Research Insights

Strategies to Overcome Superbug Invasions

Emerging Research and Opportunities

Dimple Sethi Chopra and Ankur Kaul

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Strategies to Overcome Superbug Invasions:

Emerging Research and Opportunities

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Foreword

It is a privilege for me to write the foreword for the *Strategies to Overcome Superbug Invasions: Emerging Research and Opportunities*. In today's world, antibiotic resistance in bacteria is an emerging public health problem and actually a crisis. Some bacteria have evolved to survive the most effective antibiotics in the clinical setting leading to death of increasing number of people. These bacteria are mentioned as superbug metaphorically and they can invade living tissues, systems and also our environment. The loss of effective antibiotics nowadays weakens our ability to fight infections caused by these superbugs. Recent studies analyzing the antibiotic pipeline indicates there aren't enough antibiotics in development to meet current needs. Therefore, we need novel strategies to fight with antibiotic resistant bacteria. In this book, the researchers have evaluated the current literature to probe into the antibiotic resistance problem and to review the promising approaches to overcome this public health problem. I hope, this book will contribute to this aim and will help the researchers in this field.

Mümtaz Güran
Eastern Mediterranean University, Turkey
1st June 2021

Preface

"Declare the past, diagnose the present, foretell the future." – Hippocrates

Technology in this 21st century has hugely impacted our lives in almost everything we do today. It has assisted us as humans to make 'smart' advances; our ancestors could only dream about. As we utilize intelligent devices and tools to improve our quality of life, bacteria have also evolved rapidly to become quite 'smart.' These superbugs can cause antimicrobial resistance (AMR), which is one of the reasons for failed antimicrobial chemotherapy. In fact, the superbugs have outpaced have our ability to get newer antibiotics to the market. Antibiotics had always been considered as one of the most relevant discoveries in the last 100 years. Unfortunately, the rising occurrence of AMR has caused the down sliding of our most existing antibiotics.

The phenomenon of AMR develops when bacteria acclimatize and grow in the presence of antibiotics. When many antibiotics belong to a common class of anti-bacterial agents, become resistance to a specific antibiotic drug can ultimately lead to resistance to an entire class of the bacteria. Once resistance develops in one class of organism, it can unpredictably and rapidly spread to another creature/species. Hence, it becomes increasingly challenging to treat commonly seen bacterial infections in clinical settings. Globally, it is reported that there is loss of 7 lakh lives to AMR annually, and this figure could escalate to 10 million by 2050. Undoubtedly, AMR was considered one of the most severe current threats to the global health after HIV/AIDS, Ebola and non-communicable diseases in the pre COVID-19 era.

Moreover, now, the recent COVID-19 experience has been fairly dreadful to our global public health systems that were quite ill prepared to tackle a new unpredictable viral strain. The irrational use of antimicrobial agents during COVID-19 pandemic times will further be a source for AMR snowballing.

Hence, it becomes imperative to study about development of AMR, the challenges to overcome these superbugs. This book has been structured into eleven chapters. It includes the development of novel approaches in searching for new antimicrobials, designing more effective preventative measures, and, essentially, enhanced understanding of bacterial ecosystems and antibiotic resistance. The development

of conceptual frameworks created on current expansions in the field of diagnosis of systemic bacterial disorders, and application of in-silico methodologies to pace up the speed of drug discovery for COVID-19 are also discussed herein.

We wish to thank all the contributors for their engagement and collaboration which played a monumental role in bringing this book to fruition. To conclude, we are also grateful to the publisher, IGI Global for sincere co-operation throughout the project.

We hope that this book will be enlightening source for, clinicians, and other healthcare professionals; microbiologists and students in the life sciences; and scientists and decision makers in medical research.

Dimple Sethi Chopra
India

Ankur Kaul
India
20th May 2021

Acknowledgment

We express our sincere gratitude towards the authors and their respective collaborators for their scientific contributions to this book entitled *Strategies to Overcome Superbug Invasions: Emerging Research and Opportunities*.

We are also highly grateful to the Editorial Advisory Board members for their commendable suggestions and inputs for improving the scientific content of this book. In fact, the reviewers had worked tirelessly in improving the quality of this publication.

The patience and co-operation of the entire IGI Global publishing team, who were directly or indirectly involved in the fruition of this book, is highly appreciated.

It goes without saying that we are sincerely indebted to our families for their unconditional support, either morally or physically, throughout the successful journey of this book.

Last but not least, we bow down to the power of Almighty for His blessings.

Section 1
Challenges and Antimicrobial Resistance (AMR): A Diagnostic Perspective

This section consists of chapters that contain an introduction to AMR and current trends of diagnosis of bacterial infection with special emphasis on mycobacterium tuberculosis.

Chapter 1
Resistance Phenotypes and Surveillance

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ABSTRACT

The emergence of drug resistance complicates surveillance and treatment of antimicrobial phenotypes. For example, the rise of Methicillin-resistant Staphylococcus aureus and carbapenem-resistant Enterobacteriaceae influence delivery of care. Moreover, a lack of surveillance programs in most of the developing world exacerbates the problem of MDR. Existing studies in humans are mostly retrospective single-center surveillance-based studies that look at the molecular makeup and prevalence of phenotypic resistance for several pathogens. Very few studies examined infection prevention measures or antimicrobial stewardship activities, and of those that did, none of them were multicenter. The aim of this chapter is to explore prevalent phenotypes in clinical settings and antimicrobial resistance (AMR) surveillance programs throughout the world.

INTRODUCTION

Antimicrobial resistance (AMR) threatens to upend decades of progress on managing infections. Antimicrobial drugs have saved life on earth from once-fatal diseases and risky procedures like surgeries and transplants, but their systematic misuse and overuse have transformed the bacterial population and limited the effectiveness

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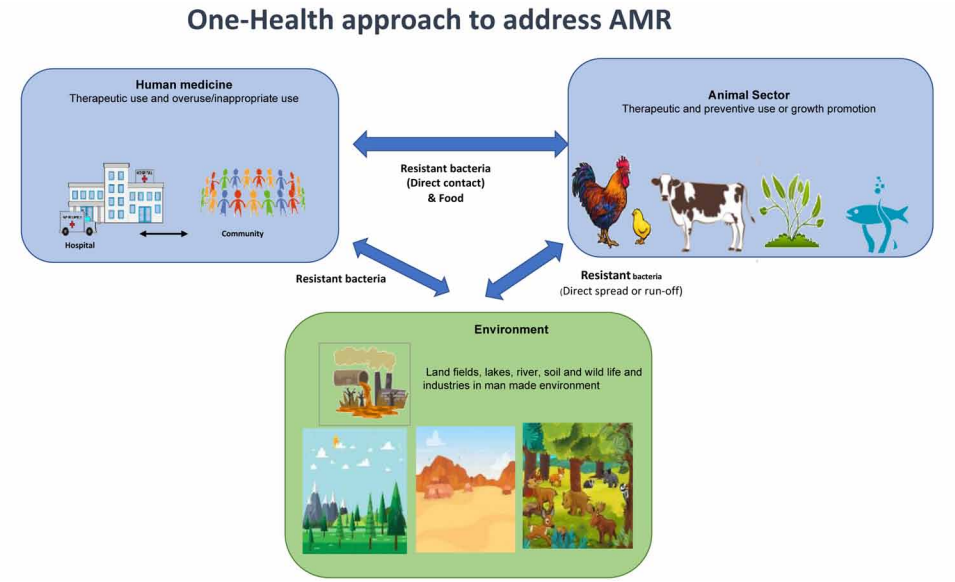
of these miracle drugs. Moreover, the interconnectedness of biomes has made the exchange of bacteria in the environment, animals and humans much easier. The challenge of AMR has led many experts to reconsider the effectiveness of current antibiotic therapies over the next 100 years with a dwindling pipeline of new drug research and development (R&D). Antibiotic usage outside of human medicine, like in agriculture for growth promotion creates another entry for antibiotics to enter our biome through our environment (Diallo, 2020). The broad scope of drug resistance requires effective collaboration among disciplines and countries to build robust surveillance program health outcomes. Similar plans to educate stakeholders and surveil infections to prevent antimicrobial resistance through hygienic infection control practices would sustainably optimize use of antimicrobial medicines in humans and animals. monitor antimicrobial resistant phenotypes to inform interventions. A "one health" approach, like the one proposed by the World Health Assembly in 2015, outlines an action plan that encourages collaboration among actors in human and veterinary medicine, finance, agriculture, environment and consumers. By creating programs, policies and research through multiple sector collaboration, a "one health" approach can achieve better public well being (GLASS WHO Report, 2017).

Figure 1. The One Health triad

Figure 2. AMR within the one health ecosystem, where One Health
Photo credit: Dr Sabiha Essack, UKZN South Africa and Paula Cray, NC state Veterinary medicine



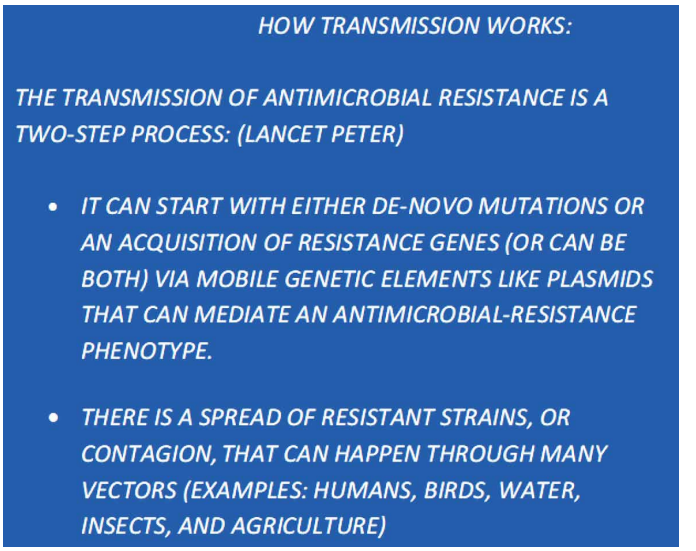
The emergence of drug resistance complicates surveillance and treatment of antimicrobial phenotypes. For example, the rise of *Methicillin-resistant Staphylococcus aureus* (MRSA) and *carbapenem-resistant Enterobacteriaceae* (CRE) exacerbate delivery of care. Most at risk to these infections are those living in developing countries, which often lack the surveillance programs necessary to track and address these infections. Additionally, very few research studies in these countries examine infection prevention measures or antimicrobial stewardship activities, and of those that do, none are multicenter (Gandra, 2017). The aim of this chapter is to explore prevalent phenotypes in clinical settings and antimicrobial resistance (AMR) surveillance programs throughout the world.

What is AMR Resistance and how Does it Develop?

Antimicrobial resistance, or AMR, refers to a microorganism's (like bacteria, viruses, and some parasites) ability to prevent an antimicrobial (like antibiotics, antivirals, and antimalarials) from being effective. Antibiotic resistant infections are associated with higher rates of medical costs and hospital stays. Accordingly, Healthcare acquired infections (HAIs) or nosocomial infections are a concerning type infection that patients get while receiving treatment for medical or surgical conditions at hospitals and long-term facilities like nursing homes. These are infections that are

unrelated to the original illness and appear within 30 days after patient discharge (Revelas, 2012). Among the most prevalent types of HAI's include infections from MRSA, Carbapenem- resistant Enterobacteriaceae (CRE), Central Line Associated Bloodstream Infection (CLABSI) and Ventilator-associated pneumonia (VAP). Even though resistant infections can impact anyone, some people are more prone to infection (immunocompromised persons such as HIV+, those suffering from chronic kidney or liver disease, congenital immunodeficiency etc).Therefore, the standard antibiotic treatments may not be effective and lead to spread of infections to others.

Figure 3.



What is Phenotypic Resistance?

When it comes to antibiotic resistance, the conversation typically involves infections caused by a genetic alteration through the acquisition of genetic genes or mutations. However, there are situations where antibiotic resistance can come about without any genetic change. This phenomenon of phenotypic resistance, or a non-inherited form of resistance, can be seen via growth in biofilms, not in classical clinical susceptibility tests at microbiology laboratories Corona, 2013). Recent research has highlighted the importance of bacterial metabolism on susceptibility of antibiotics (Corona, 2013). This illustrates the important role antibiotics play as inter-microbial signaling molecules, regulators of gene expression and mediators of host immune

response. A better understanding of these processes is essential to develop novel therapeutic solutions based on bacterial susceptibility.

History of AMR

To understand the rise of phenotypic resistance, it is imperative to revisit the origins of antibiotics. Famously, Sir Alexander Fleming discovered penicillin through a failed experiment 1928 (Ban, 2006). His discovery was a useful treatment for all kinds of bacterial infections and eventually opened the gate for fourteen other classes of antibiotics for clinical and veterinary use (Yap, 2013). Together, these innovations became the miracle drugs of the twentieth century and treated life-threatening infections for the first time. However, extensive use of antibiotics over the last few decades have dramatically reduced the efficacy of these drugs and contributed to the rise of antibiotic resistance.

Bacteria commonly acquire resistance to an antibiotic through gene mutations or by transferring genetic materials with other bacteria. They also possess other characteristics that limit portals of entry for antibiotics. These include: using enzymes to break down antibiotics; "efflux pumps" that reduce antibiotic concentrations to ineffective levels; changes to the bacterial membrane that prevent antibiotics from entering the cell, thereby reducing a core mechanism by which antibiotics like Penicillin's work (Rosenblatt-Farrell, 2009).

Bacterium that exhibit more than one of the functions above are likely resistant to more than one type of antibiotic and are known to confer multi-drug resistance. Yet, the implications of antibiotic misuse were predicated long before the first case of penicillin resistant *Staphylococcus aerus* by the very man that had discovered it. Upon winning the Nobel Prize for his discovery in 1945, Fleming said, "The time may come when penicillin can be brought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant (Yap, 2013). Not surprisingly, within 10 years of widescale production of penicillin, penicillin resistance to *Staphylococcus aureus* began to emerge (Rosenblatt-Farrell, 2009).

