibiotics should effectively kill the pathogen responsible for infections and, simultaneously, cause as little disturbance as possible to the microflora of the individual. Presently, the ideal antibiotics do not exist and the overuse of broad spectrum agents in respiratory infections and diarrhoeal diseases consequently drives resistance development in pathogenic bacteria as well as in the normal bacterial reservoir of the patient. This makes them potential carriers of resistant microbes that might be dangerous to themselves and to other patients. The reservoir for resistance mechanisms in the gut can be transferred to more virulent pathogens passing through the body and be spread to other individuals. Furthermore, the bacteria that are carrying resistance mechanisms will be lost very slowly, if at all. Thus, they will form a large reservoir of bacteria that continuously disseminates resistance genes to other microbes. Such long-term persistence of resistant commensal normal flora and the resulting spread of resistance to other bacterial species, including pathogens, have been demonstrated recently. For example, one study showed that when patients were antibiotic-treated for one week for stomach ulcer caused by *Helicobacter pylori* infections, Enterococcal bacteria that are part of the normal microflora of the intestine developed high levels of resistance. Importantly, these resistant enterococci remained in the intestine of the treated individuals for up to three years after treatmentwas finished. Furthermore, in the interconnected world of today, with increased travel and migration, wehumans will in a

sense assist the bacteria to obtain novel mechanisms for resisting antibiotics all over the world (Sjolund*et al.*, 2003).

2.11 Ecological Roles of Antibiotics Resistance

Putative antibiotic r genes are omnipresent in natural environments. This raises the question of their natural functions, a topic that has been the subject of several thought-provoking reviews (Aminov, 2007). Do they determine antibiotic resistance phenotypes in nature? Are these genes maintained for resistance or for unrelated genetic or biochemical needs? Can we assume that bacteria are constantly exposed to a wide variety of toxins or otherwise inhibitory molecules in the environment? What are the ecological roles of low-molecular-weight natural products identified to have antibiotic activity in the laboratory? They have numerous sources, such as products of the degradation of natural polymers in nutrient conversions, plant products, antibiotic compounds from insects and fungi, and general organic decay. Plants produce many compounds that inhibit bacterial growth in the rhizosphere.

In addition, the environment contains many products that are man-made and/or triggered by human contamination, e.g., petroleum chemicals, solvents, the products and waste of industrial processes, garbage, etc. Since the beginnings of the industrial revolution, humankind has dumped ever-increasing amounts of organic and inorganic toxins into streams, rivers, seas, oceans, land, and air. Heavy metals are frequently present in soils. Arsenic, mercury, and iodine were used industrially and, prior to the discovery of antibiotics, as medicinals; under some circumstances, they are still employed as such. The major bacterial solution to toxic challenges takes the form of multivalent pumping systems that prevent intracellular accumulation of structurally diverse bactericidal and bacteriostatic substances (Piddock, 2006). Actinomycetes and other microbes

producing antibiotics and bioactive small molecules invariably possess multiple efflux systems (Mendez and Salas, 2001) as demonstrated for the tetracycline-producing organism *Streptomyces rimosus* (Petkovic, 2006). The coexistence of production and resistance functions has been confirmed extensively in recent studies of antibiotic biosynthetic gene clusters and examinations of the genome sequences from producing strains (D'Costa*et al.*, 2007).

With the exception of nonspecific efflux systems, the potential antibiotic resistance determinants found in antibiotic-producing strains are generally associated with structural types or modes of action. It has been suggested that these resistance mechanisms are for "self-protection" of the host, on the assumption that the producer would self-destruct if it started to make its antibiotic product (Hopwood, 2007). However, this notion has not been proven.

The production of an antibiotic in the laboratory is routinely assayed by inhibitory activity against bacterial strains of laboratory or clinical origin. Because traditional inhibition assays showed that they are produced late in the growth phase of the microbe, antibiotics have been called "secondary metabolites"; they appear to play no role in normal growth of the host. In addition, the so-called secondary compounds appear to be produced at undetectable concentrations in early exponential phases of growth. Laboratory studies indicate that streptomycetes producing small molecules pass through a transition phase during growth in flask cultures that is associated with the onset of significant developmental changes, including sporulation and antibiotic production. Such interpretations of the relationship of growth-associated processes to small-molecule production may apply only under laboratory and industrial conditions.

The quasi-r genes associated with small-molecule biosynthetic clusters could have other metabolic and regulatory processes. Antibiotic resistance is highly pleiotropic in character. Is it possible that other selective pressures—the expression of efflux or influx systems, for example—might lead to strains resistant to an antibiotic? Pleiotropic interactions can also derive from changes in the distributed metabolic pathways that are networked in cells; an alteration in the concentration of one enzyme or protein could lead to adjustments in processes concerned with microbial community networking (Vallino, 2003).

Whether the microbial products identified to have antibiotic activity do function as antibiotics in natural environments is a moot question. As mentioned earlier, the word "antibiotic" was coined by soil microbiologist Selman Waksman, the Nobel Prize-winning discoverer of streptomycin. He and his distinguished team of researchers (including Mary and Hubert Lechevalier and Boyd Woodruff) isolated hundreds of actinomycetes from different soils and subsequently identified compounds with antibiotic activity in the laboratory (streptothricin, neomycin, actinomycin D, etc.). These discoveries were the genesis of the antibiotic industry. Waksman described an antibiotic as "a compound produced by a microbe that kills or inhibits the growth of another microbe." Subsequently, he must have realized that this was an anthropocentric viewpoint, for he stated that "one is forced to conclude that antibiotics play no role in modifying or influencing the living processes that exist in nature" (Waksman, 1973). Unfortunately, the word "antibiotic" had become fixed, defining both compounds and activities. All low-molecular-weight inhibitors from nature were called antibiotics.

Over the last century, studies of microbial natural product function have been predicated on "useful" clinical applications or chemistry. Cellular targets (receptors) have been identified and

detailed studies of the biochemistry of inhibitory action carried out (Gale *et al.*, 1981). However, biological and ecological considerations of the roles of these compounds have been rare. In the case of compounds with antibiotic activity, the principal interest was the MIC, an anthropocentric concept if ever there was one. Some efforts were made to detect antibiotic activity in soil environments (Gottlieb, 1976); however, the fact that the results were negative was possibly a disappointment but was of no concern.

In recent times, studies of microbes in natural environments have provided drastically altered concepts of the natural lifestyles and functions of bacteria (for example, they do not grow as isolated, single colonies in the wild), and questions have been asked about the possible roles of the large number of bioactive microbial compounds that are produced. Although the ability to isolate single bacterial colonies on agar was critical to bacterial identification and the study of pathogenicity, this practice has actually delayed the development of microbial ecology. Nowadays, emphasis is being placed on investigations of the interactions within complex bacterial communities (microbiomes) in different environments, as many diseases occur as the result of polymicrobial infections.

Considering again the ubiquity of the quasi-r genes found in nature, if antibiosis is not a common function, what are the roles of these genes? It is well known that antibiotic activity is only one of the biological properties of bioactive small molecules. They exhibit extensive pleiotropy/multifunctionality and most likely are involved in cell-cell signaling within and between bacteria and other organisms in the environment (fungi, plants, insects, and even human and animal hosts). Do the quasi-resistance mechanisms provide the means for attenuating cell-cell interactions, natural degradation pathways, or other functions (Davies, 2006)?

Penicillinaseshave been implicated in cell wall turnover (Jacobs *et al.*, 1994). Efflux pumps are promiscuous, and a variety of low-molecular-weight compounds with limited structural similarities may be substrates for the same pump (Poole, 2005).

2.12How to Control Antibiotic Resistance Development

By any consideration, the most serious consequence of the use of antibiotics is the concomitant development of resistant strains; this has prompted continuous efforts to exert control over antibiotic usage. Erythromycin was an early example; introduced as an alternative to penicillin for the treatment of *S. aureus* in Boston City Hospital in the early 1950s, it was completely withdrawn after less than a year because 70% of all the *S. aureus* isolates were found to have become erythromycin resistant. The same was observed with chlortetracycline and chloramphenicol and, subsequently, with other antibiotics (Finland, 1979).

It is clear that antibiotic resistance seems inevitable. What steps can be taken to prevent or at least delay this process? Over the years, many different solutions have been proposed by knowledgeable experts and all the major international health groups (e.g., WHO and the CDC). Among the proposals for action are strict controls on antibiotic use by humans, requiring accurate prescriptions (no use of antibiotics to treat colds and other viral infections), no delivery of antibiotics without a doctor's prescription (reducing needless use of antibiotics), and controlled therapeutic use in animal husbandry and agriculture. Interestingly, the Swann recommendations of 1969, were the first to call for a ban on nontherapeutic use in animals and agriculture, a reasonable but highly contentious suggestion that has been impossible to enforce in many countries to this day. Deception has played a role in this failure; many of the antimicrobials approved for treatment of humans are given to animals under the cover of

different names for different uses, as described in the *Report of the Advisory Committee on Animal Antimicrobial Use Data Collection in the United States of the Alliance for the Prudent Use of Antibiotics* (DeVincent and Viola), 2006). Although the Netherlands and Scandinavia have successfully reduced resistance levels, it is clear that restriction of antibiotic use is difficult to implement on a global scale. Universal adherence to the suggested rules for restraint could have a positive effect, but would resistance be eliminated? Almost certainly not. See the most recent report (of many), *Antibiotic Resistance*: *an Ecological Perspective on an Old Problem* (American Academy, 2009). However, if well-considered restrictions and rules for usage were supported by a pipeline of structurally novel antibiotics and semisynthetics designed to be refractory to resistance mechanisms, one could expect some significant and lasting improvements in the treatment of infectious diseases.

Past history provides recurrent warnings. Following its introduction in the United States in the 1950s, penicillin was available over the counter for almost 10 years before prescriptions were required. Thus, we can assume that a "core" population of antibiotic-resistant strains was established by the early 1960s in most industrialized nations. Transmission of plasmid-encoded resistance mechanisms that developed during that period contributed to international dissemination.

