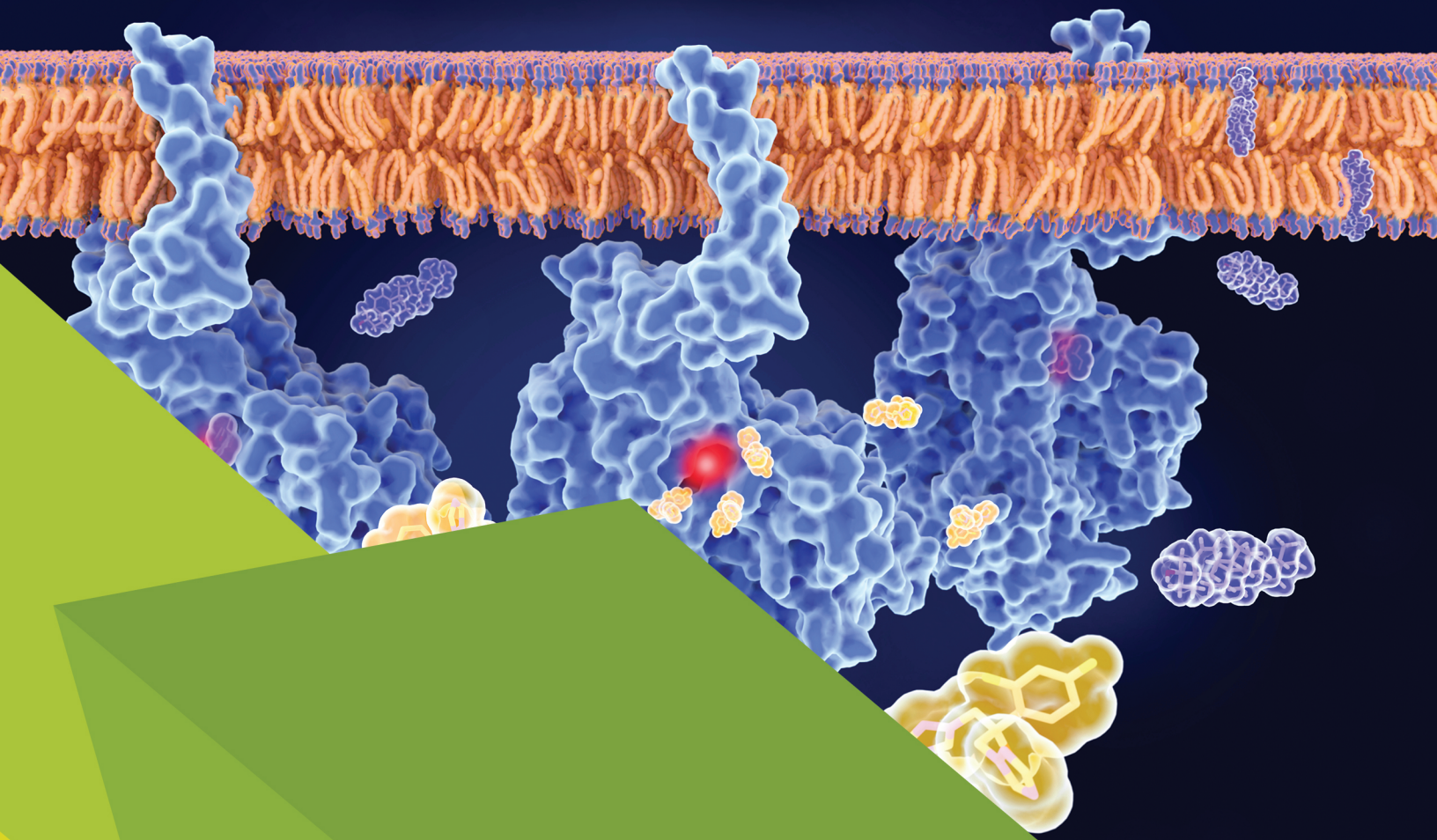
CURRENT DEVELOPMENTS IN THE DETECTION AND CONTROL OF MULTI DRUG RESISTANCE



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Current Developments in the Detection and Control of Multi Drug Resistance

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CONTENTS

PREFACE

The ability of different species of bacteria to resist the antimicrobial agent has become a global problem. Despite the development of so many antibiotic molecules during the last fifty years, increasing antibiotic resistance worldwide may lead to therapeutic dead-end. The disease burden from multidrug-resistant strains of organisms causing AIDS, tuberculosis, gonorrhoea, malaria, influenza, pneumonia, and diarrhoea is being felt in both the developed and the developing countries alike. This book summarizes the emerging trends in the field of antibiotic resistance of various MDR bacterial species.

Current strategies of detection and control of multidrug resistant bacteria provides the clear and recent information regarding strategies involved in diagnosis and treatment of MDR bacteria. Over the past decades, due to the exponential rise in the infections caused by MDR bacteria, it is necessary to elucidate the basic mechanism of antibiotic resistance to find out the effective methods for treatment and control. Primary goal of this book is to elaborate and enhance the theoretical knowledge of bacterial pathogenesis, diagnosis and treatment. It concentrates on further elaboration of current strategies of detection and control of MDR bacteria, Quantification of pathogenic bacteria, High Resolution Melting Analysis techniques, Biosynthesis pathways and Rational structure based drug design for MDR infections.

We have attempted to provide large amount of information in each chapter supplemented with in- depth knowledge of current strategies of detection and control of MDR bacteria. This book enhances the knowledge of Microbiology, Biotechnology and Life sciences in field area of MDR pathogenesis and control. The idea to encompass knowledge spanning from what is known to what is unknown in the writings from the experts in the field will result in a rigorous effort to justify the various topics.

We strongly believe that this book will be reader's delight providing comprehensive understanding of diagnosis and treatment of MDR bacteria. It will be an ideal resource for student's better understanding of MDR bacteria biology and useful for biomedical students, cellular and molecular biologists. I hope you will find this book interesting and relevant.

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CHAPTER 1

Introduction

Sanket Kaushik[1] and **Nagendra Singh**[2,*]

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Antimicrobial resistance occurs naturally over time due to changes at the genetic level of microorganisms. These microorganisms are present in food, animals, plants, humans, and the environment. Many pathogens can spread from people to people or from animals to humans. Some of the major drivers of the development of antimicrobial resistance are misuse (overuse, inappropriate selection and/or dosing, *etc*.) of antimicrobial agents, poor infection and disease management, lack of access to clean water, sanitation and hygiene for the people, poor access to affordable and quality drugs and diagnostics, lack of awareness and knowledge about health care, *etc*.

The global resistance among various pathogenic microbes to antimicrobial drugs is now becoming a serious threat to public health worldwide. The failure of treating microbial diseases is leading to prolonged illness and higher expenditure on healthcare along with a greater risk of life. Today, most infectious agents, including viruses, bacteria, fungi and parasites, have developed Multi-Drug Resistance (MDR), which is responsible for drastic increase in morbidity and mortality. In the last few decades, the administration of antibiotics has drastically increased due to a rapid rate of microbial infections; the latter owed to the emergence of MDR in various microbial strains. MDR is defined as a reduced sensitivity of a microbe to an antimicrobial drug that had been used effectively against the same microbe earlier. Such resistant organisms are able to combat the drug leading to ineffective treatment and increased persistence and spreading of the infection in an individual or population.

Antibiotics are a class of chemotherapeutic agents used in the clinical management of microbial infections. Although antibiotics have been used as magic bullets for curing various bacterial infections, their benefits are diminished due to the global emergence of resistant bacterial strains. The term antibiotics was

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first coined by Selman Walksman, an American microbiologist, for compounds that inhibit the growth of microorganisms [1]. The modern era of antibiotics started in 1928 with the discovery of penicillin from the culture of the fungus *Penicillium notatum* by Alexander Fleming, a British doctor. Although the development of antibiotic resistance is mainly attributed to the imprudent and excessive usage of antibiotics, both biosynthetic antibiotic as well as resistance-conferring genes have been known to have evolved much before the use of antibiotics [2 - 5]. The natural occurrence of antibiotics in sub- inhibitory amounts acts as signaling molecules for quorum sensing, biofilm formation, and in production of virulence factors. The elucidation of the precise roles and mechanisms of antibiotics still remains to be understood. Antibiotics are secondary metabolites of microorganisms that are produced in a very low concentration during the late stages of the stationary growth phase. The drastically different roles of antibiotics have been explored in natural conditions in comparison to the high doses used for therapeutic purposes against microbes. Nutrient starvation induces microbes to produce a large array of small compounds called parvomes [6]. So far, only a small fraction of parvomes has been characterized as antimicrobial agents.

WHO has reported rapid MDR emergence in *E. coli, K. pneumonia*, *S. pneumonia*, *Mycobacterium tuberculosis* and species of *Salmonella* and *Shigella* spp. for a number of antibiotic molecules [7]. Various fungi such as *Candida*, *Aspergillus* spp., *Cryptococcus neoformans*, *Trichosporon beigelii, Scopulariopsis* spp., and *Pseudallescheria boydii* have been reported as resistant against drugs such as polyene macrolides, azole derivatives, nucleotide synthesis inhibitors and 1,3-β-glucan synthase inhibitors [7, 8]. An extended exposure to antiviral drugs has made viruses also resistant in immunocompromised people. MDR has been observed in several viruses such as cytomegalovirus (CMV), herpes simplex (HSV), Varicella-Zoster (VZV), human immunodeficiency (HIV), influenza A, hepatitis C (HCV), and hepatitis B (HBV) viruses [9, 10]. MDR emergence has also been reported in several parasites, including *Plasmodium spp.*, *Leishmania spp.*, *Entamoeba spp.*, *Trichomonas vaginalis*, *Schistosoma* spp. and *Toxoplasma gondii* for drugs such as pentavalent antimonials, miltefosine, chloroquine, artemisinin, paromomycin, amphotericin B, atovaquone, sulfadiazine and pyrimethamine [11 - 19].