Phenotypic Resistance

Similarly, other types of antibiotic resistance began to show after the development of new antibiotics and spread quickly throughout the world. For example, infections from Methicillin- resistant *Staphlyoccoccus aureus* (MRSA) is one of the most pressing healthcare-acquired infection today. MRSA resistance began in the United Kingdom on 1961, two years after the discovery of methicillin (Molton, 2013). Over the next few decades, MRSA spread first to North America and then to Europe and

Asia (Molton, 2013). Today, rates of MRSA differ around the world. The percentage of MRSA isolates in Europe range from 0.9% in the Netherlands to 30.7% in Greece (Curtis, 2015). Likewise, a study that looked at 15 tertiary hospitals within India found that rates of MRSA varied; western India presented a 25% MRSA rate compared to 50% in South India (Joshi, 2013). The spread of MRSA is similar to the transmission of other infections in the 20th century like *Vancomycin-resistant enterococci* and *Klebsiella pneumoniae carbapenemase–producing K. pneumoniae*.

Fortunately, rates of MRSA infections are falling in Europe and the United States primarily due to improved infection control, screening and restrictions on some antibiotics (Molton, 2013). On the other hand, multi-drug resistant MRSA has become endemic in many Asian countries. Approximately 28% of clinical S. aureus isolates in Hong Kong and Indonesia and 70% contained MRSA, while two studies in India found MRSA rates to be 41% to 45% (Chen, 2014).

Another concerning bacteria is *Carbapenem-resistant Enterobacteriaceae*, which is resistant to the last resort Carbapenem class of drugs. Its rates vary across the world; from 6% in United States to 14% in China and 46% in India (resistance map). Similarly, *Acinetobacter baumannii* presents its own share of challenges with a carbapenem resistance rate of 30% in the US, 82% in China and 73% in India. On the other hand, *Pseudomonas aeruginosa* is a multi-drug resistant infection with carbapenem resistance rates of 10% in US, 25% in China and 43% in India.

Factors Contributing to AMR

There are many factors that create favorable environments for the exchange of bacteria. Many of these resistant organisms develop through inappropriate use of antibiotics and are often spread through the hands of healthcare workers (Multidrug Resistant Organisms MDROs What Are They). Objects like medication carts, bedside tables and IV poles are other ways in which these resistant organisms can be transmitted, in addition to direct contact like sores (Multidrug Resistant Organisms MDROs What Are They). Hand hygiene is the simplest way to reduce the spread of infections, but there is a lack of a compliance among healthcare providers throughout the world (Pittet, 2009). Consequently, poor hand hygiene is the leading cause of spread of multi- resistant organisms (Pittet, 2009). Several studies over the past three decades have found that implementation of a hand washing program notably reduced the percentage of patients colonized with *Klebsiella pneumoniae*, hospital acquired MRSA, urinary tract infections and other hospital acquired infections (Pittet, 2009). For example, a study done in a Russian neonatal unit estimated that one health care-associated infection would cost US$1100, which could cover 3265 patient- days of hand antiseptic (Pittet, 2009). Most importantly, hand washing programs are the most cost-effective and efficient way of limiting the spread of germs and bacteria.

Similarly, rising incomes, poor access to quality primary health care, and easy over-the-counter access to antibiotics has further led to increasing rates of resistance (Singh, 2017). Between 2000-2015, global consumption of antibiotics rose by 65% and doubled in low and middle- income countries (Klein, 2018). Among the most at risk of drug resistance are key populations like newborns suffering from sepsis and patients requiring antibiotics to prevent infection during surgery, organ transplant care or cancer treatment (Saha, 2018). This creates a dilemma where more people still die because of lack of access to antibiotics than from resistant infections.

Unrestricted and inappropriate use of antibiotics results in more infections that are tougher and more expensive to treat (Laxminarayan, 2016). With increased antibiotic use comes a greater risk to acquire a multi-drug resistant pathogen which directly correlates with prolonged hospital stay, exposure to infected or colonized patients, and receiving broad-spectrum antimicrobial agents, especially third-generation cephalosporin. It is important to comprehensively manage these risk factors to limit AMR.

Identifying risk factors for MDR is also important to diagnose the underlying factors of antimicrobial infection. Patients with preexisting conditions like diabetes, skin lesions, and previous colonization with a multi-drug resistant infection are at a higher risk of MDR. Previous prolonged use of antibiotics or hospital stays are other risk factors that can cause a variety of infections from skin to urinary tract. Once infected, multi-drug resistant infections are difficult to treat because they are unresponsive to many common and powerful antibiotics. Treatment with an inappropriate antibiotic can also make the infection difficult to cure, which underscores the importance of patient and prescriber education on antibiotic use (Multidrug Resistant Organisms MDROs What Are They).

Socioeconomic inequality is another major cause of low health among the poor. This type of inequality amplifies the perception that some people are worth more than others, fueling a sense of superiority among the rich and subordination amongst the poor. In the same way, antimicrobial resistance is linked to poverty. A country's quality of governance, public spending on health per GDP and education are known to affect health outcomes and are likely to cause antibiotic resistance as well (Collignon, 2018). For example, in Europe, poor governance and corruption are as closely associated with variances in antimicrobial resistance levels between countries as are antibiotic consumption patterns (Collignon, 2018). It is estimated that antimicrobial resistance could push 28 million people into extreme poverty by 2050 without effective measures to control AMR (Adeyi et al., 2017).

Surveillance

Surveillance data on drug resistance is extremely important. It can help assess disease burden, improve diagnostic and treatment and monitor the effectiveness and design of control programs, which in turn helps create a sufficient public health response (Zignol, 2018). However, many low- and middle-income countries (LMIC) do not have the capacity for routine testing for patients' resistant to drugs and instead rely on surveys to get estimates on drug resistance. These gaps in surveillance contribute to greater burden of AMR because healthcare professionals lack the data to make effective treatment decisions and preserve antibiotics. This has led the World Health Organization (WHO) to establish the Global Antimicrobial Resistance Surveillance System (GLASS) to monitor commonly reported resistant bacteria in high, middle and low- income countries. The purpose of GLASS was to collect AMR data from 40+ partner countries in order to report health outcomes and surveillance capacities across countries. The first report (WHO GLASS report, 2017) described high rates of resistance across the world for select bacteria like Acinetobacter spp., Escherichia coli, Klebsiella pneumoniae, Neisseria gonorrhoeae, Salmonella spp., Shigella spp., Staphylococcus aureus, and Streptococcus pneumoniae. This functional and comprehensive global surveillance system provides reliable data across socio-political and economic contexts (Iskandar et al., 2021).

To make a greater, coordinated effort to provide practical guidance on antibiotics across countries, the United Nations established the Interagency Coordination Group on Antimicrobial esistance (IACG) was created in consultation with the World Health Organization (WHO), Food and Agriculture Organization (FAO) and World Organization for Animal Health (OIE). In a recent discussion paper, the agency highlighted the importance of an effective surveillance program that covers human, animal, food and plant populations. Such a system would provide easily comparable data that can be exchanged across nations. Many low-income countries, in comparison, lack the capability to establish and maintain surveillance systems to collect data on AMR, which means they are unable to use data to study trends that can inform effective policies and programs.

Figure 4. Antibiotic Resistance of Escherichia Coli (Resistance Mapping Charts)

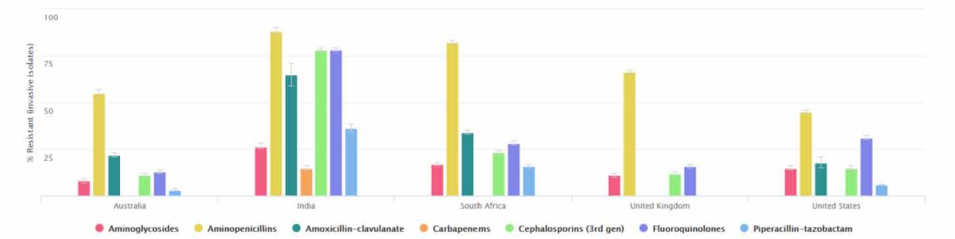


Figure 5. Antibiotic Resistance of Acinetobacter baumannii

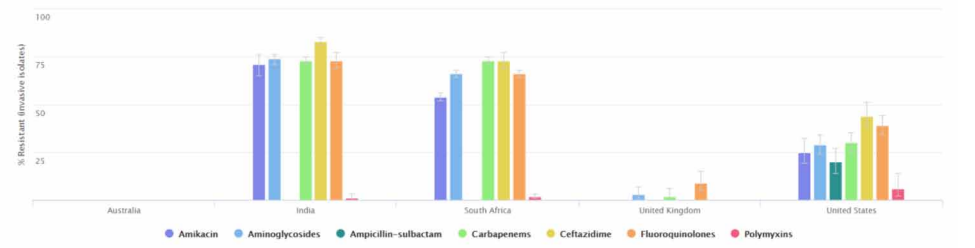


Figure 6. Antibiotic Resistance of Klebsiella pneumoniae

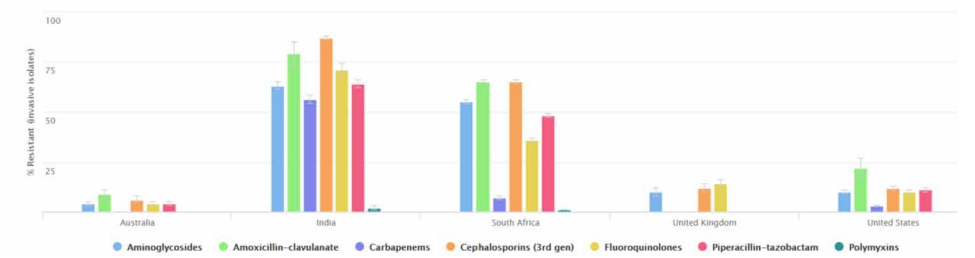


Figure 7. Antibiotic Resistance of Salmonella paratyphi

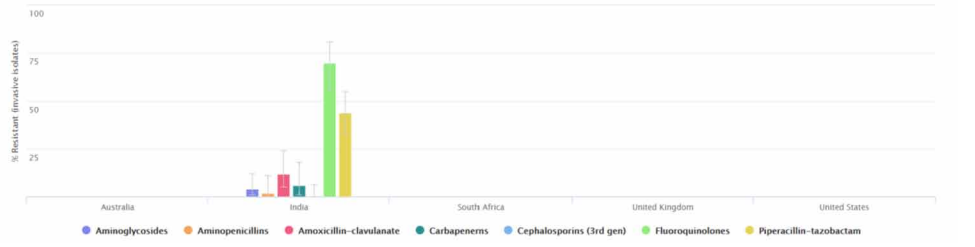


Figure 8. Antibiotic Resistance of Salmonella typhi

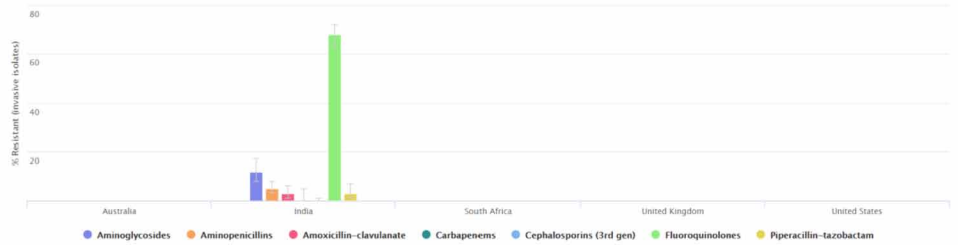


Figure 9. Antibiotic Resistance of Staphylococcus aureus

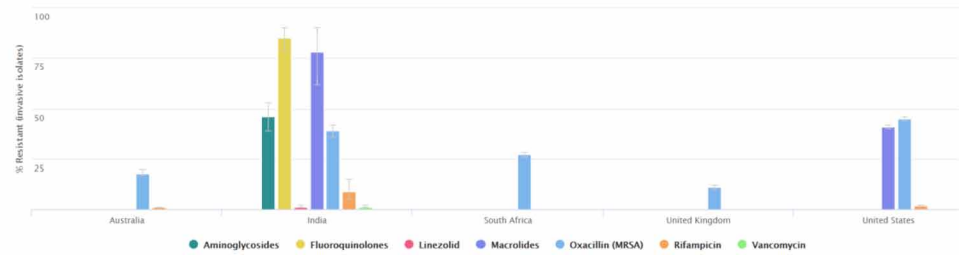
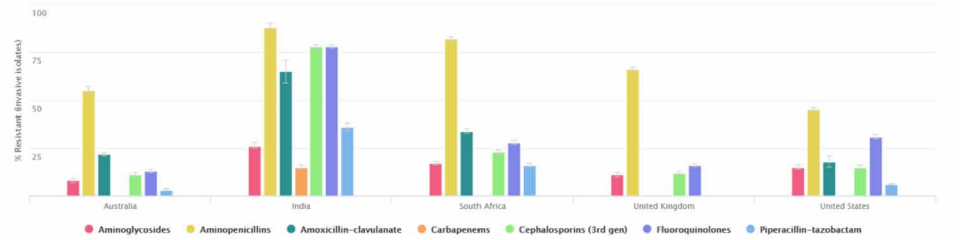


Figure 10. Antibiotic Resistance of Streptococcus pneumonia
All charts courtesy: resistance map from www.cddep.org



ROLE OF PHARMA AND ANTIBIOTICS

The pharma industry in India is growing rapidly and can deliver some of the needed innovation in diagnostic testing and treatment. Some factors for market growth include greater prevalence chronic diseases, rising income, modern medical infrastructure, expansion of health insurance coverage, and more frequent patent product launches. The Indian pharmaceutical market can potentially grow up to $55 billion by 2020 because of greater affordability and market access (McKinsey, 2020). Local players are especially well-positioned to lead because of their dominance in formulation development capabilities and early investments (McKinsey, 2020).

Yet, bottlenecks in drug discovery have added to antimicrobial challenges. Pharmaceutical companies have been reluctant to invest in antibiotic research and development because of low returns, not to mention the how difficult it is to predict when resistance can rise to an antibiotic. Therefore, the government and the pharmaceutical industry must do more to develop accessible diagnostic tools for healthcare providers to use if drug resistance is to be prevented. One solution could be for the government to provide grants or tax incentives to companies that develop better technological innovations to calculate the prevalence of and prevent antimicrobial infections (Plackett, 2020).

CURRENT PROGRESS AND UPDATES

In this section, standard practices throughout the healthcare industry will be covered, along with an overview of gaps and other potential solutions. For example, hospitals and other healthcare facilities have rules in place to prevent MDR infections. As mentioned earlier, one of the most effective rules is hand washing. Healthcare workers are encouraged to wash their hands with soap and warm water or alcohol-based hand sanitizer when handling protective clothing, after touching surfaces, and before and after every patient (Multidrug Resistant Organisms MDROs What Are They). Antibiotic control and private rooms for patients are other effective precautions.