On the side of success, mode-of-action-guided chemical modifications of compounds such as aminoglycosides, β-lactams, macrolides, and other antibiotic classes have resulted in active derivatives that are refractory to one or more of the known resistance mechanisms. However, the target for resistance function cannot be modified or removed completely without affecting antibiotic activity. Novel semisynthetic compounds generated by such chemical modifications of

antibiotic core structures have extended the useful life of several classes, such as methicillin (oxacillin), the macrolide azithromycin, and the modified aminoglycoside amikacin, among others. But this approach does little more than buy time. The r genes evolve in response to new selection pressures, and since multiple mechanisms of resistance exist for every class of antibiotic, the avoidance of each and every modification is impossible. In addition, in some cases, chemical modification of antimicrobials has led to enhanced toxicity. As mentioned earlier, the ability to pump antibiotics out of cells is a common feature of most environmental microbes and their pathogenic relatives and is the most widespread form of resistance to most classes of antibiotics. Devising compounds that interfere with efflux of active inhibitors from the cell is an attractive strategy for the design of modified or combination therapeutics (Lomovskaya*et al.*, 2007). Unfortunately, in spite of considerable effort, very few effective compounds have been obtained, and only one or two have come close to market. This approach is clearly viable, but for the time being, it remains little more than a pipe dream.

Over the years, there has been much discussion of "cycling" antibiotics to try to reduce selection pressures for resistance and thus prolong the useful life of compounds; this involves the periodic replacement of front-line antibiotics with alternative structural classes in hospitals. Cycling does not provide a long-term solution, however, since resistant strains never disappear from the resident population; when related antibiotics are reintroduced, the problem strains (or r genes) are quickly reselected. In large hospital complexes, it may be difficult to decontaminate the "infected" intensive care centers appropriately while cycling between different antibiotics. Has this approach been given a fair test? What might the experience be in more easily controlled situations(Bergstrom, 2004)?

A related tactic involves treatment with combinations of inhibitory compounds that have different modes of action. This combinatorial approach (a fluoroquinolone plus a macrolide or a β-lactam plus an aminoglycoside or tetracycline) has been used in the past to overcome resistance and has also been applied with success in the treatment of diseases such as cancer and HIV infection. However, detailed pharmacodynamic information is essential, and regulatory issues need to be resolved before standardized combinations of antibiotics can be used in routine practice. For example, how does one guarantee that in a mix of two or more active compounds, all arrive at the site of infection at the predetermined concentration range for maximum synergy (and not simply additive effects)? Nonetheless, with seriously life-threatening infections in hospitals, drastic measures must be taken, and a variety of antibiotic combinations are frequently used. Is it possible that older and/or unused (or even discarded) antibiotics might be rehabilitated for "last-resort" use in rational combinations to overcome multidrug-resistant bacterial infections, as some studies have suggested (Yeh*et al.*, 2009).

Many strategies for avoiding, inhibiting, or bypassing resistance mechanisms in pathogens have been attempted. The most notable successes in such endeavors have been with the β-lactam antibiotics. Clavulanic acid and related compounds are potent inhibitors of β-lactamase enzymes and are frequently used in combination with the β-lactam antibiotics. These combinations have been highly effective (Reading and Cole, 1977), but bacteria have found a way to outsmart us: a number of β-lactamases that are refractory to inhibition by clavulanate have appeared. To date, research to extend this approach to other classes of antibiotics has not been successful. This poses another interesting ecological question—given that β-lactamases are common in nature, what are the roles of the natural β-lactamase inhibitors, such as clavulanic acid (ThomsonAmyes, 1992).

It has also been proposed that inhibitors of bacterial virulence could be used to arrest the disease process and thus do away with the requirement for antibiotics. This elegant solution appears to have an advantage over antibiosis in that selection for resistance (survival in the host) might not occur because the growth of the infecting organism would not be impaired. Some success has been obtained in small-animal models, but more extensive studies are essential if this therapy is to be validated (Balaban*et al.*, 1998). Other nonantibiotic approaches for the treatment of bacterial diseases involve stimulation or recruitment of the innate immune system of the host (Finlay, 2004). Recent advances in our understanding of the roles of the human gut microbiome in innate immunity may lead to other therapeutic options.

This review has ignored (among other things) one of the major aspects of the control of bacterial diseases, i.e., prevention. In an ideal world with effective vaccines against all infectious diseases, the use of antibiotics would be reduced drastically and hopefully limited to surgical procedures in hospitals under strict controls. However, despite years of effort, there are few widely used antibacterial vaccines. The success of the pneumococcal vaccine is a model of what can be achieved (American Academy, 2005).

2.13 Ultraviolet Light

UV, or ultraviolet, light is an invisible form of electromagnetic radiation that has a shorter wavelength than the light humans can see. It carries more energy than visible light and can sometimes break bonds between atoms and molecules, altering the chemistry of materials exposed to it. UV light can also cause some substances to emit visible light, a phenomenon known as *fluorescence*. This form of light which is present in sunlight can be beneficial to health,

as it stimulates the production of vitamin D and can kill harmful microorganisms, but excessive exposure can cause sunburn and increase the risk of skin cancer (*www.wisegeek*,2016).

The term "ultraviolet" means "beyond violet." In the visible part of the spectrum, wavelength decreases and the energy of the electromagnetic waves increases from red through orange, yellow, green, blue and violet, so UV light has a shorter wavelength, and more energy, than violet light. Wavelengths are measured in nanometers (nm), or billionths of a meter, and ultraviolet wavelengths range between 10nm and 400nm. It can be classified as UV-A, UV-B or UV-C, in order of decreasing wavelength (*www.wisegeek*, 2016).

UV light has become a global concern due to the fact that it is a major environmental mutagen and carcinogen, leading to conditions such as skin cancer (Livneh*et al.*, 1993). Coupled with the fact that ozone layer (the atmospheric layer that filters out solar UV radiation from the sun) is continuously depleted by pollution, there is pressure to better understand the mechanics in how mutations arise so that possible remedies in the future can be devised. UV radiation may be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria. Such environmental stress caused by UV may in some way induce different evolutionary changes on bacteria that would have otherwise not been selected for. This area thus provides avenues of physiological, ecological, and genetic investigation because mutations play a key role in biological processes such as evolution, carcinogenesis, aging, and generation of somatic genetic diversity (Livneh*et al.*, 1993).

2.14Effect of UV Light Exposure on Bacteria

Ultraviolet light (opt 250 nm) (Muhammad and Najafi, 2013), exerts its mutagenic effect by exciting electrons in a bacterial DNA molecule.UV light is particularly effective at generating a specific precursor for mutation known as a pyrimidine dimer. The ring structures of two adjacent thymine bases in a polynucleotide chain will absorb UV light and form a cyclobutane ring which links carbons 5 and 6 of each pyrimidine ring to one another. Adjacent thymine and cytidine bases can also be photoactivated to form a 6-4 linkage between the two bases. If a pyrimidine dimer is left in place, the DNA replication machinery cannot replicate that region and the bacterium will die during cell division (Stephanie, 2011). The cell often tries to repair pyrimidine dimers before replication, but the repair mechanism can also lead to mutations as well (Michelle 2015).In order to reduce the effects of pyrimidine dimers, bacteria have several repair mechanisms, some of which are able to reverse the dimer crosslink while others are more error-prone and create mutations(Stephanie, 2011).

CHAPTER THREE

3.0. MATERIALS AND METHOD

3.1.Isolation and identification ofBacteria

3.1.1Isolation of *Salmonella typhi* and *Shigelladysentriae*From Stool

Clean, dry, sterile, disinfectant-free suitable wide-necked containers were used to collect the stool samples from patients. The patients were asked to avoid contaminating the faeces with urine (Cheesbrough, 2006). When the specimen was formed or semi-formed, a thick suspension of it was made in about 1 ml of sterilePeptone water.A loopful of fresh emulsified faeces or a fluid specimen was inoculated on *SalmonellaShigella* (SSA) Agar. The SS Agar plate was incubated aerobically at 35–37 °C overnight. *Salmonella* produced colorless colonies 1–2 mm in diameter with black center, while *Shigella* produced colourless colonies, 2-4 mm in diameter without black center. *Salmonella typhi*and *Shigelladysentriae*wereidentified by urease test, Indoletest,Methyl Red test,VogesProskaeur Test,Motility test, Triple sugar iron agar (TSI) test,Oxidase test, Citrate test, Lactose test, Mannitol test and H_2S production (Cheesbrough, 2006).

3.1.2.Isolation of *Proteus mirabilis* and *Staphylococcus aureus* from Urine

Sterile, dry, leak-proof containers were used to collect samples from patients. Clean-catch specimen was mixed by rotating the container. Using a sterile wire loop(one that holds 0.002 ml), a loopful of urine was inoculated on a quarter plate of CLED (Cystine Lactose Electrolyte-Deficient) Agar. The plate was incubated aerobically at 35–37 °C overnight (Cheesbrough, 2006).

Proteus mirabilis produced blue –gray translucent colonies while *Staphylococcus aureus*produced deep yellow colonies of uniform colour. *Staphylococcus aureus* which was coagulase positive was confirmed by coagulase test, Catalase test, mannitol test, oxidase test and

blood Hemolysis test. *Proteus mirabilis* was confirmed by urease test, oxidase test, indole test, citrate test, Methyl Red test, VogesProskaeur Test, Motility test, Triple sugar iron agar (TSI) test, H_2S production test (Cheesbrough, 2006).

3.2.Biochemical Tests

Biochemical test were carried out to ascertain the identity of the organisms as described by(Cheesbrough, 2006).