Most of the infectious organisms have developed high level of alarming MDR against many commonly used antibiotics, being called “super bugs”. Examples of super bugs include MDR bacteria causing urinary tract infections, skin infections and pneumonia. Resistance is known to be a naturally occurring process. A lot of research is currently being performed in order to gain better understanding of resistance mechanisms, which will further aid in tackling the resistant microbes.

The emergence of MDR is a serious threat to society in the current era, since influenza, HIV, tuberculosis, pneumonia, yeast infections and other diseases are major causes of mortality. As per the WHO's update, in 2018, 3.5% new cases and 18% old cases of tuberculosis in the world are estimated to present MDR. About 8.5% of MDR cases showed extensive drug resistance (XDR) tuberculosis. Over 40,000 cases of XDR-TB cases are projected to be emerging every year. MDR-TB is caused by bacteria that do not respond to at least two of the most potent drugs, isoniazid and rifampicin. Pneumonia is one of the most common infections globally. MDR Gram negative bacteria (MDR-GNB) are responsible for serious threats to the health care system. The most common *Enterobacteriaceae* isolated from patients with pneumonia were found to be *Klebsiella pneumoniae*, *Enterobacter* spp. and *Escherichia coli*, which accounted for 12%, 8%, and 7%, respectively, of all bacterial isolates included in a study [20]. Antiretroviral therapy for HIV also faces severe challenges due to the development of resistance. Similarly, malaria parasites have also been observed to be resistant to several drugs such as chloroquine, artemisinin, and pyrimethamine [18].

In recent times, infections by MDR microbes have become a big issue in public healthcare management. The overuse or misuse of antibiotic agents in the last few decades is responsible for the emergence of several MDR strains of bacteria, viruses, fungi and parasites. A rapid increase of severe systemic infections along with the development and spread of resistant pathogens are creating a serious challenge to the current healthcare system. Regrettably, so far, the efforts to develop new drugs have not been sufficient to tackle the emergence of new MDR strains. Along with implementing social awareness programs, there is an urgent need to understand the development of resistance mechanisms to find a therapeutic solution for the MDR microorganisms.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 2

Development of Drug Resistance

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Abstract: The phenomenon of drug resistance is a widely acknowledged problem in clinics. Drug resistance not only increases the treatment time, but also paves the way for testing the maximum limit of dose tolerance of antibiotics in the patient. There is no escaping of the fact that drug tolerance may remain a perpetual problem and bacteria will keep on evolving as a part of the natural selection process. Therefore, novel drugs targeting the novel mechanism of action could be a proposed solution for this problem. The mechanism of action includes efflux pump, alteration/modification of drug target, enzyme inactivation and prevention of drug penetration. The other thing is to avoid the unnecessary usage of antibiotics so that the bacteria living inside the body do not develop resistance. The places where antibiotics can be bought for human or animal use without a prescription, the emergence and spread of resistance are made worse. Similarly, in countries without standard treatment guidelines, antibiotics are often over-prescribed by health workers and veterinarians and over-used by the public. Therefore, this unregulated overuse of antibiotics may lead to an era where normal infection may become difficult to treat and could lead to mortality. The maintenance of hygiene is a must for everyone and it is the only way to get rid of pathogenic bacteria. So, in this chapter, we summarize recent literature on the development of drug resistance, their mechanism of actions used by microbes to develop antibiotic resistance, factors determining their development by infective agents and the spread of resistant bacteria.

Keywords: Antibiotics, Bacteria, Drug resistance.

INTRODUCTION

Drug resistance means the development of mechanisms to show less or lack of sensitivity to a particular drug or drug family. From a medical point of view, the

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increase of resistant bacterial strains is an alarming issue but at the same time, it validates the theory of adaptation in evolution. The first actual antibiotic was first discovered by Sir Alexander Fleming [1], and since then, it has been used throughout the world in medicine and plays an essential role in fighting microorganisms [2]. However, excessive use of antibiotics generated multi-resistant bacteria which pose, in recent years, a serious threat to human life [3]. A newer set of antibiotics is required to treat infections that were treated easily in the past. Bacteria surviving the hospital setup are normally drug-resistant and often require strong antibiotics with prolonged administration. Antibiotic resistance is emerging as one of the global problems to human and animal health. One year of exposure to antibacterial agents also developed resistance in many bacterial species. This is due to the constant evolution of microbes to overcome the antimicrobial compounds produced by other microorganisms and synthetic compounds [4]. The evolutionary history of pathogens reveals that antibiotics have played a pivotal role in their evolution [5]. Several important factors can accelerate the evolution of drug-resistant microbes. These include the sub-therapeutic dosing, misuse and overuse of antimicrobials, and patient noncompliance with the recommended course of treatment. This resistance develops over the due course of time due to survival mechanisms adopted by pathogen against a drug or, to a lesser extent, change in the receptor activity of a host [6, 7]. Extended usage of antibiotics has resulted in the emergence of antibiotic-resistant bacteria, which are often difficult to eradicate requiring either a higher dose or a new class of antibiotics [8].

There are several strategies used by microbes to develop antibiotic resistance which falls into the four categories such as efflux pump, alteration/modification of drug target, enzyme inactivation and prevention of drug penetration (Table **1**). Although intrinsic resistance utilises efflux pump, enzyme inactivation and limited uptake of drugs and acquired resistance utilises may be drug target modification, efflux pump and enzymatic inactivation [9].

Table 1. Mechanism of drug resistance.

S.No.	Mechanism of Drug Resistance	Drug Resistant Agents
1	Efflux pump	Fluoroquinolones, aminoglycosides, tetracycline, β-lactams, macrolides
2	Alteration/modification of drug target	Fluoroquinolones, rifamycins, vancomycin, β-lactams, β-lactams, aminoglycosides
3	Enzyme inactivation	β-lactams, macrolides, β-lactams, macrolides, rifamycins
4	Prevention of drug penetration	β-lactams, tetracycline, fluoroquinolones

Antibiotic resistant bacteria can modify their active site where antibiotics act, evolving a novel survival strategy [10, 11]. Researchers have also found several shreds of evidence where these microbes have an ability to inhibit the accumulation of antibiotics, thereby decreasing their effective concentration inside the bacterial cell. Bacteria produce efflux pumps to transport various antibiotic molecules. The efflux pump also flushes out antibiotics and prevent the accumulation of drug, thus, rendering bacterial resistance [12, 13]. Frequent mutations in the bacterial DNA can make the bacteria produce more pump molecules, increasing resistance. Bacteria also can change their membrane permeability. Membranes have pores that allow the transportation of molecules; thus, decreasing the permeability would mean lesser uptake of antibiotics [14, 15].

Bacteria seem to be an advanced version of this theory as some of them have developed an ability to produce enzymes that degrade antibiotics [11, 16]. Penicillin is known as the first discovered antibiotic and certain bacteria produce beta-lactamase enzymes, which degrade the beta-lactam ring of penicillin to inactivate it [17]. Some bacteria produce enzymes that are capable of adding a different chemical group to the antibiotic molecule, preventing binding between the antibiotic and its cell target. For example, *Mycobacterium tuberculosis* produces a protein that mimics the structure of DNA and this protein binds to fluoroquinolone antibiotics, thus preventing them from binding to the bacterial DNA. This property makes *M. tuberculosis* resistant to fluoroquinolones, sulphonamides, trimethoprim and DNA gyrase [18, 19].

Microbial resistance can occur in another manner when an antimicrobial drug functions as an antimetabolite by targeting a specific enzyme to inhibit its activity [20, 21]. First, bacteria may overproduce the enzymes, which are the drug targets and these target carry out enzymatic reactions. Secondly, the cell wall of bacteria develop a bypass mechanism which is not susceptible to binding of the antibiotics. Both of these strategies have been found as mechanisms of sulfonamide resistance [22, 23].

Many antibiotics act on a bacterial target (usually a protein), inactivating its activity. Some bacteria produce DNA mutations that modify the target so that these drugs may not bind to it [24]. Some bacteria add different chemical groups to the target, thus, shielding themselves from antibiotics, while some other bacteria express alternative proteins which can be used to inhibit the action of antibiotics. Hence, *Staphylococcus aureus* can produce a new penicillin-binding protein by acquiring the resistance gene *mecA* [25].

The targets of β-lactam antibiotics are proteins required for bacterial cell wall synthesis. This type of resistance is the basis of MRSA (methicillin-resistant

CHAPTER 3

Current Challenges in Treating MDR

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Abstract: Multiple-Drug Resistance (MDR) is a mechanism that renders the ineffectiveness of one or more than one antimicrobial agents shown by disease causing microorganisms. MDR has become one of the major public health challenges as initially, the MDR species were restricted only to hospitals, but now they are found everywhere. Due to the slow advancements in the discovery of new or different antibiotics against the MDR organisms, the medical society is facing great challenges in the treatment of infections caused by resistant microorganisms. This chapter outlines the challenges involved in drug discovery, and treatment to fight and prevent MDR infections.