Greater innovations in health care can improve AMR surveillance. For example, genomic sequencing is a valuable tool for drug resistance surveillance. It completes drug susceptibility testing in two rounds; it starts with first-line drugs and then moves to second-line drugs (Zignol, 2018). Genomic testing runs around US$130 on average per sample. The emergence of rapid molecular tests like GeneXpert MTB/RIF are improving access to susceptibility tests (Zignol, 2018). Greater affordability for these tests can expand testing and revolutionize public health.

For surveillance efforts to work, robust foundations are necessary. Therefore, it is essential for DNA extraction methods and recording of nomenclature and data to be standardized for stronger surveillance (Zignol, 2018). Likewise, targeted gene or whole-genome sequencing on sputum samples directly can overcome the need for culturing through a standardized approach (Zignol, 2018). When genomic sequencing is standardized and economically feasible, it could be a powerful tool in countries with low lab and referral capacity to better monitor antimicrobial resistance.

FUTURE TRENDS AND CONCLUSION

An important measure of success for antibiotic control would be how cost-effective genetic First sequencing, which in turn would improve testing for antimicrobial resistance by reducing lab work load through cost savings. Already, the price of genetic sequencing is lower than some and second line phenotypic testing of drugs and grouping specimens to sequence several isolates in one single run offers additional savings (Zignol, 2018). To expand genomic sequencing in low-income countries, molecular biology and bioinformatic skills need to be developed along with mentoring from supranational reference labs. Other models of antibiotic stewardship should also be adopted. For example, the United States Center for Disease Control approaches antibiotic resistant through a surveillance-based database and educating prescribers on the importance of antibiotic control. This has led to clarity on the prevalence of infections like *Clostridium Difficile* and that patients

were given powerful antibiotics more often than other drugs (Antibiotic Use in the United States). Similar programs in India and other LMIC can transform healthcare by improving treatment and public health programs.

It isn't enough to just limit antimicrobial consumption to prevent rise of phenotypic resistance. A combination of better access to quality care, robust health policies, community engagement, and intersectoral action can go a long way in changing the prevalence of AMR. For example, by improving supply chain management, inappropriate use of antimicrobials due to drug shortages can be prevented (Ganguly, 2011).

Resistant phenotypes and surveillance are difficult to monitor but the future is bright. Investments in global health particularly by LMIC would make emerging technology more accessible, improve health outcomes and control the factors that lead to poverty. Similarly, health programs would be an important tool to educate and curb multi-drug resistance rates. When these investments are made, healthcare delivery and public health will improve immensely.

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Chapter 2
Emerging Strategies for Sensing of Blood-Stream Bacterial Diseases

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ABSTRACT

Early prognosis of infection has been a major concern for clinicians worldwide. The diagnostic investigation in clinical practice focuses on resolving the complex mysteries of the deep-seated systemic infections, which are often difficult to decipher. Amongst the infectious diseases, the bacterial infections are the most ubiquitously found infections. The clinics have moved to application of molecular imaging techniques for early detection of systemic bacterial infections which even allow the follow-up during antimicrobial therapy to assess the efficacy of a particular course. Further, new age diagnostic methodology has seen a paradigm shift to the detection of biomarkers in the blood samples of patients.

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1.1 INTRODUCTION TO INFECTION: HISTORICAL AND CURRENT PERSPECTIVE

In the past, primitive human populations most probably have suffered for long time from infectious diseases similar to diseases of other wild primate populations.

The chronicle of infection control began nearly 30 years before the discovery of *Vibrio cholera* by Robert Koch, a renowned microbiologist. It was the time when John Snow, a physician had found the epidemic spread of London cholera was due to faecal contamination of drinking water facility in the golden square locality. And in the case of AIDS(Acquired Immuno Deficiency Syndrome (AIDS), where causative organism retrovirus type of Human Immunodeficiency Virus (HIV) was identified in year 1984 long after its occurrence since 1981 (Moorhead, 2002).

Thus, an important activity in the field of medical research is discovery of pathogens which cause diseases in humans, and many bacteria, fungi, viruses, helminthes, protozoa and prions are recognized as potential pathogens.

The various types of systemic infectious diseases can be classified based on the causative organism and the description along with disease associated is mentioned in Table 1.

Despite advances in the comprehension of pathophysiology of infection at the molecular level, it remains one of the major causes of morbidity and mortality world-over, particularly in developing countries (Lopez, Mathers *et al.*, 2006; Kok, Pechère *et al.,* 2004).The infections of bacterial etiology are the most common in clinical practice. As per the World Health Organisation, (WHO) report (2008), billions of money is spent on healthcare in mitigation of infectious diseases globally. Of these infections which manifest the presence of viable bacterial load in the blood pool are categorized as Blood Stream infections (BSIs). Although, currently focus is on ongoing pandemic viral infection, significant global burden still remains bacterial pathogens. (WHO Fact Sheet, 2019).

There are two main reasons to this changing global scenario with rising cases of the communicable diseases. As humans are encroaching into the biomes, at the interface, there are 'hot-spots' for human transmission of new pathogens from the wild. This phenomenon has been shown by the most recent pandemics and epidemics like SAR-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2 or COVID-19), SARS-CoV1, Ebola and Zika viral outbreaks, MERS (Middle East respiratory syndrome), chikungunya infections (CDC Report,2019). In addition to this, the looming secondary bacterial infections are result of low immune system following a viral illness(Langford BJ et al. 2020). Moreover, the irrational use of antimicrobial agents is leading us to the ever increasing number of drug resistant pathogenic bacteria.The rising rates of Antimicrobial resistance (AMR) is another area of public health threat as well as a significant cost burden to the global economy.

Table 1. Etiology Of Infectious Diseases In Humans

Infection causing microorganism	Characteristic(s)	Size range of microorganism	Disease(s)
Bacterial	Prokaryotic unicellular	0.2 - 100 µm	TB, anthrax, diphtheria, toxic shock syndrome,Meningitis,
Fungal	Eukaryotic unicellular, Eukaryotic multicellular	5.0- 10 µm 2.0-10.0 µm by several mm	Systemic mycosis, Aspergillosis
Viral	Small non-cellular parasite	0.015-0.2 µm	AIDS, dengue fever, SARS,
Protozoa	Eukaryotic unicellular	2.0- 200 µm	Chagas disease, African trypanosomiasis, Malaria
Helminthes	Parasitic worms	1.0 mm to several metres	Filariasis, Schistosomiasis, Hookworm infection
Prion	Misfolding of protein	–	Creutzfeldt–Jakobdisease

1.2 BACTERIAL INFECTIONS AND THEIR CLASSIFICATION

The bacterium is the simplest organism and most abundant of all the organisms on the earth. Life on earth cannot survive without it and these omnipresent bacterial microorganisms are mostly to blame for infections (Pfaller, Diekema et al., 2007).

Broadly classified as Gram variable and Gram Indeterminate bacteria depending upon the type of staining they take. Since, the first step in the microbiological detection of bacteria is almost always done by Gram staining, hence the classification (Pelczar M J, 1993).

Further both the types of bacteria can be classified based on their physical properties. Besides these, there are also some bacteria with unusual properties but it is not discussed here to avoid complexity. The rationale for the Gram staining is due to basic anatomical differences in the cell wall of these two groups of bacteria. The Gram indeterminate bacteria are stained by acid fast staining method.

The typical examples of the different genera of bacteria are:

Gram Positive: *Staphylococcus*, *Streptococcus*
Gram Negative: *Escherichia, Neisseria*
Acid Fast: *Mycobacteria, Nocardia*

In numerous clinical cases, it is very essential for therapeutic decision making and planning not only to establish whether or not there is an inflammatory reaction, but also to differentiate between infection and sterile cases of inflammation e.g. in case of wound healing and post surgical infections(Palestro, Love *et al.*, 2007; Das, Hall *et al*.,2002).

1.3 SYSTEMIC BACTERIAL INFECTIONS: DIAGNOSIS METHODOLOGY

The superficial infectious spots of bacterial origin can be simply accessed and diagnosed easily. The blood-stream infections pose a grim challenge to the clinical practice and result in uncertain findings through use of inappropriate procedures.

Accurate and prompt diagnosis which includes rapid and sensitive detection techniques of bacterial pathogens of the deep-seated infections is crucial for initiating appropriate therapy and patient management.

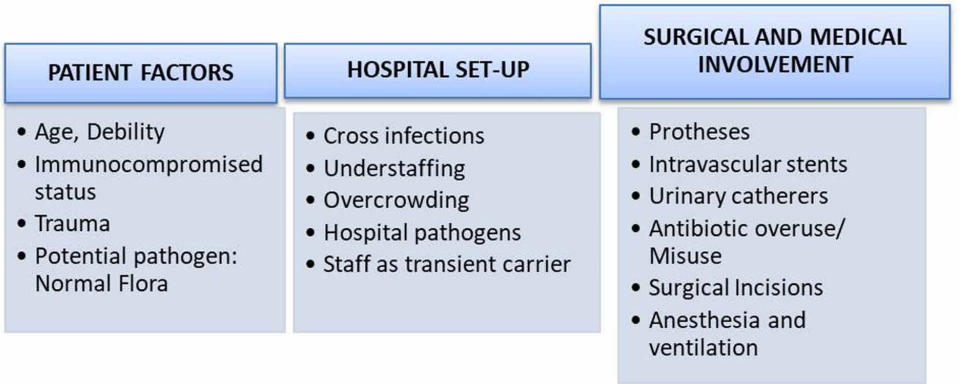
The correct evaluation by clinicians for prognosis of the Bacterial Infections is currently is in demand because of following reasons:

- Increase in number of immune-compromised individuals who are quite susceptible to even non-pathogenic microbes as a consequence of cancer chemotherapy, transplant case or case of opportunistic infections like HIV, TB etc
- The global population is aging with ever increase in demand of orthopedic grafts and cardiovascular implants and other prosthetic devices that further become the reason for microbial colonization post surgical intervention (Baldoni, 2009)
- The spectrum of the causative microorganism is changing, with the presence of antibiotic resistant bacterial strains

The various predisposing factors for the occurrence of Infectious Diseases are elaborated in Figure 1.

The early prognosis and competence to discriminate between bacterial and sterile inflammation is vital to treat patients efficiently and prevent the pathological complications (IAEA Report 2000).

Figure 1. Predisposing factors for occurrences of Infections in Humans



Presently, the diagnosis of most of the infections can be regarded as a two step procedure. The first step is based on classical assessment which includes physical examination, clinical history, and laboratory tests. The second step is based on the use of imaging techniques like Ultrasound, Computed Tomography (CT), and Magnetic Resonance Imaging (MRI) which are currently employed for pinpointing of infectious lesions.

1.3.1 Traditional Laboratory Investigations

The microbial infection on the body may be indicated by clinical manifestations such as high fever, pain, lack of appetite, general illness and abnormal laboratory reports. The routine techniques of diagnosis depend on physical examination of microbes from infectious lesions. There is a plethora of laboratory investigations available which are based on white blood cell-count, acute-phase proteins, cytokines, markers of the inflammatory response, but none of them can specifically discriminate between infection and inflammation. These techniques are time-consuming and conclusive findings often are quite late to lead a correct decision-making in clinics (Wareham, Michael *et al.*, 2005).

1.4 DIAGNOSTIC INFECTION IMAGING

The scope of diagnostic imaging modalities in terms of electromagnetic spectrum is from ultrasonic to gamma ray and X-ray frequencies. Commonly, these techniques are *in vivo* procedures, with the exception like optical projection tomography (OPT)

of optical imaging technique, where the sample is a 'fixed' *ex vivo* preparation. Commonly, these techniques are *in vivo* procedures (Wickline, Lanza *et al.*, 2002).

With the recent resurgence of significance in molecular imaging strategies, the design of target-specific molecular imaging agents based on enhanced understanding of the biochemistry of the Infectious lesions may ultimately lead to the development of an ideal agent for infection imaging.

1.4.1 Introduction to Imaging Modalities For Infection

Diagnostic Infection imaging studies can be further classified into functional and anatomic imaging technique based on the type of information they provide about the infection.

Diagnostic Infection imaging studies can be classified into **(a) Anatomic** and **(b) Functional imaging techniques** based on the type of information they provide about the infection.