.3.2.1.Catalase Production Test

This test was used demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as *streptococci*. Hydrogen peroxide 1-2ml solution was poured into a test tube.Using a sterile wooden stick or a glass rod, several colonies of the 18 to 24 hours of the test organisms were taken and immersed in the hydrogen peroxide solution.An immediate bubbling of oxygen was observed (Cheesbrough, 2006).

3.2.2. Coagulase test

This was carried out to identify *S. aureus*which produces the enzyme coagulase. Coagulase causes plasma to clot by converting fibrinogen to fibrin.The slide test was carried out using two clean slides containing a drop of water. Few colonies of the test organisms were emulsified in each of the drops to make two thick suspensions. A drop of human or rabbit plasma was added to one of the suspensions, and mixed gently. And the control has no plasma Positive results was obtained as a result of clumping of the organisms within 10 seconds (Cheesbrough, 2006).

3.2.3. Triple Sugar Iron Agar (TSI) Test

One of each colony type of the isolates was selected on plate using a sterile straight wire loop. The center of the colony was touched lightly and inoculated in the prepared TSI medium by stabbing the butt twice and streaking the slants followed by incubation at 37^{o}C for 24 hours. The medium was kept moist with the evidence of water of condensation at the slant butt junction.

A yellow butt (acid) and red or pink (alkaline) slope indicates the fermenting of glucose only. Cracks and bubbles in the medium indicate gas production from glucose fermentation. A yellow (acid) butt indicates the fermentation of lactose. A red or pink (alkaline) slope and butt indicates no fermentation of glucose or lactose. Blackening along the stab line or throughout the slant indicates hydrogen Sulphide (H_2S) production. *Salmonella* forms a red slope (alkaline) and yellow (acid) butt with or without gas or H_2S production (Oyeleke and Manga, 2008).

3.2.4. Urease Test (Christensen's (Modified) Urea Broth)

Urea agar was prepared and inoculated heavily over the entire surface of the slants in test tubes. The test tubes were incubated at 37^{o}C for 3-12 hours. A urease-positive culture produces an alkaline reaction in the medium, evidenced by pinkish-red color of the medium. Urease-negative organisms do not change the color of the medium, which is pale yellow-pink (Oyeleke and Manga, 2008).

3.2.5. Methyl Red -VogesProskaeur Test

Five ml (5ml) of MR-VP broth was inoculated with the test isolates and incubated for four days at 35^{o}C. After incubation period, the 2-3 drops of methyl-Red was added into the tube positive Methyl-Red test was identified by red colour of the indicator and the negative test was identified by yellowing of the indicator. To the other tubes, 5 drops of 40% potassium hydroxide (KOH) was added followed by addition of 15 drops of 5% Naphthol in ethanol the solution was shaken

and kept in slopping position. The development of red colour within one hour indicated positive result. No colour change indicated negative result (Oyeleke and Manga, 2008).

3.2.6. Oxidase Test

A piece of filter paper was flooded with few drops of oxidase reagent, a bit growth of the test isolated was picked and a solution was prepared and smeared on the paper using sterile tooth pick. The positive result was detected by the development of intense purple colour within 30 seconds of the smear (Oyeleke and Manga, 2008).

3.2.7. Indole Test

The test isolate was grown in 5ml peptone water for 24h at 37^{o}C. After incubation period, three to eight drops of Kovacs reagent was added to the tubes and shaken gently. Positive result was indicated by formation of red colour in the upper layer of the tube within one minute. Negative result was identified by retention of yellow colour (Oyeleke and Manga, 2008).

3.2.8. Citrate Utilization Test using Simmon's Citrate Agar

Simmon's citrate agar slopes was prepared in test tubes and solidified in slanted position the slopes were stabbed with the test organisms and incubated at 37^{o}C aerobically for 24-72 hours the development of deep blue colour indicates a positive reaction (Oyeleke and Manga, 2008).

3.2.9. Motility Test

Hanging Drop Method

Vaseline was spread on the four corner of a clean coverslip using toothpick, after thoroughly mixing the cultures small drop of bacterial suspension was aseptically placed in the center of the coverslip using sterile wireloop, thecavity slide was lowered with the cavity facing down, the drop protruded into the cavity center, the slide was pressed and sealed up the coverslip firm to the cavity slide, the hanging drop was turned over and placed on the stage of microscope, the

drop was first examined by locating the edge under low power(x10) and switched to the high power objective lens (x40) the actual motility was distinguished from Brownian movement (Cheesbrough, 2006).

3.3.Preparation of Turbidity Standard (Equivalent to 0.5 McFarlandStandard)

A 1% v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to99 ml of water and mix well. A 1% w/v solution of barium chloride was also prepared by dissolving 0.5g of dihydrate barium chloride ($BaCl_2.2H_20$) in 50 ml of distilled water. 0.6 ml of the barium chloride solution was added to 99.4 ml of the sulphuric acid solution, and mix, a small volume of the turbid solution was transfer to a capped tube bottle which was used as standard turbidity (Cheesbrough, 2006).

Using a sterile wire loop, colonies were emulsified in 2ml of sterile physiological saline and standard turbidity was obtained.

3.4.Inoculation of Samples

Mueller-Hinton Agar was prepared and autoclaved according to manufacturer. The media was poured into Petri dishes to depth of 4mm (about 20ml per plate). Care was taken to pour the plates on a level surface so that the depth of the medium was uniform. After the gel had solidified, using a sterile swab, the standard inoculum of *Salmonella typhi,Shigelladysentriae, Proteus mirabilis and Staphylococcus aureus* prepared, each was streaked onto the Muller Hinton agar plates (Cheesbrough, 2006).

3.5. Exposure to Antibiotics

Prepared Amoxicillin, Ciprofloxacin, and Gentamicin paper discs were placed at the center of the plates and the plates were incubated for 24 hours, at 35-37^{0}C. The bacteria were confirmed sensitive to the antibiotics, a zone of inhibition was formed (Cheesbrough, 2006). The zones of inhibition were measured and recorded, and then bacteria from the edges of the inhibition zones were picked up with a swabsticks, and inoculated on to new Mueller-Hinton Agar plates. This process was repeated for each of 10 exposures (Cheesbrough, 2006).

3.6. Exposure to UV Light

The bacteria (*Salmonella typhi,Shigelladisenteriae, Proteus mirabilis and Staphylococcus aureus*) isolated were exposed to ultraviolet light (UV light), by placing the petri dishes containing the bacteria under the UV light at distance of 15cm, and wave length of 254nm for 1, 3, 5, 7 and 10 minutes (Muhammad and Najafi, 2013). After each minute of the exposure, the bacteria were sub cultured in another Mueller-Hinton agar plates. Amoxicillin, ciprofloxacin, and gentamicin paper discs were placed at the center of the plates of each bacteria. Zones of inhibition were formed. The zone of inhibitions were measured and recorded (Cheesbrough, 2006).

CHAPTER FOUR

4.0RESULTS

4.1 Isolation and Identification of Bacteria

Isolation of *Salmonella typhi*was done using differential media, which is *Salmonella Shigella* agar (SSA). *Salmonellatyphi*produced colorless colonies with black center 1 mm in diameter.

Isolation of *Shigelladysentriae*was done using differential media, which is *Salmonella Shigella* agar (SSA). *Shigelladysenteriae*produced colorless colonies without black center2 mm in diameter.

Isolation of *Proteus mirabilis*was done using differential media, which is CLED agar. *Proteus mirabilis* produced blue –gray translucent colonies.

Isolation of *Staphylococcus aureus*was done using differential media, which is CLED agar. *Staphylococcus aureus*produceddeep yellow colonies of uniform colour.

Biochemical characteristics of the test bacteria were presented in Table 4.1

Table 4.1: Biochemical Characteristics of Bacteria

Mt	TSI	Ur	In	Ox	Ci	La	Ma	MR	VP	Ca	Co	Bh		Inference
+	A/k	-	-	-	-	-	+	+	-					*S typhi*
-	A/K	-	+	-	-	-	-	+	-					*S dysenteriae*
+	A/K	+	-	-	+	-	-	+	-					*Pmirabilis*
-				+			+			+	+	+		*S aureus*

Key Mt (motility test), Ur (urease test), In (indol test), La (lactose test), Ci (citrate test), Ox (oxidase test), Ma (mannitol test), Ca (catalase test), Co (coagulase test) and Bh (blood haemolysis)

4.2 Repeated Exposure of Bacteria to Antibiotics

Over the course of repeated exposure to the antibiotics, all the bacteria developed and gained resistance to the antibiotics gradually. The following results were obtained as shown in tables 4.2 to 4.2.