Keywords: Antibiotics, Drugs, MDR, Microorganisms, Pathogens, Proteins.

INTRODUCTION

MDR infectious microorganisms are the most threatening species to the public health as the infections caused by these organisms cannot be treated by first line antibiotics, thus resulting in more expensive and long-lasting treatments, *e.g.*, MDR-tuberculosis (TB). MDR is antimicrobial resistance expressed by a microbial species to at least one drug out of three or more antimicrobial categories [1]. Along with MDR bacteria, MDR viruses and parasites are also among the most threatening challenges to public health. Extensive drug resistance (XDR) is a new term given to microbes that are resistant to all antimicrobial agents except two or less antimicrobial categories with different chemical structures [2 - 4].

Thus, it results in a prolonged infection time and, also, adds an increased risk of infection spread. More expensive treatment options fallout with the emergence of

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MDR against commercially available drugs. The evolution of global trades and tourism has increased the risk of MDR spread all over world. It is important to cutdown the use of antimicrobial agents to the lowest possible levels to reduce the risk of resistant microbes. The development of novel categories of antimicrobial agents is also desired to tackle MDR pathogens. The Infectious Diseases Society of America (IDSA) has recognized antimicrobial resistance as "one of the greatest threats to human health worldwide." MDR has a crucial impact on the lives of immunocompromised patients and has been associated with increased mortality rates. A rise in MDR has challenged all the aspects of drug designing [5, 6].

The drugs which are designed to treat a disease are target specific, any mutation in these target sites reduces the effectiveness of the particular drug, further causing antibiotic resistance. Second, the development of MDR is related to the intake of broad-spectrum antibiotics for empirical as well as definitive therapy. There are two categories of drug resistance:

Natural/Intrinsic Resistance

Few microbes show resistance towards certain drugs as they lack the metabolic process or the target sites affected by drugs. For example, vancomycin, due to its large size, cannot enter Gram-negative bacteria, as drugs in these bacteria enter through small size porins. Intrinsic resistance is also acquired by *Mycobacterium tuberculosis* for fluoroquinolones, tetracycline, aminoglycosides drugs due to the presence of efflux pumps [7].

Acquired Resistance

In a few microbes, drug resistance develops due to the consistent use of antimicrobial agents (AMA) over a period of time and or non compliance with the drug regimen. Thus, the acquired resistance depends upon the particular microorganism and the drug as well. For *e.g*., *Staphylococci,* coliforms, and *Tubercle bacilli* are notorious microbes for rapid acquisition of resistance. Resistance in microbes may be developed by mutation (vertical transfer) or horizontal gene transfer. MDR in Mtb has been reported due to mutations, expression of genes encoding new proteins, or drug inactivating enzymes under the selection pressure of antibiotics [8].

MECHANISMS OF MDR DEVELOPMENT

Many microorganisms survive by developing adaptability to the antibiotic agents by altering their genetic makeup. Spontaneous mutations are introduced in the DNA of the microbes in order to nullify the effects of the antimicrobial agents.

This enables some bacteria to resist the activity of certain antibiotics to develop drug resistance. The mutated genes are also transferred to other microbes to make them resistant. MDR Organisms (MDROs) employ one or more mechanisms to become resistant to antibiotics.

Alteration of the Target Protein by Mutation

Mutation of a gene affected by a drug, rendering the drug ineffective, is a common way of developing resistance. The drug target site is altered as a result of spontaneous mutation of the bacterial gene on the chromosome, which gets selected in the presence of the antibiotic drug. Rifamycins and quinilones have been shown to be ineffective by mutations in the RNA polymerase and DNA gyrase genes, respectively. The resistance may also get acquired by the transfer of altered genes from one microorganism to another by transformation, conjugation or transduction, such as the acquisition of *mecA* genes responsible for methicillin resistance in *Staphylococcus aureus*.

Another example of drug resistance by the mutational changes is resistance to oxazolidinones (linezolid and tedizolid). These drugs are bacteriostatic antibiotics having a broad Gram-positive activity by binding to the A site of bacterial ribosomes and inhibiting the translation process. The mechanisms of linezolid resistance include mutations in genes encoding the domain V of the 23S rRNA and/or the ribosomal proteins L3 and L4 (rplC and rplD, respectively), and methylation of A2503 in the 23S rRNA mediated by the Cfr enzyme as modification in ribosome modulates its susceptibility to antibiotics [9].

Modifications of the Drug

One of the best bacterial strategies to develop resistance is by producing enzymes that act upon the drug and inactivate it either by its chemical modifications or by its complete destruction, rendering the drug unable to interact with target molecules.

Synthesis of enzymes responsible for chemical alteration of the drug is a well understood mechanism of acquiring resistance in both Gram-negative and Gram-positive bacteria. The most frequent biochemical alteration reactions catalysed by such enzymes are acetylation (*e.g.*, aminoglycosides, chloramphenicol, streptogramins), phosphorylation (*e.g.*, aminoglycosides, chloramphenicol), and adenylation (*e.g.*, aminoglycosides, lincosamides). The resultant reaction of target modification is hindrance in the drug binding, leading to higher bacterial minimal inhibitory concentrations (MICs) of the drugs. One of the best studied examples of this category is the presence of aminoglycoside modifying enzymes (AMEs) which catalyse covalent modification of hydroxyl or amino group of

CHAPTER 4

Methods of Detection of MDR

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Abstract: World health organization (WHO) has acknowledged the problem of multi-drug resistance (MDR) bacteria as an endemic and widespread problem globally. MDR in LMICs represents one of the biggest threats to global health and is one of the greatest current challenges in infectious disease research. High mortality/morbidity rates of MDR infections are mainly due to the lack of timely, rapid detection and treatment of the causative pathogen. Molecular mechanism conferring MDR against most common treatment options includes mutations in antibiotics' susceptible genes at one or many sites. A number of methods, including culture-based, nucleic acid-based amplification of resistance conferring genes, and immunological based assays have been developed to detect MDR. Each method has defined specificity, sensitivity and time around to detect MDR infections in clinical settings.

Keywords: Antimicrobial Resistance, Detection of MDR, Multi-drug resistance bacteria (MDR), MDR infections.

INTRODUCTION

Emergence of multi-drug resistance bacteria (MDR) is a serious threat to global health care system. These 'superbugs' are ubiquitously present, causing mortality worldwide. Pathogen showing insensitivity to at least one in three or more antimicrobial categories is termed to be MDR. The development of resistant strains is explained well by natural selection theory. Irrational use of antibiotics, poor sanitation, inapt food and hygiene practices cause sensitive bacteria to divide and mutate. Such mutated strains transfer their resistant genes over the generations (Table **1**). Infections due to MDR bacteria are of serious concern as

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very few treatment options are available with current antibiotic therapy. Moreover, such mutated strains are the main culprits for the spread and outbreak of antibiotic resistance. Penicillin-resistant *Streptococcus pneumoniae*, Methicillin-resistant *Staphylococcus aureus* are common Gram-positive bacteria. Vancomycin-Resistant *Enterococci*, Extended-spectrum β-lactamase *Klebsiella pneumoniae* (carbapenemase) represent Gram-negatives. Including bacteria, all other infectious agents like viruses, fungi, parasites, protozoans are capable of developing and spreading antimicrobial resistance. In primary, secondary or clinical MDR, the problems get terribly worse in the immune comprised host, subsequently leading to persistent illness and high therapeutic cost [1].

Table 1. Prevalent drug-resistant microbes.

S. No	Name of Bacterium	Drug	Genes	References
1.	*Escherichia coli*	Carbapenem and Cephalosporins	*Tem*	[3]
2.	*Enterobacter spp.*	Carbapenem	*Tem*	[4]
3.	*Klebsiella spp.*	Carbapenem, Colistin, and Cephalosporins	*tem, ctxM, vanB, shv, blaKPC1*	[5]
4.	*Acinetobacter baumannii*	Carbapenem	*Tem*	[6]
5.	*Pseudomonas aeruginosa*	Carbapenem	*Tem*	[7]
6.	*Staphylococcus aureus*	Methicillin	*vanA, mec*	[8]
7.	*Enterococcus faecium*	Vancomycin	*vanB, vanC*	[9]
8.	*Salmonella* Typhi	Fluoroquinolone	*invA,hilA*	[10]
9.	*Streptococcus pneumoniae*	Penicillin	*parC, gyrA*	[11]
10.	*Neisseria gonorrhoeae*	Cephalosporins	*gyrA*	[12]
11.	*Mycobacterium tuberculosis*	Rifampicin, isoniazid, and fluoroquinolone	*katG, rpoB*	[13]

MDR bacteria have shown a myriad of strategies to reduce the efficacy of available drugs. Genetic mutations, exchange of plasmid or chromosomal genes, reformed cell wall composition and permeability, target proteins or enzymes modifications, efflux pumps, enzymatic antimicrobial inactivation or transformation are some of them. As per the WHO's reports (2020), the mortal combat against these multi-drug resistance bacteria is a big concern and presents a multitude of challenges with very few effective and innovative antibiotics. By 2050, no treatment options will be available against these MDR bacteria. Looking at the present scenario, immediate screening of drug resistance and new alternatives for treatment hold paramount significance [2].