The morphological imaging modalities viz., X-Ray radiograph, Ultrasound, Computed Tomography, and Magnetic Resonance Imaging reveal anatomical/ structural alteration in tissues or organs as a result of microbial invasion with the inflammatory response of the host to the invasion (Brant, Helms *et al.*2006). CT and MRI have higher sensitivity and resolution and the detection of anatomical changes can help predict the precise site of the pathological condition (Scatliff, Morris *et al.*2014). However, in early phases especially in absence of any anatomical alteration infection specificity cannot be predicted. Moreover, as a result of distortion in normal anatomy due to post surgical scarring or presence of vascular grafts, the role of these diagnostic techniques is limited(Gemmel, Dumarey *et al.* 2009).Some of key characteristics and main issues related to application of these techniques in infection are listed below:

1.4.1.1 Planar X- Ray and Computed Tomography

- It is based on the application of X- Rays where the planar X-Ray is a pure 2D-form of visualization procedure while the Computed Tomography produces a 3D imaging technique
- It is relatively inexpensive, easily available for clinical practice, and their clinical findings are highly reproducible and reliable with excellent spatial resolution of 50-200 μ
- These X-ray based modalities can map only anatomical aspect. So they have a limited utility in infection imaging (Palestro, Love *et al.* 2007; Peterson, 2006)

1.4.1.2 Ultrasonography

- It is widely available in clinics as well as portable forms, quick to perform and with excellent spatial resolution attaining below 1mm
- It is versatile diagnostic tool based on use of ultrasound waves
- It is usually employed as a first line investigation by medical practitioners suspecting some internal infections around abdominal region and can be utilized to obtain functional information to a very limited extent (Gotthardt, Bleeker-Rovers *et al.*, 2010)
- The limitations of ultrasonographic results are extremely dependent on operator's discretion and the mapping and the visualization of internal body structures is intricate as a result of dense regions like intestinal gas and bony structures which hindered penetration of the ultrasonic waves (Cootney, 2001)

1.4.1.3 Magnetic Resonance Imaging

- It utilizes nuclear magnetic resonance (NMR) to image nuclei of atoms in internal organs to get detailed information
- MRI is based on the detection of molecules that contain nuclei that possess the property of nuclear spin and provides a resolution of 10 μ
- The advantage of MRI is excellent anatomic details to pinpoint the exact location, and its contraindications are scanning of patients with pacemakers, metallic implants and prostheses
- Furthermost, long duration of imaging procedures make the scans prone to movement artifacts especially for elderly and children (Olsen, 2013)

1.4.1.4 Endoscopic Techniques

- It requires a simple instrument called Endoscope which is used to scan the interior of a hollow cavity of the organ
- The main complications associated with this technique are perforation in the lining of stomach and esophagus, which may bleeding especially at the site of a biopsy removal
- The endoscopic techniques have limited utility only for gastro-intestinal tract infection (Neumann *et al.*, 2013)

1.4.1.5 Optical Tomography

- It is an another emerging modality based on detecting the transmission of photons through biological systems and a specific tissue contrast is created by different absorption or scattering patterns of photon at various wavelengths
- Its use is limited on soft tissues, like breast and brain tissue and on the organ under investigation being partly transparent or translucent (Haisch 2012; Sharpe 2004)

1.4.2 Functional Imaging Techniques

These techniques are exemplified by nuclear medicine studies make use of tracer amounts of radioactive materials (Brant, Helms *et al.,* 2012) and they assume a significant role in diagnosing the infections focus and in planning targeted antimicrobial chemotherapy. The advances in understanding pathogenesis of infectious disease combined with progress in radiopharmaceutical sciences have boosted the development of nuclear medicine (NM) techniques for the diagnoses of infection more precisely (Elgazzar, 2006). The nuclear medicine technique comprises of Single photon emission computer tomography (SPECT) and Positron emitting tomography (PET) which provide detailed localization to give crucial information in clinics. Diagnostic radiopharmaceuticals, as a component of Nuclear medicine technique, offer a methodology for the assessment of the infectious disease state and monitoring the antimicrobial chemotherapy non-invasively.

The Radionuclide Imaging techniques Like Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) can offer considerable benefit over these less sensitive diagnostic methods for imaging specific biological targets, predominantly those present in low concentration. The nuclear medicine techniques show specificity in locating the infectious focus with alterations in functional aspects that are early clinical manifestations.

Also, SPECT can further offers the prospect to extend the time-window of patient examination (due to the longer physical half-life of single photon emitters) thus, permitting clinicians to examine biological processes *in vivo* several hours or days after administration of the labeled compound. And there are plethoras of radiotracers are currently available for infection and the list is expanding every year.

The radiotracers are being utilized to prepare these radiopharmaceuticals could be Single photon emitting radioisotopes like ^{111}In, ^{67}Ga, and ^{99m}Tc and PET radiotracers can be ^{18}F, ^{68}Ga.The most widely used SPECT radioisotope for this technique is ^{99m}Tc due to its favorable physical properties. The 140 keV energy of γ emission with half-life of 6 h offers ideal nuclear medicine imaging properties (Saha, 2010).

SPECT/ PET scans are commonly employed imaging modality which provides more comprehensive information especially in patients with osteomyelitis and suspected of deep-seated infected joint prostheses or implants. Perhaps the least expensive and simplest technique to boost the specificity of 'Three-Phase Bone Scintigraphy' is the delayed imaging schedule. In contrast to normal bone scintigraphy, in which uptake of radiotracer decreases by about 4 h post injection, the tracer-uptake in condition like osteomyelitis builds up for several hours, resulting in a higher lesion-to-background ratio on the delayed scintigraphic scans (Kim *et al.,* 2016).

1.5 NEW-AGE LABORATORY BASED BLOOD TESTS

1.5.1 Biomarker Detection

Early diagnosis of systemic infections is considered complicated through the conventional blood-culture reports, as it can lead to unnecessary delay in treatment and enhance possibilities of adverse outcomes.

Most sensitive diagnostic approach based on host biomarker detection for bacterial infection include polymerase chain reaction (PCR) to detect nucleic acids (DNA or RNA), (Tkadlec *et al*., 2019) and immunological tests like ELISA that are based on antigen−antibody interactions (Tsao *et al.*, 2020).

These conventional techniques show high specificity in distinguishing viable bacterial cells. But, the complicated and laborious process requirements in such diagnostic techniques renders them unsuitable for rapid diagnostics (Samuel *et al.,* 2004).

The biomarkers have always play an important role in the prognosis and diagnosis of human disease. Since, the levels of Biomarker in the blood circulation are dynamic, they can be used to assess the changes in response to different inflammatory stimulus, especially in the case of bacterial infections. The levels of C-reactive protein (CRP),an acute phase protein, procalcitonin (PCT) and presepsin shows elevation in certain conditions such as inflammation and bacterial infections. It was found that elevated CRP levels (over 120 mg/l) and decreased PCT levels (less than 1.25 ng/l) was observed in the immunocompromised hematological patients with fungal infection (Marková *et al*., 2013). Whereas elevated PCT level and normal levels of CRP was primarily the prognosis for - blood stream infection of bacterial origin (Stoma *et al*., 2019). Recently, TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) biomarker was found to suitable for distinguishing viral infections from the bacterial infections due to higher concentration in the cases of viral dieases only (Does *et al.*, 2018).

1.5.2 Systemic Inflammation Response Syndrome (SIRS) Analysis

SIRS analysis represents the early signs of infection as an inflammatory response to any foreign invasion. The SIRS is manifested by several conditions such as:

1. Increase in core temperature above 38 °C or decrease in core temp below 36 °C.
2. Change in rhythm of heart beat, which may be more than 90 beats per minute (Tachycardia).
3. More than 20 breaths per minute or decrease in arterial Carbon dioxide tension below 32 mmHg.
4. Abnormal increase or decrease in white blood cell count, which may be more than 12000 per μL or less than 4000 per μL or greater than 10% abnormal white blood cells.

When two or more than two parameters are there and among statement (a) and (d) at least one is confirmed, indicates the systemic inflammatory response (Dellinger *et al.*, 2013). This method cannot be used as marker of infection alone because change in white blood cell count may occur by several other reasons including just the inflammation, but can be used as important prognostic parameter (Conti *et al.*, 2015).

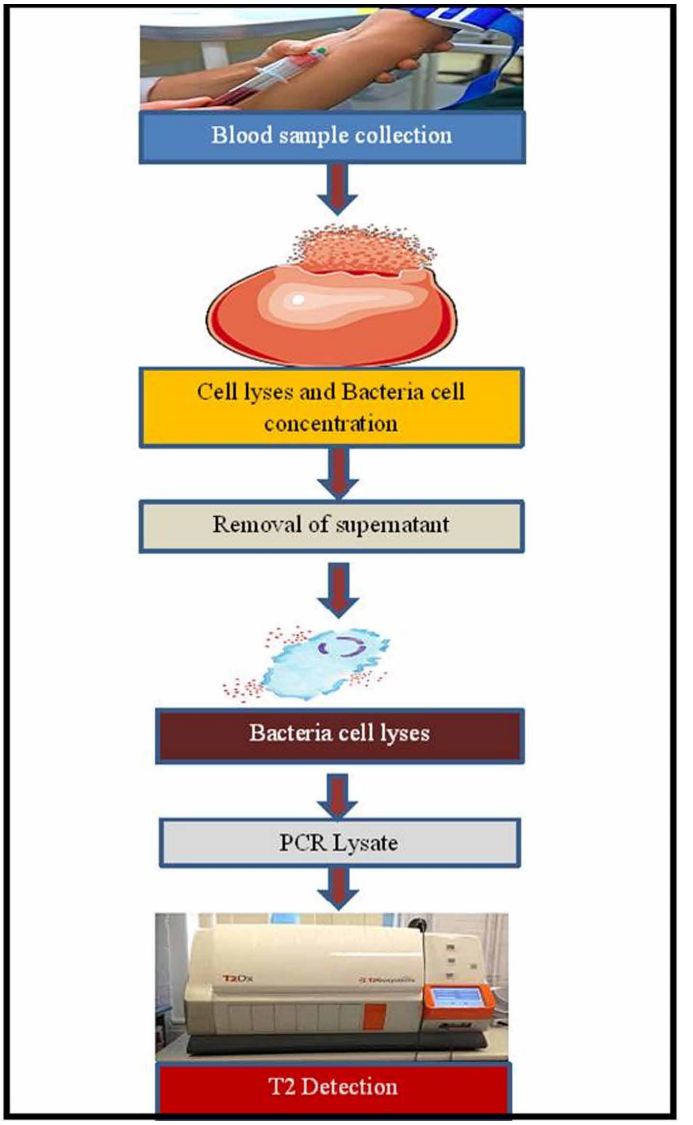
1.5.3 T2 Magnetic Resonance

T2 magnetic resonance (T2MR) offers superior tool for the early detection of systemic infection with low limit of bacterial load detection as 1 CFU/mL of whole blood (Figure 2).

This diagnostic tool is amalgamation of MRI along with the PCR based amplification of DNA infection causing pathogens.T2 Magnetic Resonance can detect various targets such as cells and nucleic acid within blood. The first medical application of T2MR in diagnosis was T2Candida and T2Bacteria panels, found successful in detection of microbial cells within whole blood (Clancy and Nguyen, 2018).

The detection of pathogenic cells from blood is based on lyses of blood cells, concentrates cellular debris and bacterial cells, breakdown of cells by bead-beating, amplification of DNA by thermostable polymerase and detection of amplified product by amplicon-induced agglomeration and T2 measurements (Mylonakis*et al.*, 2015).

Figure 2. Schematic representation of diagnosis based on T2 Magnetic Resonance



1.5.4 16s Metagenomics

16S Metagenomics is one of the newer molecular approach for the diagnosis of Infections, but this technique has not been commercialized yet due to optimization issues. The advantages offered by 16S Metagenomics over Blood culture techniques are given as (Rutanga *et al.*, 2018):

- High sensitivity
- Detect all bacteria in a given sample
- Culture independent
- Polymicrobial infections can be detected
- Low blood volume requirement for diagnosis
- Shorter turnover time

Beside these advantages offered certain loopholes are also there, owing to this it become difficult to implement this technique over Blood culture. Limitations of this technique are given below as:

- False positive results
- Difficulty in identification between pathogen and environmental contaminant
- Do not provide information about antibiotic susceptibility profile

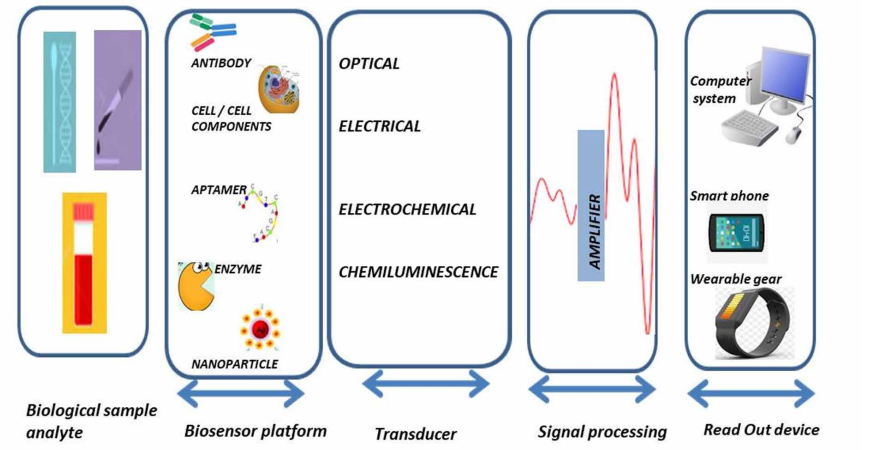
1.6 NEW AGE BIO-SENSING DIAGNOSTIC PLATFORMS

The new generation diagnostic tests aim to tap the potential of body fluids in identifying and evaluating the human well-being. The non-invasive easy collection process and convenient storage makes saliva more clinical relevance for tracking the physiological processes. (Roi *et al.* 2019) The biosensor based diagnostic platforms have the prospective to make commercially and clinically viable diagnostic systems [Figure 3]. Such biosensor based devices will convert a biological response into quantifiable signal which may be electrochemical (Simoska *et al.*, 2019), thermal (Tlili *et al.*, 2013), chemiluminescence (Wu *et al.*, 2018),or optical (Massad N *et al.*, 2016) as an output indicator.

Current approaches in investigational tools include application of nanomaterials/ nanoparticle (NP) (Tallury *et al.,* 2010) and utilizing their unique physiochemical properties for designing a biosensoring platforms. Moreover, convergence of nanotechnology with advanced fabrication techniques has created a new dimension 'nanobiosensor' hold a great promise for future application as smart POC device. (Zarei, 2018)

These 'smart' diagnostics in collaboration with AI tools (Smith *et al.*, 2020) can further augment the more accurate diagnosis and these investigative measures can be more accessible to the remote settings.

Figure 3. Representative image of new-age bio-sensing platform for detection



CONCLUSION

The early diagnosis of infection still poses great challenge to clinicians. The functional imaging techniques offer the advantages to some extent, but for the specificity of the diagnosis, the healthcare community still relies on traditional laboratory investigations like blood culture tests.

The new developments in AI enabled molecular approach based diagnostics have shown great potential to compensate for intrinsic limitations of culture-based diagnostic tests. Further, the body fluid based biomarker detection can also aid in enhancing diagnostic capabilities.

Hence, these new-age diagnostic techniques are being designed with substantial focus on robustness of the tests, and comprehensive data interpretation methodologies so that there is substantial improvement in the patient–health.

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Chapter 3
Exploring the Potential of Peptides and Peptidomimetics in Biosensing

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ABSTRACT

Biosensors are devices that capture the biological signal and convert it into a detectable electrical signal through transduction. Biological entities like DNA, RNA, and proteins/enzymes can be conjugated onto the biosensor surface to detect and observe certain biological analytes in environment, biomedical, and food industries. Peptides have been efficiently used in the fabrication of peptide-based biosensors due to their attractive properties like established synthesis protocols, diverse structures, and as highly enzyme-selective substrates. However, owing to their labile nature, peptidomimetics are the best alternatives at the bioreceptor interface due to their specificity and stability, relatively low cost and easy modifications, and capability

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to form supramolecular assemblies like nanosheets. Such bioconjugation strategies efficiently convert interaction information into a measurable signal, thus highlighting the importance in the fabrication of next-generation novel robust biosensors desirable for detection and dissemination of pathogens causing infections in the living and non-living worlds.