Salmonella typhi isolated from stool samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin Table 4.2 (*Salmonella typhi*) showed antibiotic sensitivity test, Ciprofloxacin zones gradually decreased, from 1st to 10th exposure and was observed to be from 44mm to 15mm, for Gentamycin decrease of zone diameter was observed as 27mm at 1st exposure to 18mm at 3rd exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 23mm to 15mm, at 1st to 3rd exposure respectively as shown in Table 4.2

Shigelladisenteriae isolated from stool samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin Table 4.3 (*Shigelladysenteriae*) showed the antibiotic, sensitivity test for each test antibiotic, Ciprofloxacin zones gradually decreased, from 1st to 10th exposure was observed as 35mm to 20mm respectively, for Gentamycin decreased of zone diameter was from 26mm at 1st exposure to 15mm at 4th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 25mm to 15mm, at 1st to 3rd exposure respectively as shown in Table 4.3

Proteus mirabilis isolated from urine samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin. Table 4.4 (*Proteus mirabilis*) showed the antibiotic sensitivity test, Ciprofloxacin zone gradually decrease, from 1st to 10th exposure was observed as 44mm to 25mm respectively, for Gentamycin decreased of zone diameter was observed from 30mm at 1st exposure to 15mm at 10th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 25mm to 15mm, at 1st to 4th exposure respectively as shown in Table 4.4

Staphylococcus aureus isolated from urine samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin; Table 4.5 (*Staphylococcus aureus*) showed the antibiotic sensitivity test, Ciprofloxacin zone gradually decreased, from 1st to 10th exposure was observed as 35mm to 18mm, respectively, for Gentamycin decreased of zone diameter was observed from 30mm at 1st exposure to 17mm at 7th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 22mm to 15mm, at 1st to 4th exposure as shown in Table 4.5

Table 4.2: Zone of Inhibition of *Salmonella typhi* After 10 Repeated Exposure to Antibiotics

List of Antibiotics/ No of Exposure	Diameter in (mm)									
	1st day	2nd day	3th day	4th day	5th day	6th day	7th day	8th day	9th day	10th
Ciprofloxacin	44	38	35	30	25	23	20	17	17	15
Gentamycin	27	20	18	00	00	00	00	00	00	00
Amoxicillin	23	20	15	00	00	00	00	00	00	00

Table 4.3: Zone of Inhibition of *Shigelladysentriae* After Repeated Exposure to Antibiotics

List of Antibiotics/ No of Exposure	Diameter in (mm)									
	1st day	2nd day	3th day	4th day	5th day	6th day	7th day	8th day	9th day	10th
Ciprofloxacin	35	35	30	28	28	27	24	23	22	20
Gentamycin	26	22	15	15	00	00	00	00	00	00
Amoxicillin	25	20	15	00	00	00	00	00	00	00

Table 4.4: Zone of Inhibition of *Proteus mirabilis* After Repeated Exposure to Antibiotics

List of Antibiotics/ No of Exposure	diameter in (mm) 1st day	2nd day	3th day	4th day	5th day	6th day	7th day	8th day	9th day	10th
Ciprofloxacin	44	44	44	40	30	30	28	28	27	25
Gentamycin	30	30	24	24	23	23	20	20	18	15
Amoxicillin	25	25	20	15	00	00	00	00	00	00

Table 4.5: Zone of Inhibition of *Staphylococcus aureus* After Repeated Exposure to Antibiotics

List of Antibiotics/	Diameter in (mm)									
No of Exposure	1st day	2nd day	3thday	4th day	5thday	6th day	7th day	8th day	9th day	10th
Ciprofloxacin	35	35	32	30	25	22	22	20	20	18
Gentamycin	30	29	25	22	19	15	14	00	00	00
Amoxicillin	22	20	17	15	00	00	00	00	00	00

4.3 Repeated Exposure of Bacteria to UV Light

Over the course of repeated exposure of bacteria to the UV light, the zone of inhibition for each exposure was measured and recorded as shown in appendix 1 to IV. The results obtained also presented in Figure 4.1 to 4.4.

Salmonella typhi isolated from stool samples collected from different patients was exposed to UV light at different minutes. Figure4.1 (*Salmonella typhi*) showed decrease in diameter at first minute of exposure which is from 45mm at 0 minute to 29mm at 1 minute and increased to 32mm at 10 minute of exposure. For Gentamycin, decrease of zone diameter was observed form 35mm at 0 minute to 27mm at 1 minute and also decrease to 25mm at 10 minute exposure. Amoxicillin showed decrease in zone diameter from 21mm at 0 minute to 17 mm at 1 minute exposure and increased to 22mm at 10^{th} minute exposure as shown in Figure 4.1.

Shigelladisenteriae isolated from stool samples collected from different patients was exposed to UV light at different minutes. Figure 4.2 (*Shigelladysenteriae*) also showed decrease in zone of inhibition at 1 minute exposure, as the zone diameter of ciprofloxacin decrease from 37mm at 0 minute to 30mm at 1 minute and increase to 34mm at 10minute exposure. Gentamycin showed decrease of zone diameter, which is from 25mm at 0 minute to 20mm at 1 minute exposure and increase to 24mm at 10 minute exposure. Amoxicilline showed decrease of zone diameter, from

25mm at 0 minute to 16mm at 1 minute exposure and increase to 20mm at 10 minute exposure as shown in Figure 4.2

Proteus mirabilis isolated from urine samples collected from different patients was exposed to UV light at different minutes. Figure 4.3 (*Proteus mirabilis*) showed decrease of zone of inhibition in all 1minute exposure, Ciprofloxacin zone diameter decrease from 35mm at 0 minute to 30mm at 1minute exposure and increased to 30mm at 10 minute exposure. Gentamycin zone decrease from 28mm to 25mm and increase to 26mm at 7 minute exposure. Amoxicillin also showed decrease of zone diameter, which is from 21mm at 0 minute to 18mm 1 minute exposure and increased to 21mm at 3,7 and 10 minute exposure as shown in Figure 4.3.

Staphylococcus aureus isolated from urine samples collected from different patients was exposed to UV light at different minutes Figure 4.4. (*Staphylococcus aureus*) showed decrease in zone of inhibition in ciprofloxacin and gentamycin rows. Ciprofloxacin zone of inhibition was decreased from 42mm at 0 minute to 40mm at 1,7and 10 minute exposure, and to 36mm at 3 and 5 minute. Gentamycin showed decrease in zone diameter which is from 34mm at 0 minute to 1 minute exposure and 28mm at 10minute exposure. Amoxicillin also showed decrease in zone diameter only at 3 and 5 minute exposure and increased to 25mm at 1, 7 and 10 minute exposure as shown in Figure 4.4.

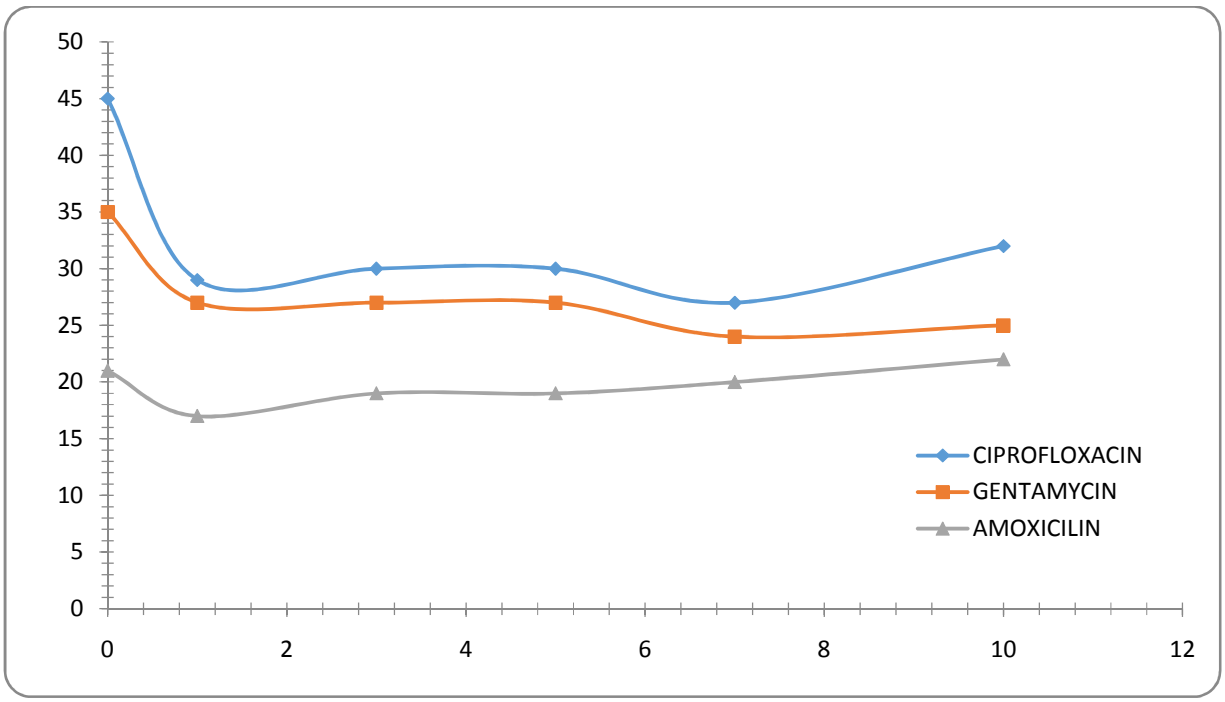

Figure 4.1: Sensitivity and Resistance Pattern of *Salmonella typhi* After Repeated Exposure to UV light and Antibiotics (Zone diameter (mm) against time of Exposure (min)).