*According to a report on anti-tuberculosis drug resistance in the world prepared by the WHO/IUATLD Global Project on anti-tuberculosis drug-resistance surveillance, MDR-TB has reached rates of up to 14% among new patients and up to 50% in previously-treated patients in some contexts (World Health Organization, 2004).

TECHNIQUES TO DETECT MDR

A number of techniques have been devised to detect MDR bacteria. This chapter will evaluate the different methods to detect MDR pathogens (Fig. **1**).

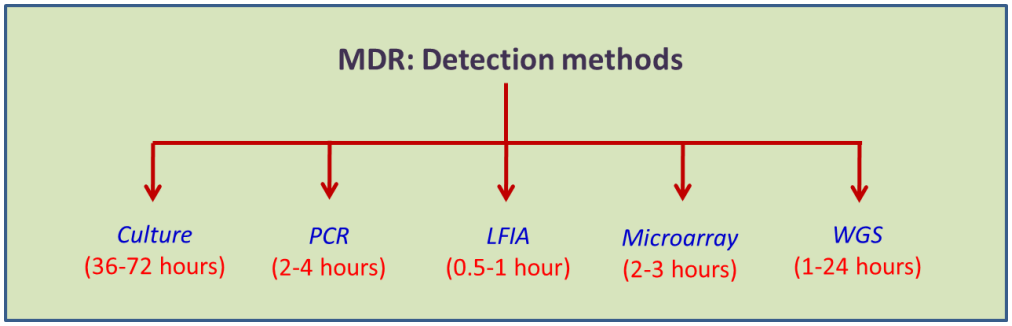

Fig. (1). Different methods to detect multi-drug resistance (MDR) along with their detection time range. PCR: Polymerase Chain Reaction; LFIA: Lateral Flow Immuno-Assay; WGS: Whole Gene Sequencing. See text for details.

Culture

Culture based methods are the gold standard for not only identification but also for the detection of MDR bacteria. Identification of bacteria is the first step in the antibacterial susceptibility test (AST). This is done by microdilution of suspected blood followed by plating to obtain a pure culture of bacteria. The bacteria are identified by Gram staining and biochemical tests as standard methods. After identification, bacteria are subjected to AST, where bacterial isolates are allowed to grow in the presence of a known concentration of antibiotics and their growth is monitored by visual detection. Nowadays, various AST cards and wells having known concentration of specific antibiotics are commercially available, which monitor the growth as well as resistivity/sensitivity pattern of bacteria against antibiotics. Despite this, culture based MDR detection method takes 3 to 5 days to get a conclusive result, and by that time, patients are administered broad-spectrum antibiotics, which further leads to the development of MDR and poor patient outcome. Hence, newer methods have been developed to improve the sensitivity and specificity as well as reduce the sample processing and MDR detection time [14].

CHAPTER 5

Molecular Probes as Diagnostic Tools

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Abstract: Diseases caused by pathogenic microbes have been a serious problem since decades. They often cause high mortality in both developing and developed countries. Diseases including diarrhea, cholera, typhoid, *etc*., are often involved in outbreaks related to pathogens surviving in water. In recent years, the management of infectious diseases has drawn much attention among scientists and researchers. Various specific, sensitive and reproducible detection methods have been developed to identify pathogen contamination in water. These advanced methods generally use probes for diagnostics of pathogens. The present chapter attempts to focus on the development of various probe chemistries for the detection of pathogens. We also intend to present here the pros and cons of different methods.

Keywords: Amplification, Annealing, Culture, Enumeration, Fluorescence, Infectious disease, Microscopic examination, Molecular Techniques, Mortality, Outbreaks, PCR, Probe, Quantification, Quencher, Real Time PCR, SYBR GREEN1, Sensitivity, Specificity, Template, Target.

INTRODUCTION

Increased population and urbanization have posed challenges to the management of infectious diseases. Water-borne infectious diseases cause major outbreaks in both developing and developed countries. These include diarrhoea, typhoid, cholera and other gastrointestinal diseases [1, 2]. Management of infectious diseases requires several measures. Identification of causative pathogens at the initial level is helpful in the therapeutic regimen of disease. A number of methods exist for the detection of pathogens, including culture-dependent and culture independent methods. All these methods have their own pros and cons. Culture-dependent methods are time consuming and labour intensive. In most laboratory setups, the disease is diagnosed on the basis of microscopic examination of culture. These methods are generally qualitative and, therefore, not applicable for

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disease management. With the span of time, molecular methods have been established and have found their applicability in diagnostics. Polymerase Chain Reaction (PCR) has been established as a standard method for the detection of pathogens. It works on the basis of *in-vitro* amplification of genes or a piece of genes using specific primer pairs. Since this method uses explicit DNA sequences, it is highly specific. Immunological techniques, such as Enzyme Linked Immunosorbent Assay (ELISA), are based on the interaction between antigen-antibody and often get limited due to non-specificity and cross-reactivity.

The development of drug-resistance among causative agents has further complicated the detection system. Various methods, including phenotypic and genotypic methods, are currently used for the quantification of these pathogens. In most of the studies, drug-resistance pattern has been performed using the standard disc-diffusion method using the Clinical Laboratory Standard Institute (CLSI) guidelines, which often give inaccurate results.

Since the last decade, Real Time/Quantitative Polymerase Chain Reaction (RTi-PCR)-based assays have been established as the gold standard in the field of pathogen diagnostics [3, 4]. The method uses molecular probes in addition to the primer pairs. Various probe chemistries are evolved to detect and quantify pathogens present in the sample. The limit of detection (LOD) of this method is very low; therefore, it is used for the diagnostic of pathogens in low doses. Variants of RTi-PCR have revolutionized the quantification of target DNA of pathogenic origin. With the advent of multiplexing, in one assay, enumeration of at least four pathogens can be achieved using different probe chemistries.

Molecular probe-based diagnostics have seen major breakthroughs, and at the same time, they face some challenges. Various methods are there that can reduce the detection time with high sensitivity, including ELISA, Polymerase Chain Reaction (PCR), and so on. Despite improvements, these methods still require more time, sophisticated instrumentation and complex sample preparation steps, which limit their use in diagnostics. In some instances, chances of false positive results occur in DNA amplification-based pathogen detection.

Pathogen detection using molecular probes has revolutionized the management of infectious diseases. The current chapter will provide an overview of the present scenario of molecular probes for pathogen diagnostics.

MOLECULAR DIAGNOSTICS FOR DETECTION OF DRUG-RESISTANT PATHOGENS

Detection of disease-causing pathogens often demands accurate and specific methods. Conventional culture-based methods often face challenges of specificity,

sensitivity and rapidity. Classical molecular methods, such as PCR have established themselves in diagnostics. Still, sensitivity and quantification are major hurdles for these techniques and methods. With the advent of RTi-PCR and its various formats, the diagnostics of pathogens have been revolutionized, saving time with improved sensitivity and ease of quantification. Various probe chemistries have been used in RTi-PCR based diagnosis of pathogens.

Detection of drug-resistant pathogens is a real challenging task. Advancements of genome sequencing and identification of genes, responsible for drug-resistance and virulence has opened new avenue towards detection of these pathogens. The development of duplex PCR-based system has shown the potential to detect pathogens in low doses. Detection of virulent gene and drug-resistant genes simultaneously in single tube format gives quick results for the identification of pathogens.

Polymerase Chain Reactions (PCR)

PCR is one of the most important disruptive technologies in molecular biology. DNA molecules are amplified in *in-vitro* conditions using specific primer pairs. Each round of PCR produces new double stranded DNA molecules. Primer pairs are designed using dedicated software to ensure specificity. Certain portions of DNA of the pathogen of interest are targeted and primers are designed accordingly. PCR consists of 3 major steps: Denaturation, Annealing and Extension. During denaturation, the double stranded (ds) DNA gets separated and will serve as templates for primers, while in annealing, the temperature is lowered down and both the primers and the DNA templates get aligned themselves at their respective complementary strands. During extension, each primer pair extends and generates a new pair of double stranded DNA. Two important factors impact the pathogen detection using PCR: a) Selection of conserved regions of genes, unique to the target organism and b) Optimization of annealing temperature for complete binding of primer pair with respective DNA template.

A number of studies exist that use PCR for the detection of pathogens. This includes pathogens causing diseases in plants, animals and human beings. Mirmajlessi *et al*. (2015) has presented review on detection of pathogens on strawberry using PCR [5]. Stockmann *et al*. (2015) has compared standard microbiologic testing with the multiplex polymerase chain reaction (PCR) system that simultaneously detects 23 diarrhoeal pathogens [6].

CHAPTER 6

Real-Time PCR High-Resolution Melting Analysis

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Abstract: High Resolution Melting (HRM) is a homogeneous, exceptionally incredible innovation for single nucleotide polymorphism (SNP) genotyping, mutation scanning and sequence scanning in DNA samples. HRM analysis works on the principle of melting (dissociation) curve methodologies of Polymerase Chain Reaction (PCR) empowered by the new accessibility of improved double-stranded DNA (dsDNA)–binding dyes and next-generation real-time PCR instrumentation. The HRM technology portrays samples of nucleic acids on the basis of their disassociation behaviors and identifies the differences in even the short sequence in amplified PCR products, just by direct melting. Samples are further distinguished according to the length of their sequence, GC content and strand complementarity. Indeed, even a single change in the base pair in the sequences of DNA samples causes differences in the HRM curve. The difference in the melting curves of different genetic sequences at distinct rates can be observed, detected and compared using these curves. Development of the melting curves after HRM analysis is basically plotted with temperature on the X axis and fluorescence on the Y axis, which resembles the real-time PCR amplification curve but with the difference of temperature for cycle number. With the use of different DNA dyes, high-end instrumentation and sophisticated analysis software, these distinctions are detected.