INTRODUCTION

In the biochemical field, sensors are usually defined as a device which includes both a receptor (bio-recognition element) and a transducer, providing specific quantitative or semi quantitative analytical information. Biosensors are powerful tunable systems capable of switching between an ON/OFF status in response to an external stimulus. In general, *biosensing techniques* can be defined as any of a variety of procedures which use biomolecular probes to measure the presence or concentration of biological molecules, biological structures, microorganisms, etc., by translating a biochemical interaction at the probe surface into a measurable physical signal. The general function of biosensors involves a receptor in the most general sense recognizing an analyte, and then a transducer either triggers a quantifiable signal or catalyzes a reaction related to the analyte concentration to generate a signal (Griffin et al., 2009). In clinical diagnosis, a sensitive, quick, convenient and versatile molecular biosensor has been desired to simplify the testing process, reduce the cost and shorten testing time (Salazar-Salinas et al., 2009). Recent advances in both disciplines allow redesigning the configuration of the sensing elements – either by modifying toggle switches and gene networks, or by producing synthetic entities mimicking the key properties of natural molecules. The primary requirement in the selection of various substances/factors as the components of biosensors includes rapid responding ability, high specificity & sensitivity, reliability, portability, productivity and long-lasting stability. In addition to this, immobilization/fabrication of bioanalyte in its native conformation, high accessibility of the receptor's sites to the species of interest and effective adsorption of the analyte to the employed support (Marx, 2007) are the main crucial factors that should be considered during the engineering of high performance biosensors. These demands need to be ardently addressed when developing the design of biosensors. Peptides and peptide based analogs possess the potential candidature for fulfilling many of these requirements.

BIOSENSORS: A GENERAL OVERVIEW

The word "sensor" is derived from the Latin word "sentire" which basically means 'to identify' anything. In terms of classification, physical sensors and chemical sensors are the two most primary and widely opted classes of sensors. Biosensors, an amalgam of physical and chemical sensing method, are the most recent type of sensors in a way that these have been recognized by only some eighteen years prior to today (Buenger et al., 2012). Basically, biosensors are receptor-transducer based devices which interpret some specific biophysical or biochemical property of the medium. Furthermore, the most interesting quality that sets them apart is the presence of biological/organic recognition element which enables the detection of particular biological molecules in the medium (Wang, 2006).

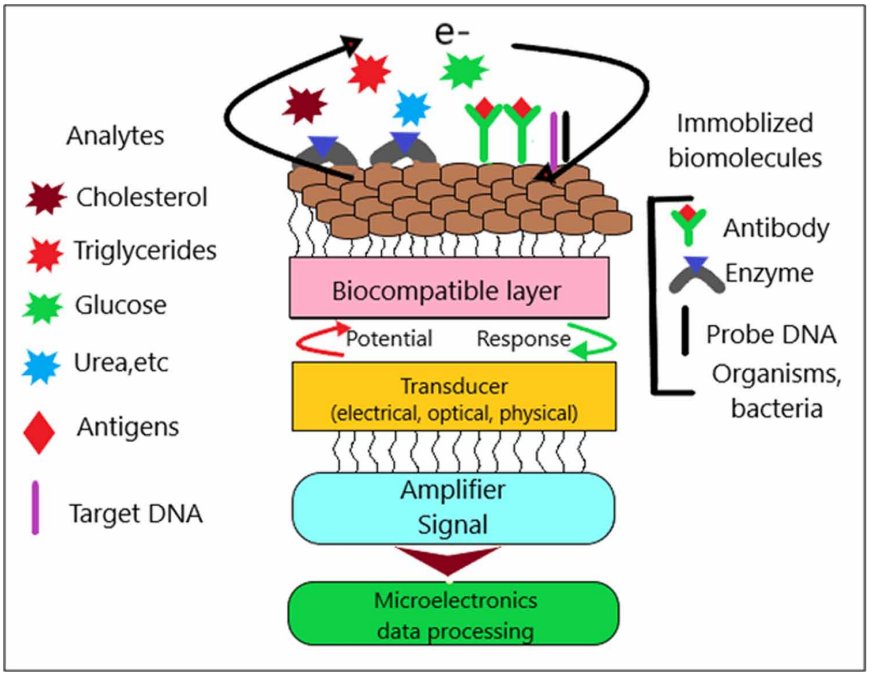
i. Major Breakthrough in the Development of the Biosensors

Ever since the development of the first biosensor as a potentiometric enzyme based electrode that was invented for the detection and measurement of glucose in any medium (Clark and Lyons, 1962) the major focus has remained on reducing the size and making the laboratories economically stable towards the development of portable, small-sized/nanosized and multi-functional biosensors (Chao et al., 2016). The bioelement is principally any organic body which is able to detect any particular analyte from the medium of interest while remaining irresponsive towards any other potentially interfering species whereas sensing element consists of the signal transducing portion of the biosensor which could be in the form of any magnetic, optical, electrical or electrochemical etc. transducing mechanism (Vidal, 2013). Elaboration of these two components is given in Figure 1.

ii. Types of Biosensors

Depending upon the type of transducer, biosensors can be categorized in the groups of electrochemical, mass dependent, optical, radiation sensitive and so on. On the basis of the choice of the bioelement such as enzyme, nucleic acid, proteins, saccharides, oligonucleotides, ligands etc. there are various sets of biosensors (Mohanty and Kougianos, 2006), that can be fabricated keeping in view the type of analyte under consideration (Turner, 2000). Peptide sequences play a substantial role in enzymatic and many other assays due to their characteristic properties such as good stability, regular synthetic protocol, and hence, these molecules are of utmost importance in the fabrication of sensitive, fast, and convenient biosensors.

Figure 1. Schematic representation of different parts of a biosensor



PEPTIDE-BASED BIOSENSORS

Peptides are formed by natural or synthetic short polymers of amino acids which are linked by peptide bonds. Peptide sequences that are specific enzyme substrates play a crucial role in assays of enzymatic activity and screening of enzymatic inhibitors. Due to these unique properties, peptides are excellent candidates for developing sensitive, fast, and convenient biosensors. Generally, peptides do not generate a measurable signal directly in response to a binding event, hence conjugating them with a signal marker is an efficient strategy to convert the information of analyte/binding into a measurable signal and till date, several methods have been utilized to construct such peptide-based biosensors. These biosensors can be used for detecting various analytes including metallic ions, proteins, proteases, kinases, bacillus species, nucleic acids and antibodies.

i. Fluorosence Based Biosensors

Environmentally sensitive fluorophores conjugated with peptides are most widely used common signal markers. Their fluorescence emission can be affected by changes in the local environment caused by the affinity or interaction between conjugated

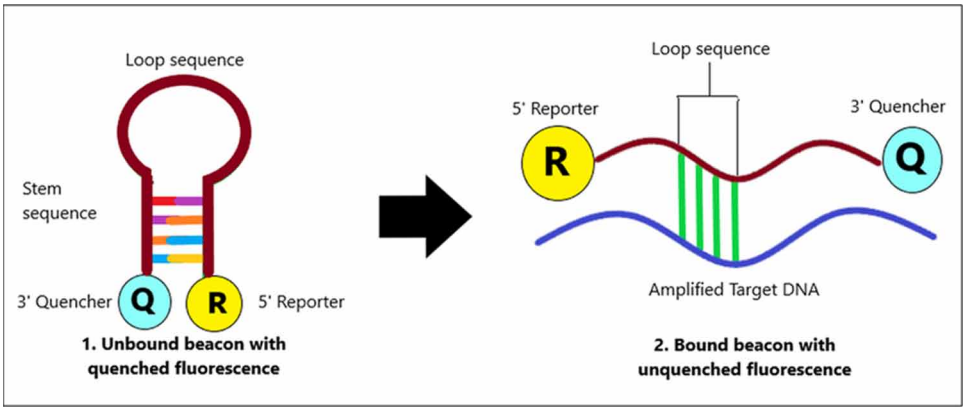
peptide and analytes. The method of conjugating peptides to environmentally-sensitive fluorophores has been utilized to develop various peptide-conjugated molecular probes/sensors (Pavan and Berti, 2012), including ion sensors, DNA sensors, redox sensors and protein sensors. Similar properties of such signal markers can also be attributed to near-infrared dyes (Peng et al., 2006), nanoparticles (Wang et al., 2010), quantum dots (Clarke et al., 2010), graphene (Feng et al., 2012; Feng et al., 2011), polydiacetylene (PDA)-liposomes (Jaworski et al., 2011), lanthanide chelators (Yan et al., 2011) and electrochemical markers (Zhao et al., 2010). Several biosensors have been widely reported in literature signifying the budding use of peptide rational design to overcome the challenges related to biosensor commercialization (Scognamiglio et al., 2013). For example, Enander et al. (2008) proved the ability of peptides combined with ratiometric fluorophores to ensure robustness in recognition and signaling in immobilized format quantification assays.

Excimer-Type Biosensors

Similarly, excimer-type protein molecular biosensors comprise of a peptide and two identical fluorophores, which are attached to the opposite ends of the peptide (Figure 2). In the presence of the target analyte, the interaction between the analyte and peptide forces the fluorophores to separate, resulting in a shift of the emission peak of the fluorophores to that of the monomer. An excimer-type peptide beacon for detection of anti-HIV antibody has been developed (Plaxco et al., 2006). The peptide beacon concept was further extended to develop an HIV sensor based on the probe–quencher pair model. In probe-quencher pair protein-sensors, fluorophores and quencher units are attached at various residues of the peptide sequence. Affinity between a peptide sequence and target protein influences the distance between the fluorophore and quencher, resulting in a change in the fluorescence emission, thus generating a signal.

In another case, the use of CPPs (Cell Penetrating Peptides) has enabled the visualization of real-time viral infection of living cells (Yeh et al., 2008). The fact that molecular beacons are able to detect viral RNA in infected cells has already been revealed (Yeh et al., 2008). These single-stranded molecular beacon oligonucleotides were specifically chosen to target a noncoding region of a viral genome and were labeled with a fluorophore at one end and a quencher at the other. Prior to encountering the virus, these constructs existed in a stem-loop structure with the quencher and fluorophore in close range, thus resulting in an absence of fluorescence as shown in Figure 2. However, as the viral particles entered cells, the molecular beacons hybridized with the viral genome and changed conformation, thereby separating the quencher and fluorophore. As a result, fluorescence was detected allowing the viral infection to be visualized. While this approach is highly

Figure 2. Working of a molecular beacon system



sensitive (detection of a single viral particle was possible), it was only successful in fixed cells permeabilized with Triton to allow uptake of the molecular beacons. In order to deliver these molecular beacons in a less invasive manner, Tat peptide was harnessed to deliver the constructs into uncompromised cells in a consecutive study which enabled real time detection of viral replication and infection (Yeh et al., 2008).

LIMITATIONS OF PEPTIDE-BASED BIOSENSORS AND THE STRATEGIES TO OVERCOME

Despite innovations and improvements in this technology, peptide-based biosensors are still limited by a number of factors, such as long term stability, sensitivity, read-out time, miniaturization and cost & productivity related challenges (Scognamiglio et al., 2010). Lowering of the detection limits and the increase of robustness are the main confrontations of the future generation of biosensors. Synthetic biology can deal with these key issues, improving biosensor potential in terms of sensitivity and stability, as well as selectivity and detection in complex mixtures, by extending the perfect response of biological systems and/or engineering them to provide new desired features (Andrianantoandro et al., 2006). Recent advances in technologies like nanotechnology, bioinformatics and molecular engineering are capable of creating new biological components alongwith decoding and rewriting genes, enzymes, and networks, modifying them to alter their core function and structure (Effendi et al., 2008). Emerging strategies are being adopted to take the advantage of engineered organisms to generate a specific response to pollutants, or to design combinatorial library of bioinspired molecules, like peptidomimetics, molecular

imprinting polymers, and aptamers, with customized features regarding specificity and stability (Checa et al., 2012).

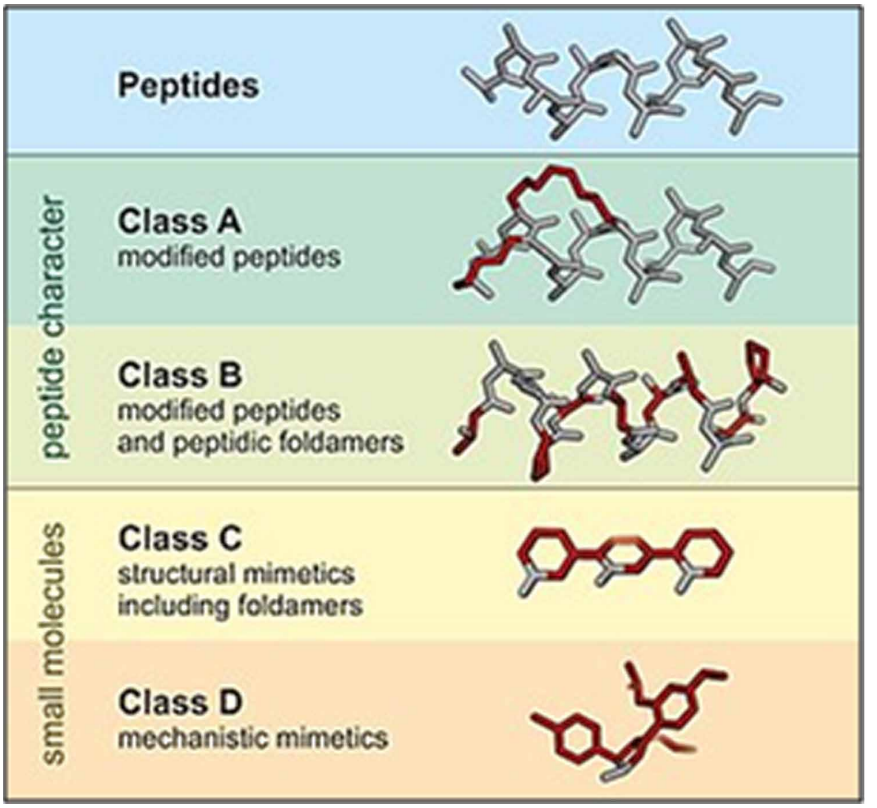
WHAT ARE PEPTIDOMIMETICS?