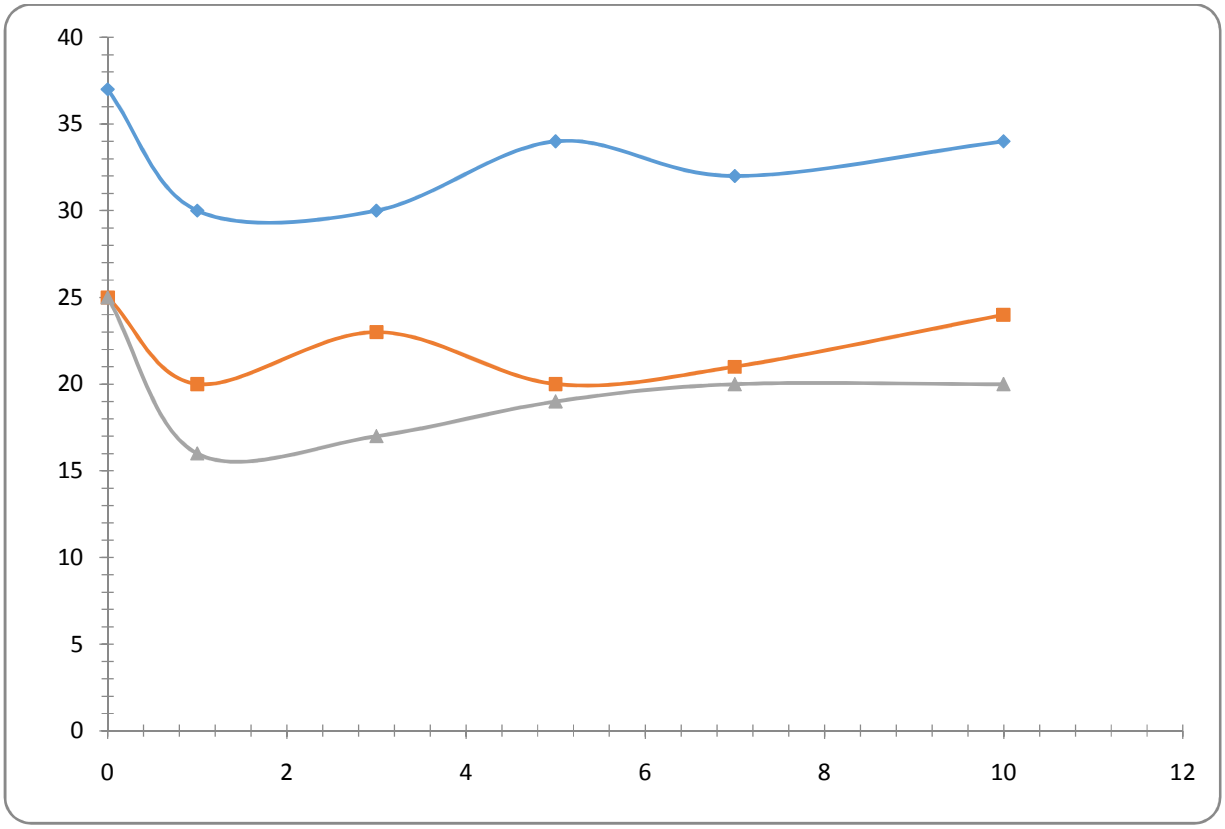

Figure 4.2: Sensitivity and Resistance Pattern of*Shigelladysentriae*After Repeated Exposure to UV light and Antibiotics(Zone diameter (mm) against time of Exposure (min)).

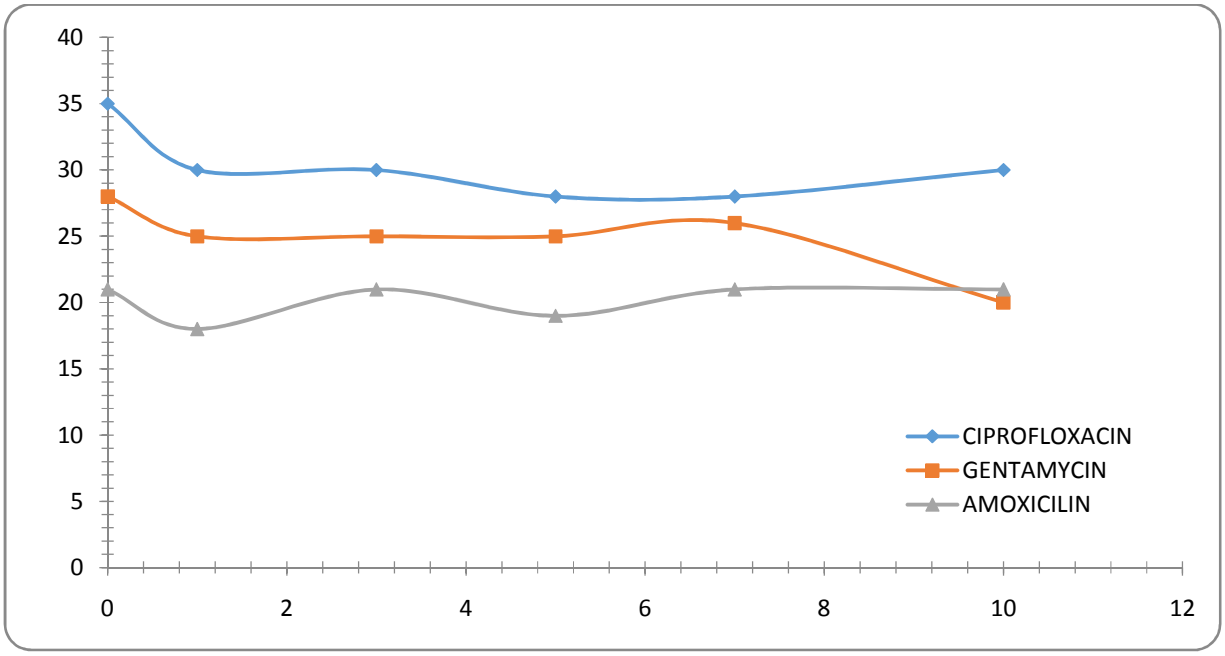

Figure 4.3: Sensitivity and Resistance Patternof *Proteus mirabilis* After Repeated Exposure to UV Lightand Antibiotics (Zone diameter (mm) against time of Exposure (min)).

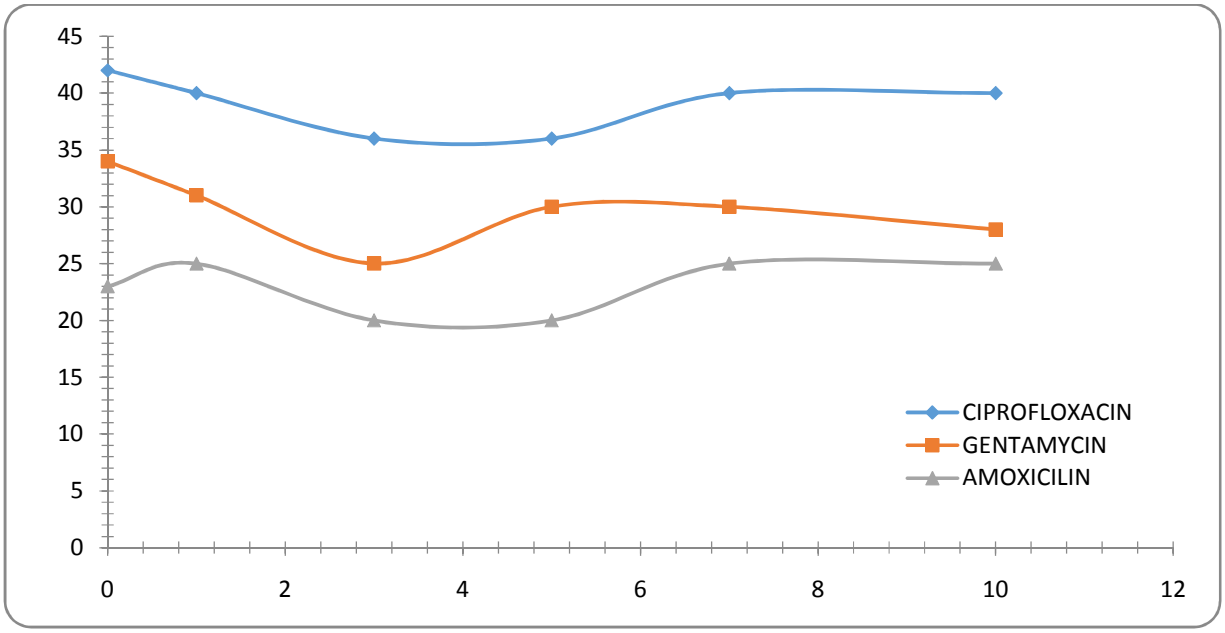

Figure 4.4: Sensitivity and Resistance Pattern of *Staphylococcus aureus*After Repeated Exposure to UV Light and Antibiotics(Zone diameter (mm) against time of Exposure (min)).

CHAPTER FIVE

5.0DISCUSSION

Out of 75 stool and 82 urine samples collected from different patients,*Salmonella typhi* (15);*Shigelladyseteriae*(9) were isolated from stool and*Proteus mirabilis*(6);*Staphylococcus aureus*(5) were isolated from urine. Only four out of the isolated pathogens(*Salmonella typhi, Shigelladysenteriae, Proteus mirabilis* and *Staphylococcus aureus,*) weresensitive to the test antibiotics (Ciprofloxacin, Gentamycin, and Amoxicillin). For the ten different exposure to test antibiotics separately, the zone of inhibition for each exposure was measured and recorded. The test organisms developed resistance to the antibiotics as seen through decrease in the diameter of their zones of inhibition. The results obtained confirmed that repeated exposure of the bacterial pathogens increased their resistance to the antibiotics they were exposed to as reported by James(2015). This means that the bacteria developed resistance when repeatedly exposed to a particular antibiotic. This could be due to the following reasons:Antibiotic modification; bacterial enzymes like *Beta lactamase* alter the structure of the antibiotic and thereby render the antibiotic ineffective. The mechanism of resistance in Gram positive and negative bacterial species to *Beta lactam* antibiotics, bypreventing the antibiotic from entering the bacterial cell or pumping it out quicker than it floods. Antibiotic is unable to inhibit the activity of the target structure in the bacteria because of structural changes in the bacterial molecule.The bacteria produced an alternative target like an enzyme that is resistant to inhibition by the antibiotic while continuing to produce the original sensitive target. This allows the bacteria to survive in the face of selection as reported byHawkey(1998) or as result of mutation in the bacterial chromosome or the acquisition of extra-chromosomal DNA as reported by Alekshun(2007).

It is also observable from the results that Ciprofloxacin is the most active antibiotic against the test organism as it has notable zone of inhibition in all the organisms upon repeated exposure. This is due to its very good spectrum of activity against several clinically important aerobic Gram negative bacilli like those belonging to *Enterobacteriaceae* (eg*E coli*) and *Pseudomonas aeruginosa*. They are also active against Gram positive cocci like *S pneumoniae*, *S aureus* and beta haemolytic*streptococci.H influenzae*, *Chlamydia pneumoniae*, and *Mycoplasma pneumonia* (Velissariou 2006).While Amoxicillin was the least active antibiotic against the test bacteria with exhibition of full resistance on 4th exposure for *shigelladysenteriae* and *Salmonella typhi* and on 5th exposure for *Proteus mirabilis* and *Staphylococcus aureus*. This could be due to the ability of bacteria to produce β-lactamase when encountered with β-lactam antibiotics as reported by (Allen *et al.*, 2009).