Keywords: Antimicrobial resistance, Genotyping, Genetic variants, HRMA, Intercalating dyes, Melting curve.

INTRODUCTION

Because of the high demand for brisk, highly-throughput examination and genetic study of microbes in addition to data handling and research, focus should be on

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the significant interpretation and improvement of advanced mechanism of detection that counteracts the requirement for electrophoretic analysis, decrease the exposure of contamination and significantly reduce labor time and reagent expenses [1]. Thus, new advances in methods, instruments, fluorescent dyes and different operating systems for melting analysis of DNA have devised a more flexible novel technique for an alternative scanning and genotyping, *i.e*., a comparatively novel approach of post-PCR analysis allowing the analysis of characterization of PCR amplicons in a closed structure [2].

Earlier analysis of the melting curves of PCR product together along-with real-time was recommended with the Light Cyclers [3]. No processing or separations were required like many DNA analysis methods. The DNA duplexes resistance was scanned as the temperature was increased in the solution by the fluorescence of SYBRs Green I. Even though large variation in PCR products was differentiated by melting temperature (Tm), refined sequence fluctuations were considered beyond the reach of fluorescent melting analysis. HRMA (high resolution DNA melting) was initially reported in 2002, and it has since been widely utilised as a research tool [4] and as a quick approach for genotyping known variations or scanning for undiscovered variants [5].

HRMA is logical, economical and rapid approach that requires labelled probes. The sensitivity of heterozygote scanning reaches 100% with the expansion of saturating dye prior to PCR and rapid melting curve analysis of the amplicons. To reduce the burden of sequencing, researchers might examine common heterogeneity with the melting of shorter amplicons and unlabeled probes. Different heterozygotes create comparable melting curves, but unlike homozygous variants, they do not discriminate [2].

For discerning variances and heterogeneity in nucleic acid sequencing, HRM analysis is a more sophisticated, inventive, robust, and closed-tube, high-throughput method [6, 7]. HRM allows for the discovery of genetic variants by analysing PCR melting curves of DNA with spectacular temperature resolution while using a saturating DNA-binding dye of high grade and advanced instrumentation software [6]. Its procedures also create unique and sensitive melting profiles, which may be used in genotyping, mutation screening, and methylation studies using heterozygozygosity, length, and GC content [8]. HRM utilizes cheap dyes and needs low optimization that works on similar platforms like TaqMan along with fluorescence resonance energy transfer (FRET) probes. HRM, on comparison to such approaches, gives better results as it is uncomplicated, coherent and more economical way to characterize more than one sample. HRMA is an elementary PCR that lacks the necessity for any big alterations in the laboratory with any specific skills. It performs under customized

conditions in a close vicinity of a specific dye. The most expensive element is the recovery of the instrument [9].

To some extent, HRM is a recent post-PCR approach that analyses the shape of the melting curve followed by amplification of PCR with saturating DNA dye, resulting in a specific amplicon melting curve, allowing the detection of small sequence variants. The present technique is obsolete used effectively for mutation scanning, SNPs genotyping, and identifying several bacterial species, as well as screening for drug resistance in pathogens [10 - 12]. Besides genotyping, HRM real-time PCR has been revealed to have an interesting capability of SNP identification across the genes which are involved in drug resistance [13]. Many researches have demonstrated HRM real-time PCR for the identification of mutations associated with drug resistance throughout the last decade. Resistant to antibiotics is assessed by utilizing culture based techniques *i.e*. the agar percentage methods or liquid media methods such as the BACTEC MGIT 960. However, these approaches are not efficient and need therapeutic susceptibility testing which takes at least a week, as they are effective [14]. It is clinically critical for patients to have an early drug susceptibility result in order to begin an appropriate therapeutic regimen that will result in improved outcomes. It also enables the monitoring of antibiotic resistance rates, which are important in the fight against infection [15].

PRINCIPLE OF THE MELTING PROFILE

Various intercalating dyes are utilized by the Post-PCR melt curve analysis to identify primer-dimmers or any other non-specific products. This process is known as Low Resolution Melting. While the temperature rises, a curve is generated in increments with 0.5 °C, thus constantly denaturing of an amplified DNA target. The dye used is primarily fluorescent, but when the duplex DNA is denatured, the fluorescence gradually decreases when attached to dsDNA. The length, GC content, sequencing, and heterozygosity of the amplified target all influence the melting profile [16].

At the melting temperature of a DNA sample, the highest rate of fluorescence declination is usually measured. As a result, the temperature at which half of a DNA sample is double stranded and the other half is single stranded is known as the Tm temperature. For fragmented DNA that is longer and/or has a higher GC content, the Tm temperature is generally greater [17]. By analyzing the curve of fluorescence *vs*. Temperature (-dF/dT versus T), data from weak fluorescence resolution melting curves may be utilised to determine the temperature (Fig. **1**).

Rise in the temperature during a low-resolution melt curve analysis is in 0.5 °C steps and this is reduced to 0.008 - 0.2 °C increments for HRM analysis. That is

CHAPTER 7

Current Therapeutic Options and Challenges for MDR

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Abstract: Multiple-Drug Resistance (MDR) against many antibiotics and other therapeutic agents is a major concern for health care providers and researchers in the field. Due to tremendous rise in MDR cases, researchers are in search of potent therapeutic options or alternatives to overcome MDR. Here, in this chapter, we will discuss the current status of the common as well as advanced methods which have been developed so far for the treatment of MDR and also the challenges and opportunities in each of those methods. This chapter discusses common methods used for the treatment of MDR, *i.e.*, major antibiotics used for the treatment of MDR bacteria and synergistic approaches by the combination of different antibiotics. Along with common treatments used against MDR bacteria, this chapter also discusses current treatments like anti-microbial peptides, anti-virulence compounds, phage therapy and drug repurposing approaches for MDR treatment.

Keywords: Allopathic medicines, Anti-microbial peptide, Antibiotics, Carbapenems, Multiple-drug resistance, Phage therapy.

INTRODUCTION

The relationship between bacterial species and humans is age old, with one affecting the other in their life cycle. Many bacterial species are essential part of the human body and its metabolism; they are referred to as the normal flora. However, many more have been identified as causative agents of numerous diseases. The use of traditional medicines and antibiotics for the cure of these infections has been consistent with emerging diseases and scientific advancements. However, the identification of novel antibiotics is being rendered

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ineffective with the emerging resistance to the available drugs. This antibiotic resistance is increasing at a very rapid rate, thereby classifying the resistant bacteria into three different categories, *i.e*. multidrug resistant, extreme drug resistant and pan drug resistant. Considering the severity of the situation, drug resistance is one of the major thrusts in the health sector. The focus on drug resistance globally initiated in 2011, when WHO declared "combat drug resistance: no action today, no cure tomorrow" (WHO Report 2011). This was later revised in 2015 when World Health Organization focussed on all the aspects of drug resistance, including awareness and understanding of the antimicrobial resistance in addition to its prevention and cure. Needless to say, antimicrobial resistance (AMR) has become a global health concern in the present scenario. It has affected the global progress in multiple ways, which include affecting the economically weaker sections to a great extent, causing untreatable infections even in animals in turn affecting sustainable food production and affecting healthcare systems. According to the World Bank Group Report on Drug-Resistant Infections (March 2017), the economic burden of AMR is expected to rise to about US $ 120 trillion by 2050, suggesting a dire and urgent need to focus on the issue [1]. The worldwide attempt to keep a check on the rising AMR was made in 2015 by WHO with the objective to create awareness, understanding, strengthening knowledge and evidence towards AMR; reducing the infection rates and optimizing antibiotic use of the antimicrobial agents. However, lack of funds has been the major cause of the huge difference in the national action planning vs. implementation. India is one of the countries enrolled in the Global Antimicrobial Resistance Surveillance System (GLASS) program to create awareness and keep a check on AMR. While understanding the mechanism of the emerging resistance, it has been observed that bacteria can evade the antimicrobial activity of antibiotics *via* three different but related mechanisms: resistance, tolerance and persistence. Therefore, this chapter is focussed on the current status of the traditional as well as advanced methods, which have been developed so far for the treatment of MDR and also the challenges involved in each of those methods.

TRADITIONAL METHODS USED FOR TREATMENT OF MDR

It is a well-known fact that the number of bacteria on earth greatly outnumbers the total humans and many of them are causative agents of infectious diseases. Multiple approaches have been employed since ages for the treatment of these infections. which can mostly be categorized in home remedies; *i.e*., Ayurveda/Unani systems of medicines, homeopathy and antibiotics. The first three systems of medicines are basically focussed on the symptom derived treatment rather than focusing on the pathogen involved. On the other hand, a wide range of antibiotics has been developed since the discovery of penicillin by

Alexander Fleming in 1928, most of which have been specific to the target bacterial species. This advent of antibiotics towards bacterial species, on the one hand, had overwhelmed all the existing systems of infection treatment back then and also revolutionized the treatment process for multiple infections, but, on the other hand, its misuse and overuse have led to the emergence of drug-resistant species. The more the number of antibiotics has been discovered, the greater the emerging resistance in isolates leading to the formation of multidrug (MDR), extensive drug (XDR) and pan-drug (PDR) resistant species. The ever-rising number of such species has urged a dire need for evaluation of existing and alternative strategies to combat them. The developed antibiotics as well as the ones in the development pipeline, are discussed in great detail in next section.