Peptidomimetics are the structural and functional mimics of bioactive peptides using non-canonical amino acids or non-peptide scaffolds which comprise an important class of drug molecules (Mizuno et al., 2017; Floris and Moro, 2012; Avan et al., 2014). These compounds have the ability to interact with the biological target and also produce the same biological effect as of protein (Vagner et al., 2008). Peptidomimetics are used to counter the undesirable effects of peptides while retaining their beneficial characteristics. Peptidomimetics have been subdivided into three types (Azzarito et al., 2013; Pelay-Gimeno et al., 2015) i.e. Type I mimetics or structural mimetics are short peptides that replicate the topography of a secondary structure and distinguish themselves from their parent peptide only by substitutions introduced to stabilize the desired conformation. Type II mimetics or functional mimetics are non-peptidic functional molecules that have a small molecular scaffold and do not recapitulate all the side chain interactions of the parent protein. Type III mimetics or functional-structural mimetics are the molecules which include nonpeptide templates that are topologically similar to the parent peptide but do not show atom-by-atom analogy. Further, these three categories have been sub-divided by Pelay-Gimeno et al. into four different classes based on the degree of their similarity to the natural peptide precursor i.e. Classes A–D where Class A mimetics are most similar to the parent peptide whereas Class D mimetics show the least similarities (Pelay-Gimeno et al., 2015). Classes A and B include peptide-like structures whereas classes C and D encompass small molecular scaffolds:

- Class A mimetics or Type I mimetics are defined as peptides that mainly consist of the parent peptide amino acid sequence. They have minimal alterations as the backbone a side chains of this class align closely with the bioactive conformation of the precursor peptide.
- Class B mimetics or Type II mimetics are the modified class A mimetics with various isolated non-natural amino acids acting as the building blocks and with major backbone alterations. These comprise of foldamers such as β- and α/β-peptides as well as peptoids in which their side chains align topologically similar to the precursor peptide.
- Class C mimetics or Type III mimetics include highly modified structures with small molecule replacing the peptide backbone completely.

- Class D mimetics or Type IV mimetics are molecules that mimic the mode of action of a bioactive peptide without a direct link to its side chain functionalities and they can be generated by affinity optimization of a class C molecule or they can be identified in screenings of compound libraries or by *in silico* screening of virtual libraries.

Figure 3. Various classes of molecular modifications



Peptidomimetics can be a promising class of molecules to regulate protein–protein interactions (PPI) in cells and hence, they play an important role in current drug-development research (Whitby and Boger, 2012). They have various properties like antimicrobial, antiviral, anticancer, antihypersensitive, antimalarial and antifouling that make them attractive in the field of therapeutics as well as diagnostics (Tenenbaum & Segal, 2015; Zhang, 2015).

TYPES OF PEPTIDOMIMETICS

a. Global Restrictions

Restricting the peptide globally means altering the final conformation of the peptide. This can be achieved by numerous ways. For instance, cyclization is the simplest way to introduce a conformational constraint into a peptide sequence. This results in the modification of the overall conformational profile of the target peptide compound and also increases the *in vivo* stability of the cyclic peptides compared to their linear analogs. Cyclization improves the quality of the bioactive compound i.e. bioavailability and potency as the high proportion of *cis* amide bonds and the absence of free *C*- and *N*-termini confer higher metabolic resistance. The limited conformational freedom results in higher receptor selectivity and binding affinity by reducing entropic effects.

Backbone cyclization combines with *N*-alkylation to enhance the stability of peptides (Gilon et al., 1991). The most popular approach which has been also extensively studied is the head-to-tail generation of cyclic peptide via amide bond formation according to standard peptide chemistry (Li et al., 2002). Another popular approach is the generation of cyclic peptidomimetics through a chemical bond involving two side-chains and that too by utilizing basic amino acid residues to form an amide bond through disulfide bridges between the two side-chains.

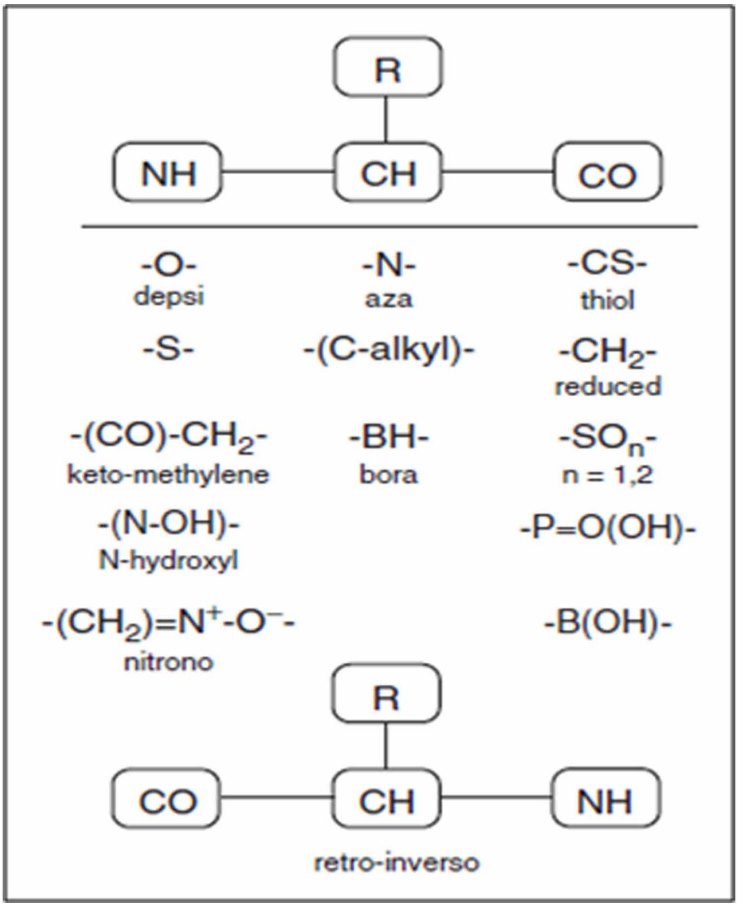
b. Local Modifications

The local modifications are restricted to single amino acids and are local alterations of the peptide structure. They can be grouped into side-chain and backbone modifications with the aim of introducing conformational restrictions and to stabilize the molecule towards protease-mediated degradation as shown in Figure 4.

The backbone modification is introduced by amide bond surrogates which improves the stability of the peptide *in vivo* (Figure 5). The amide bond isosteres mimic the structural features of the peptide bond, modify the conformational profile and the hydrogen-bonding capability (Wolff, 1995; Choudhary and Raines, 2011).

The local modifications around side-chains are achieved by modulating the conformational profile of the peptide and thus altering all the rotatable bonds present in the amino acid unit. The side-chain modifications have explored pharmacophoric steric and electronic interactions such as modulation of the hydrophobic content by adding aromatic moieties or the introduction of polar appendages to address any polar or hydrogen-bonding interactions with the target receptor. Such localized modifications give rise to unique secondary structures which can be exploited to deliver compounds of utmost importance in therapeutics (Nandel & Jaswal, 2007).

Figure 4. Isosteric local modification



Peptoids

Peptoids can be defined as mimetics of α-peptides in which the side chain is attached to the backbone amide nitrogen instead of the α-carbon and results in the formal shift of the position of the side chain with respect to the parent peptide backbone. They were first reported by Bartlett and coworkers in 1992 as oligomers of N-substituted glycine (Simon et al., 1992). Peptoids were initially recommended as an accessible class of molecules from which lead compounds could be identified for drug discovery. The sequence-specific peptoid oligomers are easily constructed from primary amines by the solid-phase submonomer method and their applications are of particular interest to study the peptoid secondary structures and drug design. Peptoids are advantageous as research and pharmaceutical tools which include the

ease and economy of synthesis, highly variable backbone and side-chain chemistry possibilities (Nandel & Saini, 2007; Nandel & Saini, 2007; Nandel & Saini, 2011; Saini et al., 2013; Nandel et al., 2014; Saini et al., 2015; Saini, 2016; Saini & Verma, 2017; Tripathi et al., 2017).

Figure 5. Isosteres of the peptide bond

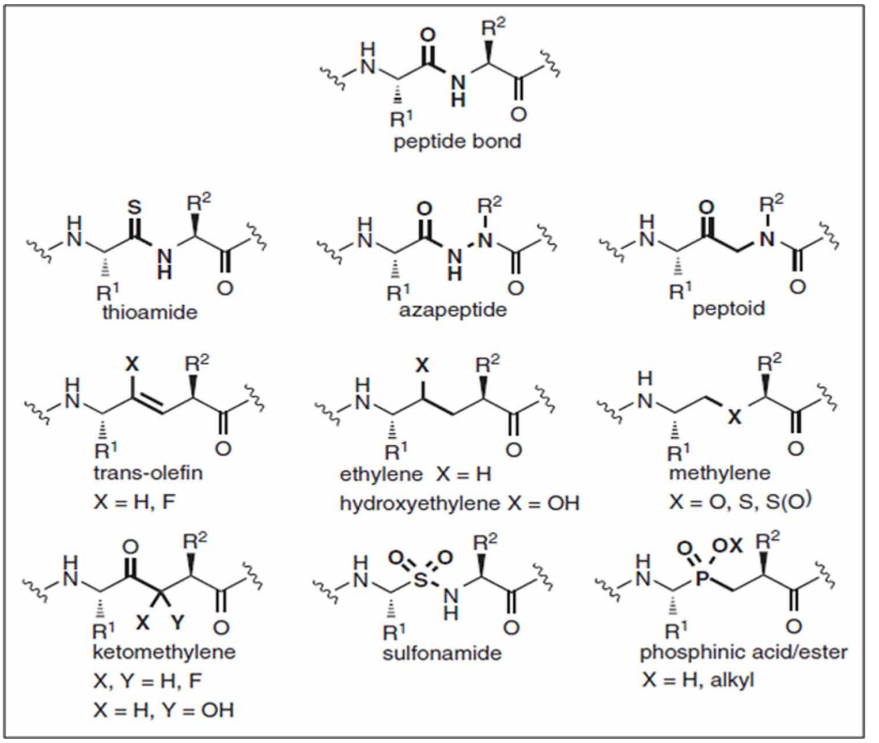
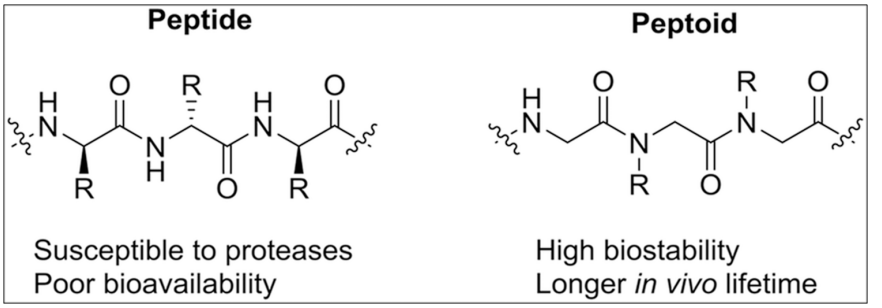


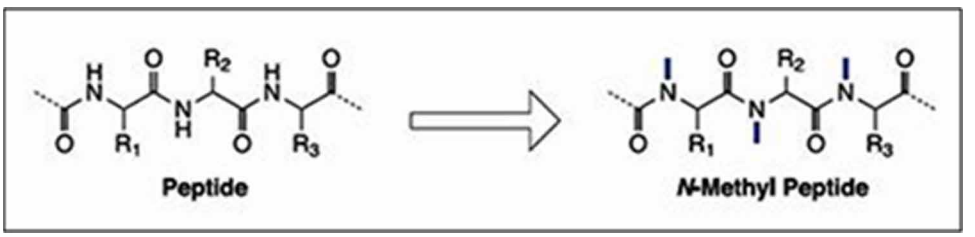
Figure 6. Schematic representation of a peptide and a peptoid monomer



N-Methylated Peptides (NMe Peptides)

It is a well-established approach for understanding the role of each amino acid constituting the bioactive peptide and strategic in identifying which amino acids act as hydrogen-bonding donors in the interaction with the target enzyme/receptor. This approach provides insight into the relationship between the conformational preferences of the peptide and bioactivity. The group of Kessler envisaged a simple approach of N-methylation to overcome various obstacles of peptides as a 'rational' way toward drug development (Chatterjee et al., 2013) and also to explore the reduction of conformational space while transforming a peptide into a peptidomimetic drug. Mono- and Multiple-N-methylations (Figure 7) of cyclic peptides were investigated to elucidate their remarkable conformational modulation ability by imparting steric constraints in the peptidic backbone and in improving the pharmacokinetic profile of the peptides to be used as drug leads. In this respect, N-methylation introduces another dimension to this 'spatial screening' (Kessler et al., 1996) due to the remarkable property of conformational modulation. In fact, N-methylation facilitates the occurrence of a cis-peptide bond and allows us to study the role of amide protons in establishing potential hydrogen bonds, resulting in optimization of the conformational and structural profile of a peptidomimetic which can be used to design potential drugs in pharmaceutical industry (Nandel & Jaswal, 2014)

Figure 7. Structural representation of N-methylated peptide



Retro-Inverso Peptides

The retro-inverso isomerization is a common method for chemically modifying the peptidomimetic structure around amide bonds to prevent the protease recognizing the peptide-based inhibitor as a substrate inhibiting protease degradation. This isomerization can be achieved by replacing one or more l-amino acids with the parent enantiomer and at the same time inverting the backbone direction from N→C

to C→N. The retro-inverso modification increases the *in vivo* stability due to the modification of amide bonds which are recognized by proteases for their hydrolysis.