The implications of antibiotic resistance is that many of the available treatment options for common bacterial infections are becoming more and more ineffective. As a consequence, there are situations where infected patients cannot be treated adequately by any of the available antibiotics. This resistance may delay and hinder treatment, resulting in complications or even death. Moreover, a patient may need more care, as well as the use of alternative and more expensive antibiotics, which may have more severe side effects, or may need more invasive treatments, such as intravenous injection, to be given in hospitalsaccording to (WHO, 2014).

Exposure of micro flora to antibiotics may increase the number of resistant factors which can transfer resistance to pathogenic bacteria (Mathew *et al.,* 2007). There is a strong association between consumption of antibiotic and antibiotic resistance of bacteria. It is evident-based with the B-lactamases. Horizontal gene transfer (HGT) has a main role in the progress and diffusion of the resistance to the B-lactam antibiotic among the enteric bacteria in both community and

hospital level infections. Regular mutations in the genome of DNA create resistance to Fluoroquinolones and other antibiotics by transfer of DNA between bacterial strains (Davies and Davies, 2010).

The findings in this research are in line with the findings of James(2015) who confirmed that repeated exposure leads to antibiotic resistance in some bacteria. This was supported by Betty *et al,*(1993) who stated thatrepeated and improper uses of antibiotics are primary causes of the increase in drug-resistant bacteria. The results obtained also conforms with the findings of (Sule*etal,* 2002) which specified that floroquinolones are very effective against most of the bacteria which are resistant to other antibiotics.

The results obtained in Figure 4.1 to 4.4 showed that antibiotics response of the test organisms after repeated exposure to UV light with 254nm wave length at a distance of 15cm for 1,3,5,7 and 10 minutes. The results indicated that exposure of the test organisms to UV light for 1 minute, increased their resistance to the antibiotics they were exposed to. The result also showed that the tests organisms become more susceptible to the test antibiotics mostly for 5,7 and 10 minute of exposure. This means that the short time exposure of the test organisms to ultraviolet light during the sensitivity testing, increased their resistance as seen by decrease in the diameter of their zones of inhibition. It is also observable that the longer the organisms were exposed to UV light, the bigger are their zones of inhibition. This means prolonged exposure to UV light makes the bacteria to be more susceptible to the test antibiotics. This may be due to the effect of the UV light on the genetic material of the organisms, that is,the excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines (specifically thymine) in DNA. When two pyrimidines are bound together in this way, it is called a pyrimidine dimer (Stephanie, 2011). These dimers often change the shape of the DNA in the

cell and can cause problems during replication. The cell often tries to repair pyrimidine dimers before replication, but the repair mechanism can also lead to mutations as well (Michelle, 2015). Cells have mechanisms to repair this damage, but if the duration of exposure to UV light is sufficient, the repair mechanisms are unable to keep up with the rate of DNA modification (Goodsell, 2001).

Extended exposure to UV radiation can alter the genetic material of a cell leading to unfavorable mutations and even cell death. Typically, the shorter the UV wavelength, the greater the damage to an organism. From a molecular standpoint, cell death could be due to the fusion of thymine bases that are located next to each other. This new bond between two of the same nitrogenous bases negatively impacts the structure and overall shape of the DNA molecule, subsequently altering the genetic material of the cell. These cells are unable to reproduce because the dimerization prevents proper DNA replication (Parkin, 2011). For this reason, UV radiation is constantly used in medical environments to sterilize instruments and machinery because of its ability to disrupt bacterial division (Kodoth and Jones, 2015).

Several research studies have investigated the effectiveness of UV light on bacteria. A study conducted by Kodoth and Janes(2015) found that UV light exposure at 254 nm from a small school laboratory light altered bacterial growth.Alyssa(2004) also stated thatUltraviolet light exposure for short time periods, such as two or five minutes, was not expected to completely destroy the bacteria.

5.2 Conclusion

After ten time exposure of the test bacteria to antibiotics,the results obtained confirmed that repeated exposure lead to antibiotic resistance in *Salmonella typhi, Shigelladysenteriae, Proteus mirabilis and Staphylococcus aureus,* but opposed the prediction that repeated exposure of the test bacteria to ultraviolet light does lead to development of resistance, it rather make the surviving bacteria more susceptible to the test antibiotics.

5.3 Recommendations

In view of the results obtained, it is strongly recommend that:

Antibiotics should only be administered to patients if proven to be effective through antibiotics sensitivity testing.

The same drug should not be repeatedly administered for the treatment of a particular disease in case of reinfection.

UV light with low wave length should not be used for a short time disinfection because of its less effect on bacterial DNA.

REFERENCES

Abraham, E. P., and E. Chain. 1940. An enzyme from bacteria able to destroy penicillin. *Rev. Infect. Dis.* 10:677-678.

Alekshun, MN and Levy, SB. 2007."Molecular mechanisms of antibacterial multidrug resistance*". Cell 128 (6): 1037–50. doi:*10.1016/j.*cell.*2007.03.004. PMID 17382878.

Allen, H. K., L. A. Moe, J. Rodbumrer, A. Gaarder, and J. Handelsman. 2009. Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J.* 3:243-251.

Alyssa, E. B. 2004. What are the effects of ultraviolet light on bacteria mortality? *California State Science Fair Project Summary*. Project Number J1303.

American Academy of Microbiology. 2009. Antibiotic resistance: an ecological perspective on an old problem. Based on a colloquium held in the FondationMérieux Conference Center in Annecy, France, 12 to 14 October 2008.*ASM Press, Washington, DC.*

American Academy of Microbiology. 2005. Vaccine development: current status and future needs. Based on a colloquium held in Washington, DC, 4 to 6 March 2005.*ASM Press, Washington, DC.*

Aminov, R. I., and R. I. Mackie. 2007. Evolution and ecology of antibiotic resistance genes. FEMS*Microbiol.Lett.***271**:147-161.

Andersson, D.I. 2003.Persistence of antibiotic resistant bacteria, *Curr.Opin.Microbiol.***6**(2003), 452–456.

Andersson, D. I. 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr.Opin.*Microbiol.***9**:461-465.

Antibiotic Resistance *Questions & Answers* [Internet]. Atlanta (GA): Centers for Disease Control and Prevention (US); [updated 2013 Dec 18; cited 2014 Jan 15].

Austin, D.J. K.G. Kristinsson and R.M. Anderson. 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance, *Proc. Natl. Acad. Sci*. 96 (1999), 1152–1156.

Balaban, N., T. Goldkorn, R. T. Nhan, L. B. Dang, S. Scott, R. M. Ridgley, A. Rasooly, S. C. Wright, J. W. Larrick, R. Rasooly, and J. R. Carlson. 1998. Autoinducer of virulence as a target for vaccine and therapy against Staphylococcus aureus. *Science* 280:438.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. 2006. "Co-selection of antibiotic and metal resistance". Trends Microbiol.14 (4): 176–82.

Barbe, V., D. Vallenet, N. Fonknechten, A. Kreimeyer, S. Oztas, L. Labarre, S. Cruveiller, C. Robert, S. Duprat, P. Wincker, L. N. Ornston, J. Weissenbach, P. Marlière, G. N. Cohen, and C. Médigue. 2004. Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res. 32:5766-5779. [*PMC free article]*.

Bergstrom, C. T., M. Lo, and M. Lipsitch. 2004. Ecological theory suggests that antimicrobial cycling will not reduce antimicrobial resistance in hospitals. *Proc. Natl. Acad. Sci. U. S.* A. 101:13285-13290. [PMC free article].

Betty J. Mcgrath,T C. Randall Marchbanks, Deborah Gilbert, And Michael N. 1993. Dudley* Antimicrobial Agents And Chemotherapy, p. 1723-1725 0066- 4804/93/081723-03$02.00/0. Copyright © 1993, *American Society for Microbiology.* In Vitro Post antibiotic Effect Following Repeated Exposure to Imipenem, Temafloxacin, and Tobramycin.

Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks E, Johnston M, Barton H, Wright G. 2012. Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. *PLoS ONE.* 7(4):1-13.

Brazas, M. D., and Hancock.R. E. 2005. Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance.*Drug Discov. Today* 10**:**1245-1252. [PubMed]

Brisson-Noel, A., M. Arthur, and P. Courvalin. 1988. Evidence for natural gene transfer from gram-positive cocci to Escherichia coli. *J. Bacteriol.* 170:1739-1745. [PMC free article]

Canton, R. 2009. Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting.*Clin.Microbiol. Infect.* **15**(Suppl. 1):20-25.

Canton R, and Morosini M-I.2011 Emergence and spread of antibiotic resistance following exposure to antibiotics.*FEMS Microbiology Reviews*; 35:977–991.

Carlsson, G., S. Orn, and D. G. J. Larsson. 2009. Effluent from bulk drug production is toxic to aquatic vertebrates. *Environ. Toxicol. Chem.* 28:2656-2662.

Cassir, N; Rolain, JM; Brouqui, P. (2014). "A new strategy to fight antimicrobial resistance: the revival of old antibiotics.".*Frontiers in Microbiology*5: 551.

Cases, I., and V. de Lorenzo. 2005. Promoters in the environment: transcriptional regulation in its natural context. *Nat. Rev. Microbiol.* 3:105-118. [PubMed]

Chan CX, Beiko RG, Ragan MA; Beiko, RG; Ragan, MA. 2011. "Lateral Transfer of Genes and Gene Fragments in Staphylococcus Extends beyond Mobile Elements". *J Bacteriol* 193 (15): 3964–3977.

Cheesbrough, M. 2006.District laboratory practice in tropical countries, ECBS edition *Cambridge University Press.*

Cirz RT, Chin JK, Andes DR, de Crécy-Lagard V, Craig WA, Romesberg FE. 2005. "Inhibition of Mutation and Combating the Evolution of Antibiotic Resistance". PLoS Biol. 3 (6): e176.