The use of plant-based traditional medicines for health care can be dated to as old as 200 BCE and are responsible for meeting healthcare needs across the world. For instance, healthcare needs up to 80% of African population and 40% of Chinese population are met *via* traditional medicines (WHO report 2002). The use of herbal formulations as antibiotics is gaining attention and mixed results have been obtained for the same. Certain studies have shown these formulations to be effective [2], while others have shown these formulations to be ineffective [3]. Medicinal extracts of various plants have been evaluated against resistant species of some of the most common pathogens, such as MRSA, *Klebsiella* spp., *etc*., and have shown promising results. Irrespective of the efficacy of the formulation, one of the most common side effects of these formulations is hepatotoxicity, which is observed due to unregulated doses of the formulations by oral route [4]. In spite of the above-mentioned concerns, the role of Ayurveda, medicinal plants and herbal formulations have been exploited as alternative strategies in the global market for a number of diseases. Together with home remedies, these alternatives have been the primary source of healthcare for a long time, but owing to their unascertained quality, lack of accurate chemical composition of the formulation, safety, efficacy, lack of regulation and unknown side effects, their use has been limited so far [3].

Major Antibiotics used for Treatment of MDR Bacteria

The discovery of antibiotics has been a long journey, and, in the process, extensive classes of antibiotics have been exploited affecting different pathways of the bacterial life cycle. The latest generation of antibiotics that are in use are discussed in this section. Depending on their mechanism of action on the bacterial life cycle, the antibiotics have been classified in the following categories:

CHAPTER 8

Phytomedicines for Bacterial Infections

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Abstract: Microbial pathogens have always been a great threat to many life forms, including human. To control microbial infection, especially bacterial infection antibiotics have been a boon. However, with the changing scenario, the bacteria have also evolved and developed resistance against many antibiotics and these pathogens have become more fatal. On the other hand, plants and plant products have been used as a natural resource to control these microbes. The plant seed oil has also been explored for the same; however, comparatively less literature is available on antimicrobial activities of seed oil derived from plants. Looking at the importance of seed oil in this field, the present review article presents a brief discussion about various aspects of seed oil and their application against bacteria.

Keywords: Multidrug resistance, Phytochemicals, Plants oil, Secondary metabolites, Traditional methods.

INTRODUCTION

The discovery of a wide range of antibiotics has been considered one of the biggest accomplishments in the field of medicine. These are pivotal for treating various bacterial infections in patients, including those undergoing surgery, in intensive care units, undergoing organ grafting or in cancer treatment [1, 2]. Antibiotics are generally target specific as they affect cell wall synthesis, DNA replication, and translational machinery of the bacterial cell. Alternatively, bacteria have also developed resistance mechanisms due to the increasing human population, overuse of antimicrobials, over prescription by clinicians, public perception, and behaviour (Non-prescription purchase, not completing the antibiotic dosage as prescribed by clinicians) and commercial pressure [3]. The development of antimicrobial resistance (AMR), which is a matter of grave concern as it results in the huge loss to individuals and economy [3, 4]. Antimicrobial resistance (AMR) has been identified by the World Health Organis-

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ation as a global threat because ~30,000 women and ~400,000 new-born babies lose their lives due to bacterial infection (WHO, 2016). According to the AR Threats (Report-2019 of CDC) antibiotic-resistant microorganisms can lead to > 2.8 million infections and 35,000 deaths in the United States each year [5]. Antibiotic resistance also increases the challenge of recalcitrant infections.

In today's era, the rapid emergence of multidrug resistance (MDR) in microorganisms is an increasing global crisis as it poses a challenge to the treatment of infectious diseases [2, 6]. This alarming situation has enforced the scientists and medical professionals to shift from broad-spectrum empirical therapy (use of known antibiotics), which are less effective against MDR bacteria, to the personalized and strategic approach [4]. Natural compounds have always been relied upon and widely searched for the novel drug discoveries for more than 5,000 years. Plants have been extensively used as a source of antibiotics, antineoplastic, analgesics, and cardioprotective. About 70–90% of the human population in developing countries still use medicines derived from plant extracts [3, 7, 8].

In the last two decades, many persistent efforts have been made to discover novel therapeutics for combating MDR. Natural products and their derivatives contribute to more than half of the Food and Drug Administration (FDA) approved drugs [3, 9]. A variety of plants extracts have antimicrobial properties [10]. These may be bactericidal as well as bacteriostatic against microbial flux pump, quorum sensing and bio-film formation [10].

Diterpenes, extracted from root bark of *Berberis aristata* were found to have antimicrobial properties against several bacterial strains including *Enterococcus faecalis, Staphylococcus spp.*, *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Shigella fexneri* at a low concentration (0.05–5 mg/mL) at the same time these diterpenes had IC_{50} value of 245 to 473 µg/mL against L20B, RD and Hep 2 cell lines, indicating higher biosafety level [11]. Similar to this Arora and Sood (2017) [12] reported the leaf extracts of *Gymnema sylvester* to have antimicrobial activities against *Klebsiella pneumoniae* and *Staphylococcus epidermidis*, further they reported the safety of these extracts based on MTT assay and Ame's test. So, it can be stated that the herbal drugs are more efficient and less toxic to various cell lines; hence, they may be explored to replace the less effective antibiotics and/or antibiotics with threatening side effects. Therefore, herbal compounds provide a novel and promising alternative source of antibiotics against microbial agents. The current review highlights the antibacterial activity of the various seed oil extracted from plants against different bacteria.

TREES AND SEED OIL

Plants mainly propagate through seeds. The seeds may contain nucellus and/or endosperm along with embryo. The nucellar and endosperm tissue contains good amount of stored nutrients, which facilitates the germination of the seed and development of the seedling till the development of first leaf. This reserve food includes carbohydrates, proteins, and lipids. Some tree seeds are rich in oil content.

The lipids are high energy molecules stored by the plants. In perennial trees, the seed oil has been reported from many plants like *Balanites aegyptiaca*, *Azadirachta indica*, *Jatropha curcas*, *Citrus spp*., and *Paeonia spp* [13 - 17]. The amount of the oil in seeds may vary from 13.74% to 54.37% in plants like *Jatropha curcas* and *Paeonia ostia* [15, 17]. The oil mainly consist of fatty acids, but it also possesses a small quantity of secondary metabolites, which may serve as nutraceuticals and/or drugs to treat various diseases, including bacterial infections.

SEED OIL CHEMICAL COMPOSITION

Neem seed oil contains the following fatty acids: linoleic acid (34.69%), oleic acid (20.46%), stearic acid (20.42%), palmitic acid (18.66%), arachidic acid (3.59%) behenic acid (0.80%), lignoceric acid (0.55%) and palmitic-oleic acid 0.17% [18]. According to Cesa *et al*. (2019), high pressure liquid chromatography-based chemical analysis of the pure neem oil revealed that it had no phenolic acids [19]. Neem oil had ~24 μg/mL polyphenols, where benzoic acid (12.6μg/mL) and t-cinnamic acid (1.9μg/mL) were found to be the most abundant.

Ahmed *et al*. (2008) studied seed oil composition based on gas chromatography/ mass spectrometry (GC/MS) in *Salvadora persica* collected from Gabal Elba, a mountain in East South Egypt [20]. They found that the oil contains mainly heptadecene-8-carbonic acid (~39%), indole (18%), hexadecanoic acid (13%) and octadecanoic acid (8%). Table **1** depicts the detail of seed oils obtained from some trees and their composition.

CHAPTER 9

Molecular Mechanisms of Antimicrobial Resistance and New Targets to Address Current Drug Resistance

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Abstract: Penicillin discovery has put forward great expectations and hope for the treatment of several infectious diseases. Inappropriate and excess use of antibiotics has led to the emergence of antibiotic-resistant (AMR) worldwide, which has become one of the greatest threats to global health. However, in the late 1940s, after approval, mass production (lead to reduced cost) and supply (lead to easy access to all people) led to the emergence of Antimicrobial Resistance (AMR). A similar behavioral pattern ensued as other classes of antibiotics were discovered (through increasing utilization to resistance). Substandard infection control practices in public healthcare settings eased the spread and transmission of resistant organisms and intensified antimicrobials' effect. The healthcare community responded with two major programs – Infection Control in the 1980s and Antimicrobial Stewardship (in the last decade). These programs depend on the end-user; however, while the importance of such global control and prevention programs cannot be disputed, these efforts alone are insufficient against the advent of AMR. Also, drug discovery has suffered from a shortage of exploitable bacterial target sites, leading to the slow evolution of novel potent drugs.

Keywords: Antibiotics, Antimicrobials, Antimicrobial resistance, Colistin-resistant, ESKAPE organism, Pan-resistant bacteria, Superbugs.

INTRODUCTION

A pathogen or an infectious agent is a biological agent that can be catastrophic and sometimes lethal to the host (both unicellular and multicellular organisms). These infectious diseases are caused by varied living agents (pathogens), which can be categorized into 5 groups namely bacteria, viruses, protozoa, fungi, and

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helminths (worms). Protozoa and worms are usually associated as parasites, whereas bacteria, viruses, and fungi are collectively known as microbes. They can grow in diverse body sections, out of which the major two can be delineated as intracellular and extracellular [1].