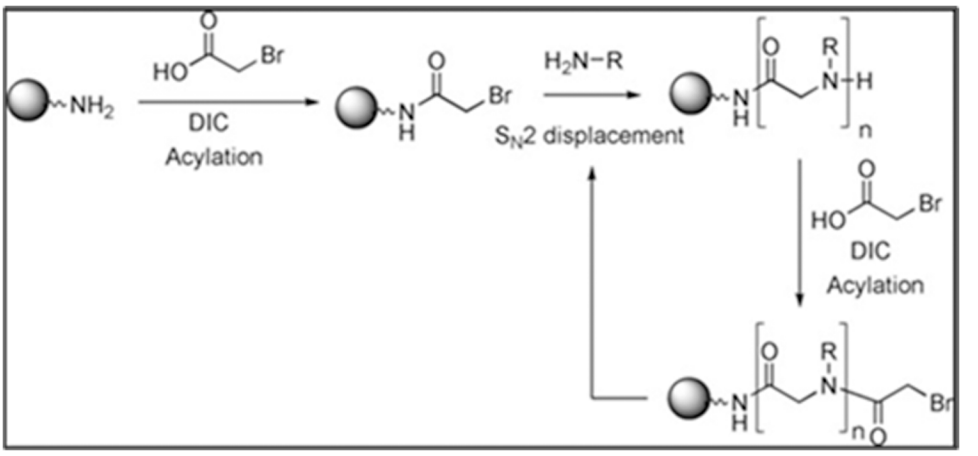
SYNTHESIS OF PEPTIDOMIMETICS AND THEIR ROLE IN BIOSENSING

The conversion of peptides to peptidomimetics is an attractive strategy where peptides are transformed to stable mimics that represent similar effects to their peptide analog in addition to increased uniformity in structure, target specificity, improved stability to proteolytic digestion and better cell membrane permeability. Consequently, incorporation of unusual amino acid substitutions, backbone amide bond modifications, stiff scaffolds or the addition of hydrophobic residues in such peptide analogs has been studied (Vagner et al., 2008; Vlieghe et al., 2010).

Synthesis of Peptoids

Peptoids are normally synthesized using the highly flexible submonomer method (Zuckermann et al., 1992). This is a solid phase synthesis methodology (Figure 8) which includes two steps: Acylation using a halo-acetic acid also called as haloacetylation of an amine functionality attached to a solid support and then displacement of a halogen using a primary amine. This method allows high coupling efficiency and good final product yields and synthesis on solid-phase allows excess reactants to be removed easily at the end of each step.

Figure 8. Schematic representation of synthesis process of peptoids



Synthesis of N-Methylated Amino Acids

Although several efficient methods are available for the synthesis of N-methylated amino acids, the most commonly used conventional method is the one developed by Freidinger et al., involving the reductive cleavage of 5-oxazolidinones to obtain the enantiomerically pure N-methyl amino acids (Freidinger et al., 1983). However, this method is not applicable for some side-chain functionalized amino acids, such as Trp, His, or Cys and showed that the base-catalyzed N-alkylation of o-nitrobenzenesulfonamide (o-NBS) protected ⟨-amino groups using dimethylsulphate as the methylating agent is a better alternative (Miller and Scanlan, 1997). A further modification of this method employs a Mitsunobu reaction, which was optimized to obtain all possible N-methylated amino acids in solution (Biron and Kessler, 2005) or on solid support (Chatterjee et al., 2012).

Synthesis of Specific Peptidomimetics

Similarly, N-protected amino alkyl thiols were treated with carbon disulphide in the presence of triethylamine (TEA) to generate trithiocarbonate salt which upon reaction with appropriate halides afforded dipeptidomimetics in good yields. This procedure was extended for the synthesis of N, N′-orthogonally protected trithiocarbonate-linked dipeptidomimetics (Marchiani et al., 2009). Pyrrolidinones were synthesized by the oxidation of the hydroxy function with tetrapropylammonium perruthenate/ N-methylmorpholine-N-oxide and concomitant cyclization. Pyrrolidines were synthesized by tosylation of the hydroxy group and subsequent intramolecular nucleophilic substitution. Further, accessible substrates were transferred into peptidomimetics by attachment of amino acid moieties at both termini (Mayer et al., 1988).

The mono- and bis-glyoxylamide peptidomimetics were prepared via the facile ring-opening of N-acylisatins with amino acids and peptide derivatives. The ring-opening of N-acylisatins with dipeptides and tripeptides was one of the most effective strategies for the synthesis of second and third generation glyoxylamides (Merrifield, 2006).

Artificial peptides and their peptidomimetics is a great prospective that can be harnessed to develop the desired molecular biosensor because of their desirable properties such as diversified structure, high protein affinity, established synthesis protocol and modified approach (Choulier and Enander, 2010; Pazos et al., 2009). A range of peptidomimetics with specific sequences can be used to deliver high affinity to particular analytes, and can be acquired by screening and optimization of such artificial libraries. In addition, peptidomimetics like peptoids are blessed with high stability, easy modification and large chemical flexibility. For example,

peptoids can be prepared with arbitrary sequences according to standard solid-phase submonomer synthesis method (Zuckermann et al., 1992). Also, peptidomimetics alongwith mass spectrometry can be used to design biosensors which can better analyze reaction mechanisms, assign enzyme substrates, detect ligand interactions etc. (Marholz et al., 2019).

The clinical potential of peptidomimetics was also confirmed by authors who projected a protein/peptide array to detect anti-Delta genotype 1,6,8 specific antibodies among Hepatitis D Virus infected patients by surface plasmon resonance imaging. An array of recombinant proteins and mimetic peptides has been defined which provided a high throughput viral hepatitis diagnostic, combining the potential of artificial molecules with the Surface Plasmon Resonance Imaging (SPRi), a technique to harness a wide sample screening. The peptidomimetics of small analytes isolated from phage display libraries were also synthesized as chimeric constructs with the NanoLuc to produce recombinant reagents for producing phage-free immunoassays, a significant contribution to the field of small-molecule immunoassays with no need of chemical conjugation of the actual analyte, the stoichiometry (peptide/enzyme) is fixed with no batch-to-batch variations, and the final tracer helps in the development of single-step immunoassays with fast readouts (Scognamiglio et al., 2015).

PEPTOIDS AT THE BIORECEPTOR INTERFACE

The detection of bacteria in complex matrices containing many undesired interferents like human cells, commensal bacteria alongwith many proteins and metabolites requires the development of novel bioreceptors, including bacteriophages, non-antibody binding proteins, half-antibodies, and single-chain (camelid) antibodies which offer higher specificity. While antibodies are the most widely used bioreceptors in affinity biosensor research, but problems in their production, purification costs, stability during & after immobilization on sensor surface and decreased binding efficiency with time are the main limiting factors although recently antimicrobial peptides has also been exploited for the detection of whole bacteria (Pardoux et al., 2020). For the simultaneous detection of many bacteria, regeneration of the sensor surface and multiplexing are the two other crucial aspects.

To overcome some of these deficiencies, recent advances in engineered antibody mimetics include peptoid nanosheets (Olivier et al., 2013), where antibody mimetic peptoids self-assemble to form 3 to 5 nm thick sheets with surface loops expressing antigen binding sites. Such nanosheets exhibit remarkable chemical and biological stablility and can be produced with ease and precise control. Other notable designed antibody alternatives include single-chain variable fragments (ScFv) (Ahmad et al., 2012), camelid-derived heavy variable chain (VHH) antibodies (nanobodies)

(Muyldermans, 2013; Hassanzadeh-Ghassabeh et al, 2013), and single-chain antibodies expressed via yeast surface display (Richman et al., 2009), DARPins (Stumpp and Amstutz, 2007), and other artificial proteins such as adhirons (Tiede et al., 2014). These alternatives are comparatively small, easy to customize, and convenient to produce in bulk in bacterial systems, avoiding the traditional antibody production route in mammals or birds. Successful regeneration is possible with stable bioreceptors, since they can often withstand harsh regeneration buffers without compromising binding capacity. Parallel multiplexing on a single chip also reduces detection costs, providing multiple items of information from a single-shot analysis (Ahmed et al., 2014).

Two-dimensional (2D) atomically defined organic nanomaterials are an essential class of materials with broad applications. The nanostructured carbons such as carbon nanotubes (CNTs) or graphene (GF) are widely employed as electronic or electrochemical transducer in biosensor devices (Valentini et al., 2013). Graphene (GF) is the emerging choice of the nanomaterial in this perspective in biosensors because of its remarkable two-dimensional (2D) layered character. Similarly, emerging graphene-like 2D materials comprise of multiple elements that possess more versatility, greater flexibility and better functionality with a wide range of potential applications. Recently, a graphene composite biosensor has been designed to detect microcystin-LR (MC-LR), a seriously hazardous and bioaccumulative cyanotoxin for water quality monitoring assessment. A three-step linking protocol was used to immobilize MC-LR onto the GF electrodes and then incubating with conjugated monoclonal antibodies to detect MC-LR toxin. The increase of electron transfer resistance upon bioconjugation of MC-LR and antibodies on GF electrodes was used to detect the change of MC-LR concentration. A great linear sensing response ($R2 = 0.99$) of EIS change was established over a wide MC-LR concentration range of 0.005 to 10 μg/L with good reproducibility. Environmental water samples were collected from different locations like Tokyo metropolitan tap, Sanshiro pond (Tokyo, Japan), Shinobazu pond (Tokyo, Japan) and Inba lake water (Chiba prefecture, Japan). This discovery is one step close to the *in-situ* detection of even very less amount of MC-LR (i.e. 2.3 ng/L) in assessing the severity of contamination of the water source with very low detection limits (Zhang et al., 2018). Such graphene film composite biosensors deliver a promising prospect of large-scale manufacture of sensor tips due to their macroscopic free-standing nature, scalable fabrication route and easily tunable size. Similarly, this attribute of orderdness can also be extended other molecules.

One of the ways to design ordered 2D organic nanomaterials is through the supramolecular assembly of sequence-defined synthetic polymers. Individual peptoid polymers with a simple sequence of alternating hydrophobic and ionic monomers can self-assemble into highly ordered, free-floating nanosheets. A wide range of

experimental characterization techniques (e.g., scanning probe, electron, and optical microscopy, X-ray diffraction, surface-selective vibrational spectroscopy, and surface tensiometry) and computational techniques (coarse-grained and atomistic modeling) have established the fundamental properties of peptoid nanosheets, their mechanism of formation, and their application as robust scaffolds for molecular recognition and as templates for the growth of inorganic minerals. Peptoid nanosheets are supramolecular assemblies of 16-42-mer chains freely floating in water that form molecular bilayers spanning many microns in lateral dimensions. Hydrophobic and electrostatic interactions result in the tight packing of the highly ordered, fully extended and packed parallel to one another component chains. Nanosheets form via a novel interface-catalyzed monolayer collapse mechanism. These nanosheets are readily engineerable, as functional monomers can be easily integrated onto the nanosheet surface or into the interior. For instance, functional hydrophilic "loops" have been displayed on the surfaces of nanosheets which can interact with specific protein targets, providing a potential platform for the molecular recognition events. Such nanosheets can also bind metal ions and serve as 2D templates for mineral growth (Robertson et al., 2016).

The performance of biosensors lies in the efficient signal capture of the biological recognition event (transduction). Transducers translate the interaction of the analyte with the biological element and generate electrochemical, electrochemiluminescent, magnetic, gravimetric, or optical signals. Nanomaterials can immobilize an enhanced quantity of bioreceptor units at reduced volumes and even act as transduction element thus increasing sensitivity and lowering the detection limits (Holzinger et al., 2014). Because of their trending potential, Olivier et al. has developed peptoid nanosheets in a way identical to the action of Human immune system. These nanosheets resembling tiny sheets of Velcro can be designed to identify varied kind of molecules, each just one-hundred nanometers across and could lead to a new class of biosensors. Antibodies have a typical architectural design: a structural scaffold which remains the same irrespective of the severity of the disease, and incessantly variable functional loops which is mimicked in a two-dimensional nanosheet scaffold covered with little functional loops like Velcro.

Functional loops on nanosheets were achieved by inserting short molecular segments into nanosheet-forming peptoid polymers. As the peptoids knit themselves together into sheets, the inserted segments expelled from the fold. These functional loops can be engineered to selectively bind certain enzymes or inorganic materials, which impart these novel materials with promising chemical sensing and catalysis with an additional advantage of a very high yield. Nanosheets with loops of varying composition, length, and density were designed to pick and sense specific enzymes out of a solution, tolerating much harsher conditions than peptides, and hence becoming the choice of material for building of a diagnostic device. This finding

also represents an important step towards extending the rules of protein folding to the world of synthetic materials (Olivier et al., 2013).

In the biosensing area, small sized coumarins are one of the broadly used fluorophores because their high quantum yield, and efficient membrane permeability. Metal-complexed coumarin derivatives are multipurpose sensor molecules (Hou et al., 2013; Yeh et al., 2014; Garcia-Beltran et al., 2014). Recently, increasing numbers of triazolyl coumarin motifs have been utilized for various biological applications, such as the development of anti-inflammatory (Stefani et al., 2012), anticancer (Zhang et al., 2014), and antibacterial agents (Shi and Zhou, 2011). These compounds have also been explored as selective chemosensors (Stefani et al., 2012; Ho et al., 2012; Shi et al., 2014). Among the anions, efficient detection of the cyanide anion is particularly useful due to its severe toxic issues related to human health (Li et al., 2014; Peng et al., 2014) and upon release to environment. Lim and Lee explored the development and utility of peptoid-derived fluorescence sensors (Fuller et al., 2013) and reported the synthesis and characterization of a novel peptoid-based fluorescence probe. Authors incorporated the coumarin moiety into the peptoid backbone via copper (I) catalyzed azide-alkyne [3+2] cycloaddition reaction and the quenching of the complex was achieved by coordination with Cu^{2+} ion. The peptoid-Cu^{2+} complex exhibited significant Turn-ON fluorescence upon the addition of CN^-. In view of its utility in rapid, selective, and reversible detection of CN^-, novelty of the newly-generated peptoid molecule as a potential CN^- sensor was established (Lim and Lee, 2016).

Peptoids can also be exploited for imaging applications to add specificity to contrast and imaging agents. For example, a potent peptoid antagonist for vascular endothelial growth factor (VEGF) receptor 2 was labeled with a positron emitter for successful positron emission tomography (PET) imaging of the receptor *in vivo*. Although, this technique was applied for tumor detection, similar approaches can be developed using peptoid ligands for neurodegeneration biomarkers, which may range from altered receptor expression to amyloid deposition (Hao et al., 2011).