Criswell, D, 2004. "The "Evolution" of Antibiotic Resistance."*Institute for Creation Research. N.p.*,

Davies J, and Davies D, 2010.Origin and evolution of Antibiotic Resistance, *Microbiology and Molecular Biology reviews*.p: 417-433.

Davies**,** J., G. B. Spiegelman, and G. Yim. 2006. The world of sub inhibitory antibiotic concentrations. *Curr.Opin.Microbiol.* 9**:**1-9. [PubMed]

Datta, N., and V. M. Hughes. 1983. Plasmids of the same Inc groups in enterobacteria before and after the medical use of antibiotics. *Nature***306**:616-617.

Depardieu, F., I. Podglajen, R. Leclercq, E. Collatz, and P. Courvalin. 2007. Modes and modulations of antibiotic resistance gene expression.*Clin.Microbiol.Rev.* **20**:79-114.

DeLeo, F. R., and Chambers.H. F. 2009. Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era. *J. Clin. Invest.***119**:2464-2474.

DeVincent, S. J., and C. Viola. 2006. Deliberations of an advisory committee regarding priorities, sources, and methods for collecting animal antimicrobial use data in the United States. Prev. *Vet. Med.* 73:133-151.

Dionisio, F., I. Matic, M. Radman, O. R. Rodrigues, and F. Taddei. 2002. Plasmids spread very fast in heterogeneous bacterial communities. *Genetics* 162:1525.

D'Costa, V. M., E. Griffiths, and G. D. Wright. 2007. Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr.Opin.Microbiol*. 10:481-489.

D'Costa, V. M., K. M. McGrann, D. W. Hughes and G. D. Wright. 2006. Sampling the antibiotic resistome. *Science* 311:374-377.

Enne, V.I. D.M. Livermore, P. Stephens and L.M. Hall. 2001. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction, *Lancet* **357** 1325–1328.

Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt. 2002. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proc. Natl. Acad. Sci*. U. S. A. 99:7687-7692.

Fajardo, A., N. Martinez-Martin, M. Mercadillo, J. C. Galan, B. Ghysels, S. Matthijs, P. Cornelis, L. Wiehlmann, B. Tummler, F. Baquero, and J. L. Martinez. 2008. The neglected intrinsic resistome of bacterial pathogens. *PloS One* 3**:**e1619.

Fick, J., H. Soderstrom, R. H. Lindberg, C. Phan, M. Tysklind, and D. G. J. Larsson. 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ. *Toxicol. Chem*. 28:2522-2527.

Finlay, B. B., and R. E. Hancock. 2004. Can innate immunity be enhanced to treat microbial infections? *Nat. Rev. Microbiol*. 2:497-504.

Finland, M. 1979. Emergence of antibiotic resistance in hospitals, 1935-1975. *Rev. Infect. Dis.* 1:4-22.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring (ed.). 1981. The molecular basis of antibiotic action, 2nd ed. John Wiley, Chichester, United Kingdom.

Gillings, M. R., M. P. Holley, and H. W. Stokes. 2009. Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. FEMS *Microbiol*.Lett. 296:282-288.

Gomez, M. J., and A. A. Neyfakh. 2006. Genes involved in intrinsic antibiotic resistance of Acinetobacterbaylyi. Antimicrob.*Agents Chemother*. 50**:**3562-3567.

Goodsell, D.S., 2001. "The Molecular Perspective: Ultraviolet Light and Pyrimidine Dimers," The Oncologist 6 (No. 3, June, 2001): 298-299, *http://theoncologist.alphamedpress.org/cgi/content/full/6/3/298*

Gottlieb**,** D. 1976. The production and role of antibiotics in soil.*Antibiot*.29**:**987- 1000.

Gniadkowski, M. 2008. Evolution of extended-spectrum beta-lactamases by mutation.*Clin. Microbiol. Infect.* 14(Suppl. 1):11-32.

Guerin, E., G. Cambray, S. Da Re, D. Mazel, and M. C. Ploy. 2010. The SOS response controls antibiotic resistance by regulating the integrase of integrons. *Med. Sci. (*Paris) 1:28-30.

Hawkey PM.1998.The origin and molecular basis of antibiotic resistance .BMJ; 317 (7159): 657-60.

Helinski, D. R. 2004. Introduction to plasmids: a selective view of their history, p. 1-21. In B. E. Funnell and G. J. Phillips (ed.), Plasmid biology.*ASM Press*, Washington, DC.

Hoffman, SJ; Outterson, K; Røttingen, JA; Cars, O; Clift, C; Rizvi, Z; Rotberg, F; Tomson, G; Zorzet, A. 2015. "An international legal framework to address antimicrobial resistance" (PDF).*Bulletin of the World Health Organization* 93 (2): 66. doi:10.2471/BLT.15.152710. PMID 25883395.Retrieved 8 July 2015.

Hopwood, D. A. 2007. How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? *Mol. Microbiol.* 63:937-940. [PubMed]

Horrevorts, A. M., J. Borst, R. J. T. Puyk, R. D. Ridder, G. Dzoljic-Danilovic, J. E. Degener, K. F. Kerrebijn, and M. F. Michel. 1990. Ecology of Pseudomonas aeruginosa in patients with cystic fibrosis. *J. Med. Microbiol.* 31:119-124.

Jacobs, C., L.-J.Huang, E. Bartowsky, S. Normark, and J. T. Park. 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. *EMBO J.* 13**:**4684-4694.

James C. B, (2015). Antibiotic Resistance by Repeated Exposure.*Science FairCalifornia State Project Summary* 2015.Project no. j1603

Kodoth, V and Jones, M. 2015. The Effects of Ultraviolet Light on Escherichia coli. West Windsor-Plainsboro High School South, New Jersey *Journal of Emerging Investigators* (P) 2

Kashmiri, S. V. S., and R. D. Hotchkiss. 1975. Evidence of tandem duplication of genes in a merodiploid region of pneumococcal mutants resistant to sulfonamide. *Genetics***81**:21.

Kevin, L and Alice, W, 2001.UV mutagenesis in Escherichia coli K-12: Cell survival and mutation frequency of the chromosomal genes lacZ, rpoB, ompF, and ampA.*Journal of Experimental Microbiology and Immunology* (JEMI) Vol. 1:32-46, M&I UBC

Levy, S and Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine.* 10(2):122-129.

Leekha, Surbhi; Terrell, Christine L.; Edson, Randall S. 2011."General Principles of Antimicrobial Therapy".*Mayo Clinic Proceedings* 86 (2). doi:10.4065/mcp.2010.0639. PMC 3031442.PMID 21282489.

Levin, B.R. 2001. How can we predict the ecologic impact of an antimicrobial: the opinions of a population and evolutionary biologist,*Clin.Microbiol. Infect*. **7** (2001), 24–28.

Levin B. R, V Perrot, Nina Walker. 2000. Compensatory Mutations, Antibiotic Resistance and the Population Genetics of Adaptive Evolution in Bacteria. *Genetics*.**154:** (3); 985–997

Lipp, E. K., A. Huq, and R. R. Colwell. 2002. Effects of global climate on infectious disease: the cholera model. *Clin.Microbiol.Rev.* **15**:757-770.

Lipsitch, M. C.T. Bergstrom and B.R. Levin. 2000. The epidemiology of antibiotic resistance in hospitals: paradoxes and prescriptions, *Proc. Natl. Acad. Sci.*97, 1938–1943.

Livneh, Z., O. Cohen-Fix, R. Skaliter, and T. Elizur. 1993. Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Critical Reviews in Biochemistry and Molecular Biology*. 28(6):465-513.

Livermore, D.M. 2004. Can better prescribing turn the tide of resistance?*Nat. Rev. Microbiol*. 2 (2004), 73–78.

Livermore, D.M. 2003.Bacterial resistance: origins, epidemiology, and impact, *Infect. Dis*. 36, S11–S23.

Li, X. Z.Nikaido H; Nikadio, H. 2009."Efflux-Mediated Drug Resistance in Bacteria: *an Updat*e". *Drug***69** (12): 1555–623.

Lomovskaya, O., H. I. Zgurskaya, M. Totrov, and W. J. Watkins. 2007. Waltzing transporters and 'the dance macabre' between humans and bacteria. *Nat. Rev. Drug Discov.* 6:56-65.

Long, K. S., J. Poehlsgaard, C. Kehrenberg, S. Schwartz, and B. Vester. 2006. The CfrrRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptograminA antibiotics. Antimicrob.*Agents Chemothe*r.50**:**2500-2505.

Marshall, B. M., D. J. Ochieng, and S. B. Levy. 2009. Commensals: unappreciated reservoir of antibiotic resistance. *Microbe* 4:231-238.

Mathew, A. G;Cissell, R. andLiamthong, S.(2007). Antibiotic resistance in bacteria associated with food animal: a United States perspective of livestock production.*Foodborne pathogens and Disease;* 4 (2).

Mendez, C., and J. Salas. 2001. The role of ABC transporters in antibiotic-producing organisms: drug secretion and resistance mechanisms. *Res. Microbiol.* 152:341-350.

Michelle F, 2015. Effects of UV Light Exposure on Bacteria Microbiology for Health Science Lab (Lab 8)

Mohammad B. and H. Najafi, (2013).Bacterial mutation; types, mechanisms and mutant detection methods: a review.*European Scientific Journal* vol.4 (p) 631.

Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, Tsuchiya T. 1998. "NorM, a Putative Multidrug Efflux Protein, of *Vibrio parahaemolyticus* and Its Homolog in *Escherichia coli*".Antimicrob.*Agents Chemother*. 42 (7): 1778–82. PMC 105682.

Mwangi, M. M., S. W. Wu, Y. Zhou, K. Sieradzki, H. de Lencastre, P. Richardson, D. Bruce, E. Rubin, E. Myers, E. D. Siggia, and A. Tomasz. 2007. Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. *Proc. Natl. Acad. Sci.* U. S. A. 104:9451-9456.