To subdue the effects caused by these pathogens (micro-organisms), drugs such as antimicrobials are prescribed. Antimicrobials are the broad classes of substances (natural, semi-synthetic, and synthetic) that act against microorganisms. As a part of antimicrobials, there is also another class of drugs called antibiotics [2], which attributes to the materials generated only by the microbes that work in contradictor to another microbe [3]. Antimicrobials comprise antibiotics, antivirals, antifungals, and antiparasitics. These agents have been in use for so many years to treat infections and wounds [4]. The introduction of these agents, such as sulphonamides (1935), penicillin (in 1941), streptomycin (1943), combination drugs, such as para-aminosalicylic acid (1944) and isoniazid (1952) into clinical practices contributed to a sharp decline in the death of patients affected by diseases such as pneumonia, influenza, and tuberculosis (8.2% per year in the USA) [5, 6].

The beginning of the modernized “antibiotic era” is linked with the names of renowned scientists Paul Ehrlich and Alexander Fleming. The idea by Paul Ehrlich, known as “magic bullet”, selectively exerts its full action only on the infectious microbes and not on the host organism [7, 8]. Since its inception, this became the mainspring of drug discoveries for the last 60 years, where a massive amount of antibiotics have been manufactured and utilized for a wide variety of purposes [9]. Their bombastic production due to technological advances has led to an ever-increasing demand that resulted in non-prescription and off-labeled use. This oversaturation of the globe because of these toxic agents significantly contributed to the selection of resistant strains of microorganisms and their circulation in microbial populations throughout the terrain. This generates an unremitting selection pressure which is not a usual natural process, but a fabricated circumstance overlaid on the environment called Antimicrobial Resistance (AMR) [9]. Ergo, antimicrobial medicines turn out to be feeble and infections become harder or impossible to treat and also increased the probability of disease spread, stern ailment, at last, heading towards the end of life (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance).

ANTIQUITY OF ANTIBIOTIC EXPLORATION AND CONCOMITANT PROGRESS OF ANTIBIOTIC RESISTANCE

Since the introduction of the foremost effective antimicrobial in 1937 (sulphonamides), the maturation of peculiar mechanisms of resistance has haunted

their medicinal application [9]. In the pre-antibiotic era, the presence of antimicrobials (such as artemisinin, a potent anti-malarial drug) [10] in many herbs from Traditional Chinese Medicine (TCM) could be thought of as a means of remedies (used for millennia) [11]. The discovery of such antimicrobial active components during ancient times might have enriched the depository of antimicrobials used by conventional medicine. Simultaneously, selective pressures forced by these antimicrobials through the deep-rooted past of TCM may be one of the components putting up to the aggregation of antibiotic resistance genes (ARGs) in our community [7].

For example, the late 1930s reported the resistance of Sulfonamide and the same mechanism operates even 70 years later also [9]. Even before the entry of penicillin into the market in 1943 as a therapeutic agent, penicillinase, which is of bacterial origin (discovered in 1940) was already identified [12]. With the excessive use of antibiotics, resistant strains (having drug inactivating capability) became widespread, thereafter, artificial studies were ventured to improve the chemical nature of penicillin to block cleavage by penicillinases (β-lactamases).

The streptomycin-resistant mutant strains of *M.tuberculosis* were detected to have risen during the treatment of the disease. In the mid-1950s, in Japan, the unanticipated identification of genetically transferable antibiotic resistance [13] changed the whole picture. Introduction of the concept of skeptical genetics, collections of ARGs could be propagated by bacterial conjugation mostly throughout the community of bacterial pathogens [14].

Over several decades, AMR caused severe infections that developed resistance and are incurable with available and new anti-microbial agents. The techniques like phylogenetic reconstruction help in revealing the history of these resistance genes and recommends the abiding existence of genes favoring resistance to varied antibiotics classes in the environment ahead of antibiotic ages [15]. The usage of molecular biology serves as the means of knowing the provenance and proliferation of drug resistance genes [16, 17].

In recent years, AMR has emerged as one of the key public health issues of the 21st century that menaces the effective avoidance and remedy of an ever-expanding range of infections caused by infectious agents that are no longer prone to the customary medicines used to cure them. The complication of AMR, specifically in the case of multi-resistant and pan-resistant bacteria (which are also called “superbugs”), requires urgent attention and solution. Also, the clinical interface of the medical pipeline of new antimicrobials is almost dry. In 2019, the World Health Organization (WHO) addressed the list of priority pathogens and identified 32 antibiotics in clinical development, of which only 6 were thought to

CHAPTER 10

Drug Discovery for MDR

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Abstract: Infections caused by MDR (Multi-drug resistant) strains are increasing with time due to the selection pressure posed by the use of antibiotics. The mechanisms that confer antibiotic resistance to bacteria are gene mutation, change in cell envelop, over expression of efflux pumps and biofilm formation. Drug development for MDR has become one of the major challenges globally. MDR infections associated with health care facilities are difficult to treat due to the limited therapeutic approaches or even no treatment options. Therefore, there is an emergency to develop new therapeutic approaches against MDR pathogens. It is possible to identify proteins that are responsible for the survival of pathogenic MDR bacteria for the purpose of drug discovery. Rational-structure based drug design is an inventive process of finding new drug targets, which relies on the knowledge of three-dimensional structure of biological targets. The three-dimension structure is obtained by high throughput techniques such as X-ray diffraction (XRD), Nuclear Magnetic Resonance (NMR) and Cryo-electron microscopy (Cryo- EM). Structure biology plays an important role in the characterization of new therapeutic targets and assessment of drug targets. Computational methods boost drug development and discovery process against MDR pathogens and analyse efficient therapies.

Keywords: Antibiotic resistance, Cryo-EM, Drug discovery, Multidrug resistance, NMR, Rational structure based drug design, Structure biology, X-ray diffraction.

INTRODUCTION

Over the past decade, emergence of antimicrobial resistance in pathogenic bacteria has become a major public health concern all over the world. Treatment

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of several bacterial infections has become increasingly difficult due to the emergence of multidrug resistant strains [1 - 3]. Antibiotics resistance may arise as a result of mutations in the gene, changes in cell envelop, over-expression of efflux pumps, biofilm formation, *etc*. [4, 5]. Multi-drug resistance to different antibiotics develops due to inappropriate use of antibiotics, poor infection prevention and insufficient conditions of sanity [4, 6, 7]. There are several clinical isolates that have developed antibiotic resistant and are more commonly found in hospital environments, such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and the members of the *Enterobacteriaceae* family [8, 9]. Drug development against such multi-drug resistant bacterial strains has become one of the major challenges for medicinal industries [10]. In this regard, it is possible to target essential proteins for the survival of the bacteria for the purpose of drug discovery [11, 12]. Rational structural-based drug design is the process of finding small molecule inhibitors based on the knowledge of three-dimensional structure of biological target proteins [13]. The three-dimensional structures of the targets are obtained by high throughput techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), spectroscopy or cryo-electron microscopy (cryo-EM) [14, 15]. The three-dimensional structure of the target molecule is used to predict candidate drug molecules that selectively and tightly interact with binding sites of the protein or enzyme of the pathogenic microbe. The small molecule inhibitors are chosen as lead molecules against the target [16]. Dorzolamide, saquinavir, zanamivir and imatinib which target carbonic anhydrase, HIV protease, neuraminidase and BCR-ABL respectively, are some of the examples of successful cases of structural-based drug design [16].

EMERGENCE OF MULTIDRUG RESISTANCE IN BACTERIA

Multi-drug resistance associated with unselective use of prolonged exposure to antibiotics led to excess morbidity, mortality and high cost in clinical settings. It also increased the prevalence of pathogenic multi-drug resistant bacteria globally [17]. It has been noted that over the last few decade plasmid-borne resistance genes and cross resistance are the major factors that facilitates the process of resistance in bacteria [18]. The Infectious Disease Society of America, documented that management of several infectious diseases is extremely challenging such as vancomycin-resistant *Enterococcus faecium*, multi-drug resistant *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterobacteriaecae* [19]. The development of antibiotic resistance occurs mainly due to gene mutation, horizontal gene transfers via conjugation, transformation and recombination events [20]. Broadly there are four mechanisms of multi-drug resistance in bacteria. They are:

- Modification of drug target;
- Drug inactivation;
- Drug target modification; and
- Bypass of target site.

Bacterial resistance leads to difficulty in treatment, causing serious complications in critically ill patients. These resistant bacteria spread not only in healthcare settings but also in communities [21]. Due to the limited options available for the treatment of infections caused by multi-drug resistant pathogens, there is an urgent need to explore new strategies to develop new drug candidates [22].

ROLE OF STRUCTURAL BIOLOGY IN DRUG DISCOVERY

Structure biology has a huge potential in drug discovery, structural information obtained from native structures of proteins and also their complexes with ligands can be used in designing of the lead compounds [23, 24]. Various industries utilize high-throughput and fragment screening approaches to identify the initial stage of small molecules in the research and development of drug discovery [25]. X-ray crystallography, electron crystallography, neutron crystallography, cryo-electron microscopy, small- angle neutron scattering and electron tomography are commonly used direct imaging techniques in structural biology for drug discovery [26 - 29]. Amongst these techniques, the ones which provide higher resolution in the structural determination of macromolecules complexes are X-ray crystallography, cryo-EM; hence are used more often in structural determination of target enzyme and their complexes with ligands (Table **1**) [30].

On the basis of structural knowledge, three dimensional structure of macromolecules provides biological mechanisms and also suggests a new therapeutic mode for rational drug design [31, 32].

Table 1. Comparison of X-ray crystallography, NMR and Cryo-EM.

S. No.	X-ray Diffraction (XRD)	Nuclear Magnetic Resonance (NMR)	Cryo-EM
Technique	Involves arrangement of atoms within a crystal.	Involves change in nuclear spin energy in the presence of magnetic field.	Involves analysis of native structure in cryogenic condition which are sensitive towards radiation.
Based	Based on X-ray diffraction pattern.	Based on absorption or electromagnetic radiation in radio frequency range.	Based on native cryogenic condition.

CHAPTER 11

Recent Developments to Fight Multidrug Resistance (MDR) in Protozoa

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Abstract: This chapter focuses on the solutions to emerging multidrug resistance in the major parasitic protozoa plaguing the world. These neglected pathogens have seized the developing nations in a vice-like grip and are seeping into the industrialised world with the dramatic increase in global travel. The alarming rise in resistance to most antiparasitic drugs has left even the wealthiest nations vulnerable. Multidrug resistance occurs to give a survival advantage to the parasite; it has been hastened by the uncontrolled use of chemotherapeutics. This chapter categorises the recent developments to overcome the MDR hurdle under different approaches. The synthesis of novel organic compounds and high-throughput screenings of new chemical entities are two major approaches. Protease and topoisomerase inhibitors of parasitic protozoa prove as worthy drug targets. *In-silico* and proteomics-based methods also accelerate drug discovery by creating potential drug libraries specific to tropical protozoa. A cost-effective and rapid method of combating drug resistance is the repurposing of licensed medicines. This approach also accounts for the established safety of drugs and high commercial availability. Molecular advancements have introduced small interfering RNAs (siRNAs) at preclinical levels as therapeutics functioning *via* a unique mechanism. The nanoparticle and cell-penetrating peptides (CPP) based delivery of siRNAs has facilitated a stable and low toxic way to silence genes providing pathogenicity and resistance. This will help in reversing MDR and breathing new life into the existing licensed antiprotozoal chemotherapies.

Keywords: Antimicrobial peptides, Antiprotozoal therapeutics, Aystems biology, Drug repurposing, *In-silico*, Multidrug resistance, Protease inhibitors, siRNA, Synthetic organic compounds.

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INTRODUCTION

The blight of protozoan disease is no longer limited to developing and poor nations; it has found a hold in the industrialised world as well due to increased global travel and multidrug resistance. Multidrug resistance (MDR) is defined as the insensitivity of an organism to the administered chemotherapeutics (that have different molecular targets) despite earlier sensitivity to it.

The fitness model of natural evolution promotes MDR. The phenomenon has accelerated in the last two decades with extensive and unnecessary use of chemotherapy. This situation is further complicated by incomplete clearance of pathogens because of viral and lifestyle-induced immune-insufficiency, which creates recalcitrant and recurring infections. The vicious cycle of resistance fuels the further spread of MDR as a consequence of chronic infections and long term hospitalisations that enable hospital-acquired infections to become a hotspot for the exchange of resistance mechanisms between parasites [1].

Pathogenic protozoa have created several MDR adaptations. These include rapid efflux of the drug and the reduction in drug uptake due to modifications in drug transporters. These transporters often hide in plain sight under other simplistic functions. Drug modification, drug degradation and sequestration, genetic mutations and target enzyme modulations, all augment MDR. These mechanisms are illustrated in Fig. (**1**). Thus, it is essential to formulate nontoxic and cost-effective chemotherapies for managing protozoan infections. Moreover, the new drugs should be mechanistically novel so that existing resistance mechanisms do not render the new drugs ineffective [2, 3].

Scientific and political advancements in developing nations have helped alleviate the obstacles in the drug discovery against parasitic protozoa. A multidisciplinary approach based on structural studies of protozoal proteins, genomic analysis and high-throughput screening of compound libraries has driven this change. In this chapter, you will learn about the recent developments in antiprotozoal therapies to overcome the multidrug resistance to conventional treatments in major pathogenic protozoa.

NEWLY SYNTHESIZED ORGANIC COMPOUNDS

Researchers have accumulated data about parasitic biochemistry over the last half-century to develop antiprotozoal drugs. They have used this biochemical data to synthesize organic molecules that block the parasitic activities of protozoa [4]. Some of these are first-line drugs, such as metronidazole for gastric protozoa, and pentavalent antimonials for leishmaniasis. However, most conventional drugs are increasingly becoming redundant with the emergence of multidrug resistance [5].

The decades of data from protozoan pathogenicity and metabolomic studies have brought to light, new targets for drug discovery. The researchers have exploited this knowledge to synthesize new organic compounds to launch mechanistically novel drugs in the market. All the new formulations have been compared to standard drugs to check for efficacy and toxicity, before declaring them as potential antiprotozoal therapies [6, 7].

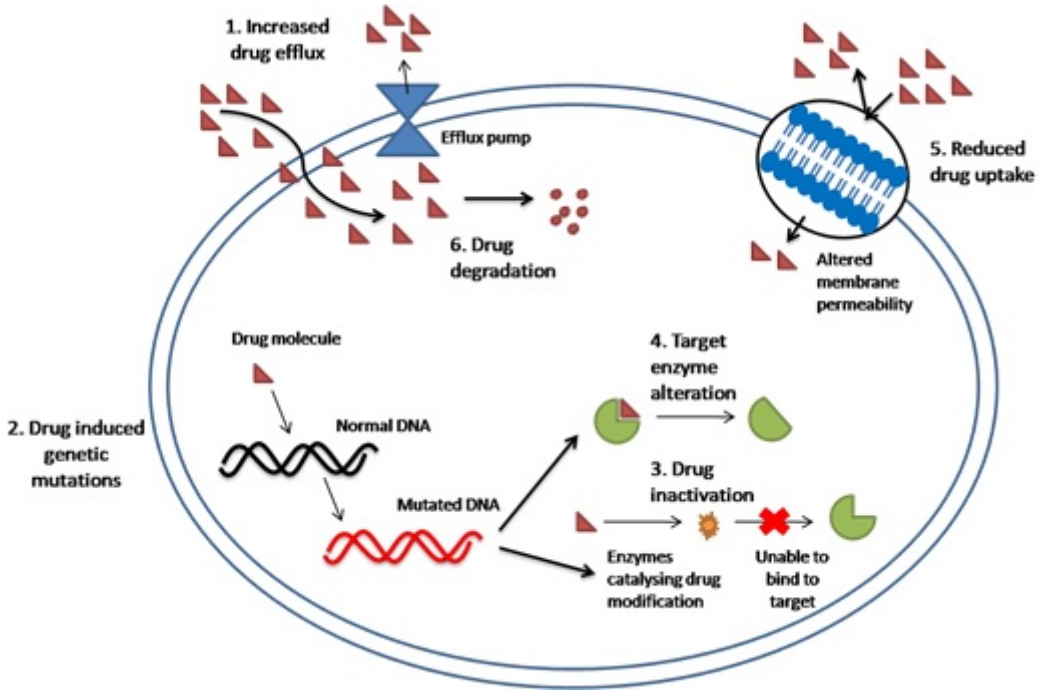

Fig. (1). Schematic diagram of mechanisms of MDR.

Different series of heterocyclic compounds have been studied and their derivatives were synthesized. Among these, a hybrid compound designed by linking molecular scaffolds of pyrazoline and pyrimidine showed excellent activity against *Leishmania donovani* and *Leishmania major* [8]. The Drugs for Neglected Diseases *initiative* (DND*i*) has introduced a highly effective aminopyrazole series for the treatment of visceral and cutaneous leishmaniasis. The aminopyrazole based medicines were not affected by the activity of MDR efflux pumps thus exhibiting a low potential for resistance [9]. The series of 1,3-dipyridylbenzene and terphenyl diamide derivatives revealed good drug candidates against *Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi* strains with IC_{50} values comparable to the standard drug, melarsoprol. Low IC_{50} values of 0.16 μM and 0.10 μM were displayed by two imidazolic compounds, against human African trypanosomiasis (HAT) or sleeping sickness [10, 11].

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Dr Sanket Kaushik is an A/Professor at the Amity Institute of Biotechnology, Amity University, Rajasthan. After obtaining his master's degree in 2008 from the University of Rajasthan, he received his Ph.D. degree in Biophysics from the Department of Biophysics, All India Institute of medical sciences, New Delhi. Currently, he is working on structural studies of protein drug targets from Multi Drug Resistant bacteria Enterococcus faecalis in its native state and complex with known and designed inhibitors. Enterococcus faecalis is a Gram positive multidrug resistant bacterium that is a common cause of hospital-born infections. His major attention lies in investigating the details of host-pathogen interface through structural biology tools for understanding the mechanism of biofilm formation leading to the development of multi drug resistance. He has authored more than 50 research papers and many book chapters. Dr Kaushik has received research grant from several funding agencies in India. He has also worked as a member of the Biotech research society of India and as one of the founder members in Bio footprints. Dr. Kaushik is also working as an academic editor and a reviewer in many scientific journals of international repute.

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