Nikaido H. 2009. "Multidrug Resistance in Bacteria".Annu. Rev. Biochem. **78**: 119–46. (doi:10.1146/*annurev.biochem.*78.082907.145923. PMC 2839888.PMID 19231985).

Norman, A., L. H. Hansen, and S. J. Sorensen. 2009. Conjugative plasmids: vessels of the communal gene pool. Philos. Trans. R. Soc. Lond. B. *Biol. Sci.* 364:2275-2289.

Novick, R. P., and E. Geisinger. 2008. Quorum sensing in staphylococci. *Annu. Rev. Genet.* 42:541-564.

Ochiai, K.; Yamanaka, T; Kimura, K; Sawada, O. 1959."Inheritance of drug resistance (and its transfer) between *Shigella* strains and Between *Shigella* and *E.coli* strains".HihonIji Shimpor, (in Japanese) 34: 1861.

Ogawara, H., N. Kawamura, T. Kudo, K.-I.Suzuki, and T. Nakase. 1999. Distribution of β-lactamases in actinomycetes. Antimicrob.*Agents Chemother*. 43:3014-3017.

Oyeleke S.B. And manga S.B. 2008.*Essential laboratory practical in microbiology*.First edition.Tobest publisher's minnanigeriap20-65s

Parkin.DM, D Mesher, & P Sasieni. 2011. Cancers attributable to solar (Ultraviolet) radiation exposure in the UK in 2010. *British Journal of Cancer*. 105, 66-69

Peleg, A. Y., H. Seifert, and D. L. Paterson. 2008. Acinetobacterbaumannii: emergence of a successful pathogen. *Clin.Microbiol.*Rev. 21:538-582.

Petkovic, H., J. Cullum, D. Hranueli, I. S. Hunter, N. Peric-Concha, J. Pigac, A. Thamchaipenet, D. Vujaklija, and P. F. Long. 2006. Genetics of Streptomyces rimosus, the oxytetracycline producer. *Microbiol.Mol. Biol*. Rev. 70:704-728.

Perez, F., A. M. Hujer, K. M. Hujer, B. K. Decker, P. N. Rather, and R. A. Bonomo. 2007. Global challenge of multidrug-resistant Acinetobacterbaumannii. Antimicrob.*Agents Chemother.* 51:3471-3484.

Piddock, L. J. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin.Microbiol.*Rev. 19:382-402.

Poole**,** K**.** 2005. Efflux-mediated antimicrobial resistance. J. *Antimicrob. Chemother.* 56**:**20-51.

Reading, C., and M. Cole. 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from Streptomyces clavuligerus. Antimicrob.*Agents Chemother*. 11:852-857.

Roberts, M. C. 2008. Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS Microbiol.Lett*. 282**:**147-159. [PubMed]

Robicsek A, Jacoby GA, Hooper DC; Jacoby, GA; Hooper, DC. 2006. "The worldwide emergence of plasmid-mediated quinolone resistance". *LancetInfectDis*6 (10): 629–40.

Ryan, F. 1992. The forgotten plague: how the battle against tuberculosis was won—and lost. Little, Brown and Company, Boston, MA.1st Edition. ISBN-13: 978-0316763813, ISBN-10: 0316763810.

Seppala, H. T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager and P. Huovinen. 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance, *N. Engl*. *J. Med*. **337**: 441–446.

Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers. 2001. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. *Appl. Environ. Microbiol.* 67:561-568.

Shah, N. S., A. Wright, G. H. Bai, L. Barrera, F. Boulahbal, N. Martin-Casabona, F. Drobniewski, C. Gilpin, M. Havelkova, R. Lepe, R. Lumb, B. Metchock, F. Portaels, M. F. Rodrigues, S. Rusch-Gerdes, A. V. Deun, V. Vincent, K. Laserson, C. Wells, and J. P. Cegielski. 2007. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg.Infect*. *Dis*. **13**:380-387.

Sjolund, M. K. Wreiber, D.I. Andersson, M.J. Blaser and L. Engstrand.2003. Long-term persistence of resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori, Ann. *Intern*. *Med*. **139**, 483–487.

Skurray, R. A., and N. Firth. 1997. Molecular evolution of multiply-antibiotic-resistant staphylococci, p. 167-191. In D. J. Chadwick (ed.), *Antibiotic resistance: origins, evolution, selection and spread*. *Wiley*, *Chichester*, United Kingdom.

Sorensen, S. J., M. Bailey, L. H. Hansen, N. Kroer, and S. Wuertz. 2005. Studying plasmid horizontal transfer in situ: a critical review. *Nat*. *Rev*. *Microbiol*. 3:700-710.

Sommer, M. O. A., G. Dantas, and G. M. Church. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325:1128-1131.

Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher H, Schled W, Bartlett J, Edwards J. 2008. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *OxfordJournals*. 46(2):155-164.

Springman, A. C., D. W. Lacher, G. Wu, N. Milton, T. S. Whittam, H. D. Davies, and S. D. Manning. 2009. Selection, recombination, and virulence gene diversity among group B streptococcal genotypes. J. *Bacteriol*. 191:5419-5427.

Sule A. M, Thanni L. A,SuleOdu, A and Olusanya, O. 2002. Bacterial Pathogens associated with wound infections in Ogun state university Teaching hospital. *African Journal of Chemical and Experimental Microbiology*.**3**(1)15.

Stephanie H. 2011. DNA Repair Mechanisms.Optimization of Genetic Biology Laboratories. *URES –Fall* 2011 Summary.

Strahilevitz, J., G. A. Jacoby, D. C. Hooper, and A. Robicsek.2009. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin.Microbiol.*Rev. **22**:664-689.

Swann, M. 1969. *Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine*.Her Majesty's Stationery Office, London, United Kingdom.

Thomson, C. J., and S. G. Amyes. 1992. TRC-1: emergence of a clavulanic acid-resistant TEM beta-lactamase in a clinical strain. *FEMSMicrobiol*.Lett. 70:113-117.

Vallino, J. J. 2003. Modeling microbial consortiums as distributed metabolic networks. *Biol*. *Bull*. 204:174.

Velayati, A. A., M. R. Masjedi, P. Farnia, P. Tabarsi, J. Ghanavi, A. H. ZiaZarifi, and S. E. Hoffner. 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest* **136**:420-425.

Velissariou, I.M. 2006.The use of fluoroquinolones in children: recent advances. *Expert Rev Anti Infect Ther,* 4(5): p. 853-60.

Ventura, M., C. Canchaya, A. Tauch, G. Chandra, G. F. Fitzgerald, K. F. Chater, and D. van Sinderen. 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol*.*Mol*. *Biol*. *Rev*. **71**:495-548.

Vernaz, N., K. Hill, S. Leggeat, D. Nathwani, G. Philips, P. Bonnabry, and P. Davey. 2009. Temporal effects of antibiotic use and Clostridium difficile infections. *J. Antimicrob. Chemothe*r.**63**:1272-1275.

Waksman**,** S. A. 1973. History of the word 'antibiotic.' J. *Hist. Med. Allied Sci*. 28**:**284-286.

White, D. G., M. N. Alekshun, and P. F. McDermott (ed.). 2005. Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy. *ASM Press,* Washington, DC.

WHO, 2014. First global report on antibiotic resistance reveals serious, worldwide threat to public health" Retrieved 2014 -05-02.

Witte W. 2004."International dissemination of antibiotic resistant strains of bacterial pathogens".Infect.Genet.Evol.4 (3): 187–91. doi:10.1016/*j.meegid.*2003.12.005. PMID 15450197.

Woodford N and Ellington MJ. 2007. "The emergence of antibiotic resistance by mutation". Clinical Microbiology and Infection:*the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 13 (1): 5–18.

Wright, G. D. 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5:175-186.

Yeh, P. J., M. J. Hegreness, A. P. Aiden, and R. Kishony.2009. Drug interactions and the evolution of antibiotic resistance. *Nat. Rev. Microbiol.* 7:460-466.

*http://www.rxlist.com/antibiotic_resistance-page3/drugs-condition.htm*10 oct, 2016

*http://www.wisegeek.org/what-is-uv-light.htm*Sep 6, 2016

APPENDIX

i. **Sensitivity and Resistance Pattern of *Salmonella typhi* After Repeated Exposure to**

List of Antibiotics/	Diameter in (mm)					
Duration of Exposure(min)	0.00	1.00	3.00	5.00	7.00	10.00
Ciprofloxacin	45	29	30	30	27	32
Gentamycin	35	27	27	27	24	25
Amoxicillin	21	17	19	19	20	22

UV light and Antibiotics

ii. **Sensitivity and Resistance Pattern of*Shigelladysentriae*After Repeated Exposure to UV light and Antibiotics**

List of Antibiotics/	Diameter in (mm)					
Duration of Exposure (min)	0.00	1.00	3.00	5.00	7.00	10.00
Ciprofloxacin	37	30	30	34	32	34
Gentamycin	25	20	23	20	21	24
Amoxicillin	25	16	17	19	20	20

iii. **Sensitivity and Resistance Pattern of *Proteus mirabilis* After Repeated Exposure to UV Lightand Antibiotics**

List of Antibiotics/	Diameter in (mm)					
Duration of Exposure (min)	0.00	1.00	3.00	5.00	7.00	10.00
Ciprofloxacin	35	30	30	28	28	30
Gentamycin	28	25	25	25	26	20
Amoxicillin	21	18	21	19	21	21

iv. **Sensitivity and Resistance Pattern of *Staphylococcus aureus*After Repeated Exposure to UV Light and Antibiotics**

List of Antibiotics/	Diameter in (mm)					
Duration of Exposure (min)	0.00	1.00	3.00	5.00	7.00	10.00
Ciprofloxacin	42	40	36	36	40	40
Gentamycin	34	31	25	30	30	28
Amoxicillin	23	25	20	20	25	25