OTHER BIOMOLECULAR ANALOGS IN RANGE

Immobilized molecular receptors on self-assembled monolayers are generally used to detect biomolecules with surface plasmon resonance (SPR) based sensors (Wink et al., 1997). SPR has evolved into a useful bioanalytical tool to detect proteins, DNA, enzymes, and other biomolecules because of its excellent sensitivity to detect proteins. However, the potential of these biosensors is limited by the nonspecific interactions of biomolecules in complex matrixes (such as cell lysate, serum, and blood). Nonspecific proteins interact with the surface of biosensors creating a false

positive response, obstructing the detection of analytes in crude biological fluids. Therefore, it necessitates the need to reduce nonspecific interactions so as to allow the use of biosensors for direct monitoring of biomolecules in biological fluids. This will eliminate the need of cleaning steps, signal amplification, labeling, or indirect detection of the analyte of interest. Preferably, it is the role of the monolayer to protect the SPR surface from nonspecific adsorption and provide an anchoring point for the molecular receptor. Hence, extensive research in surface chemistry has been undertaken in the past decade to overcome nonspecific adsorption. In fact, the high sensitivity of SPR to protein adsorption also makes it an excellent tool to monitor nonspecific adsorption on self-assembled monolayers (Mrksich et al., 1995). Among layers studied polyethyleneglycol (PEG), also known as polyethylene oxide (PEO), exhibits optimal performances in limiting nonspecific adsorption (Ostuni et al., 2001). Numerous variants of PEG monolayers have been investigated for nonspecific adsorption (Trmcic-Cvitas et al., 2009). Although PEG monolayers have been used for detection of proteins in serum (Teramura and Iwata, 2007), oxidative damage and the need for carboxylic acid functionalization of PEG to immobilize the molecular receptor are their two main disadvantages. To overcome these limitations, hybrid materials have been engineered to improve on the properties of PEG. For example, PEG copolymers of maleimide-PEG monolayers (Lee et al., 2007), polypropylene sulfide-PEG (Feller et al., 2005) and poly (lysine)-PEG18 were synthesized and successfully resisted the nonspecific adsorption. This approach was further modified by Messersmith and coworkers, who successfully developed a peptidomimetic polymer with ethylene glycol brushes (Statz et al., 2008) resulting in the reduced nonspecific adsorption of cells on a metallic surface. Similarly, a poly (aspartic acid) peptide with ethylene glycol (EG)-biotin brushes showed decreased nonspecific adsorption and can immobilize molecules via biotin-streptavidin-modified EG and biotinylated antibodies (Jeong et al., 2004; Jeong et al., 2006).

Similarly, phage display peptide libraries are useful tools for rapid selection of specific ligands for the development of immunoassay, pathogenic bacteria detection, cells targeted imaging, and so on (Arola et al., 2016; Ono et al., 2014; Ma et al., 2013; Gunay et al., 2017; Arevalo et al., 2012). For small molecular immunoassays, phage borne peptides are lucrative reagents for the development of competitive tests, in which an analyte peptidomimetic, a mimotope, substitutes for the chemical hapten and/or the analyte (Arevalo et al., 2012; Kim et al., 2008) or the noncompetitive tests, where these peptides react specifically with the analyte-antibody immunocomplex but not with the uncomplexed antibody (Arola et al., 2016). In these applications, the strong signal associated with the detection of the large phage surface and the possibility of noncompetitive detection in general result in assays with improved sensitivity, in the latter, the two-site recognition also provides better specificity (Hua et al., 2015).

However, because of their large size, low diffusion rate, and biological nature, there are some limitations to the use of phage particles and thus, require secondary reagents for its detection. Recently, the use of the Nano luciferase (NanoLuc) as a fusion partner to generate recombinant tracers has been explored for the development of such immunoassay. The imidaclothiz peptidomimetic C2-15 that specifically binds to the anti-imidaclothiz monoclonal antibody (mAb) 1E7 was fused to NanoLuc, both at the N terminus (C2-15-NanoLuc) and C terminus (NanoLuc-C2-15). The conjugation at C-terminus showed better performance than that at the N-terminus and was exploited to develop a bioluminescent enzyme immunoassay (BLEIA) and a bioluminescence lateral flow immunoassay (BLLFIA) for imidaclothiz. The luminescence signal of NanoLuc-C2-15 rapidly reaches high intensity with slow attenuation, which enabled one to capture the BLLFIA readout by using a smartphone without an external light source. The IC_{50} of the BLEIA and BLLFIA were 3.3 ± 0.2 and 6.4 ± 0.4 ng mL^{-1}, respectively. Both immunoassays exhibited good accuracy for the detection of imidaclothiz in environmental and agricultural samples (Ding et al., 2018).

CONCLUSION

The field of biosensing has emerged at a very rapid pace particularly in the last two decades and proved its potential through single analyte or multiple array based detection technology. Synthetic biology has profoundly affected the strategies to synthesize novel bioinspired materials. Furthermore, the application of nanotechnology in biosensing technology in combination with synthetic biology, is creating a deep impact on the design of biological circuits, bioproduction systems, biopolymers, biological machines, and consequently on the development of diagnostic tools for agri food, environmental, pharmaceutical and biomedical application. However, commercially robust biosensors are constantly being researched on. Therefore, various peptidomimetics together with nanotechnology offer more reliable biological recognition elements with enhanced sensitivity and stability, which actually represents the main challenges to fabricate a commercially successful biosensor.

FUTURE PERSPECTIVES

Peptidomimetics has arisen as an exciting and promising research field, receiving a noteworthy attention from the scientific community. This consideration of various peptidomimetics, particularly peptoids arises from a number of remarkable characteristics of easy synthesis, tunable properties with scaffolds that can tolerate

tough laboratory conditions and thus can serve as a solution for challenges in different application fields. More studies need to focus on the potentiality of peptidomimetics like peptoids that can be further harnessed for the development of various kinds of engineered biomaterials. The design and production of such tunable systems, based on the use of interdisciplinary technologies, has created important technological breakthroughs, allowing the expansion in the development of a wide range of research and application fields, from pharmaceutical to biomedical diagnosis and therapeutics, as well as in the biotechnological and industrial sector.

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Chapter 4
Advances in Clinical Diagnosis of Tuberculosis: Past, Present, and Future

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ABSTRACT

Tuberculosis (TB) holds a central and deadly platform around the globe, affecting mankind with around one-third of the world being affected by latent TB. TB progresses in the body through inhalation process and has a critical discrimination in terms of affecting individuals depending upon age, sex, socio-economic status, and even the stature of nation (developed or developing). The biggest challenge in TB management is accurate, direct, early diagnosis, and an ability to differentiate the type of mycobacterium. The most common and reliable direct methods include tuberculosis skin test (TST), smear microscopy, nucleic acid amplification tests (NAAT), and immuno-chromatographic-based methods. However, culturing the specimen on a mycobacterium specific media is considered the 'gold standard' for diagnosis of TB by the WHO. Mycobacterium cultures are used extensively for bacilli differentiation and also for predicting drug susceptibility testing in multi-drug-resistant TB. This chapter discusses the merits and demerits of many approaches to distinguish and identify the type of mycobacterium.

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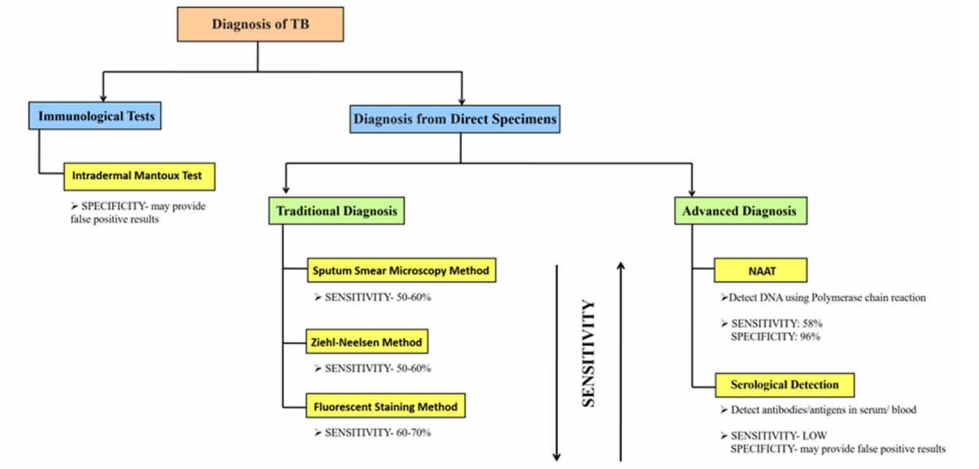
INTRODUCTION

Tuberculosis (TB) disease is a global public health malady, claiming almost 1.5 million lives annually . Putting India on a global scenario, it is found that one-fifth of the global incidence of TB occurs in India (Central TB division 2010). Tuberculosis is caused by members of the *Mycobacterium tuberculosis* (Fig. 1) complex [MTBC], which includes *M. tuberculosis, M. bovis, M. africanum, M. canetti*, *and M. microti.* TB is a major concern in industrialized countries due to their socio-economic factors such as immigration, a rapid increase in poverty, malnutrition, war, and limited medication access. To address this grave disease, the most important aspects are its effective treatment, and its proper diagnosis. The management of TB includes a 4-drug regime, and the treatment period is about six months long. To reduce transmission of this disease, and to achieve disease elimination, early, and accurate diagnosis followed by proper treatment is vital. However, there are still a lot of challenges associated with the diagnosis of TB with specificity, and sensitivity.

Figure 1. Structure of Mycobacterium



1. MOST COMMON IMMUNOLOGICAL TESTS OF TB

The most commonly used method for TB diagnosis is the tuberculosis Skin Test. This test is popularly known as the intradermal Mantoux test. It is the oldest diagnostic test, which is still included in the WHO latest recommendations for TB control (Lalvani 2007). The tuberculin fraction most widely used in this test is purified protein derivative (PPD), derived from cultures of *M. tuberculosis*. The reaction to

intra-cutaneous injected tuberculin is the classic example of a delayed hypersensitivity reaction. A person who has been exposed to this bacterium is expected to mount an immune response in the skin. However, various factors both in the host, and inherent in the test lower its specificity, and sensitivity. PPD contains over 200 antigens present in the bacilli Chalmette-Guerin vaccine (BCG) and most non-tuberculosis bacteria also. Moreover, this test also has a poor ability to distinguish latent TB infection (Nayak and Acharjya 2012). This test gives both false-positive and false-negative results. It is found that about 20% of immuno-compromised children with culture-confirmed TB disease do not react initially to the TST (Dunn, Starke et al. 2016) thus giving false-negative results. Moreover, individuals vaccinated with BCG tend to show false positive results with this test. Therefore better alternatives are followed in high incidence TB countries.

2. DIAGNOSIS OF TB FROM DIRECT SPECIMENS

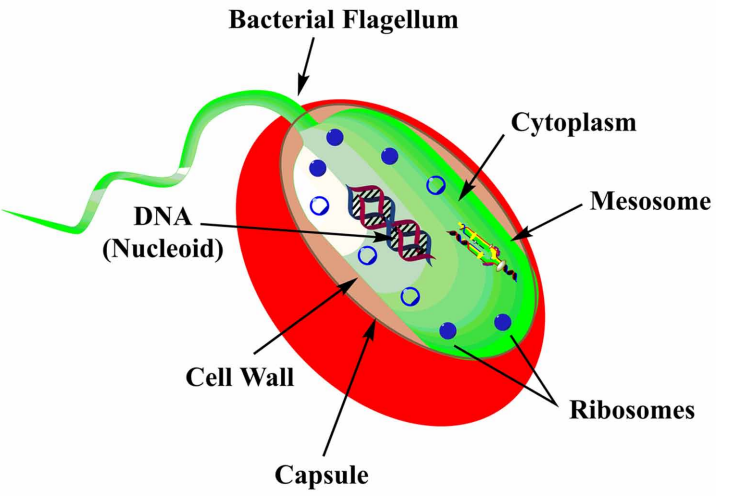
For the most effective treatment of TB, an early diagnosis becomes a pre-requisite. In patients with active TB, the infection of *M. tuberculosis* is high in the samples which can be directly analyzed for its presence. Any diagnostic method which can give the result on the same day and make a diagnosis at the point where patient consultation and presentation occurs is said to be a point-of-care (POC) diagnosis (Gondil and Chhibber 2018, Doctor, Mehra et al. 2019). The WHO, and Stop TB Partnership are emphasizing on developing a POC for the diagnosis of TB. So far, there are certain diagnostic methods available that can be translated as POC diagnosis of TB and they are represented in Figure 1.

2.1. Traditional Diagnosis of TB from Direct Specimens

Pulmonary TB, which accounts for approximately 85% of TB burden, is mainly diagnosed by expectorated sputum sample. Sputum smear microscopy has been the primary method for the diagnosis of pulmonary tuberculosis in low and middle-income countries . The smear of sputum is stained to identify acid-fast bacilli which further confirm the presence of Mycobacteria in a given clinical specimen. For direct microscopy, the Ziehl-Neelsen method is commonly used in low-income countries because of its low cost, and high specificity. However, this method has low sensitivity (50-60%) and cannot detect extra pulmonary TB cases. Another similar smear staining method is fluorescent staining with auramine O stain. This method is also rapid, specific, and has an enhanced sensitivity than the Ziehl-Neelsen method (by ~10% better). However, the usage of this assay is limited owing to its cost affectivity as it requires the employment of a sophisticated laboratory infrastructure

(*viz.* fluorescent microscope, and a darkroom) which adds a toll on the existing cost (Steingart, Henry et al. 2006).

Figure 2. Schematic representation showing varied POC diagnostic methods for TB detection



2.2. Advancements in the Diagnosis of TB from Direct Specimens: NAAT

NAAT is one of the most accurate known methods of detecting TB in direct samples and is an excellent choice for point-of-care diagnosis of TB. This method allows rapid detection of bacillus DNA (even in minuscule amounts) using polymerase chain reaction. Amidst all the NAAT available to date, Amplified Mycobacterium direct test (AMTD) (Hologic, San Diego, CA) was the first nucleic acid-based amplification test (NAAT) to be cleared by the FDA for the detection, and identification of *M. tuberculosis* from direct specimens. This assay utilizes transcription-mediated amplification of a portion of the 16S rRNA gene (specific to the *M. tuberculosis* complex) for the rapid detection and identification of TB bacilli. The sensitivity and specificity of this test are found to be 58% and 96%, respectively (Dunn, Starke et al. 2016). Apart from AMTD, various other NAAT has so far clear FDA standards and are being commercialized for laboratory testing.

Some commercially available NAAT kits include: (i) Amplicor MTB, Cobas Amplicor, and Light Cycler Mycobacterium Detection kits (Roche, San Diego, CA, USA), Amplified *M. tuberculosis* Direct Test (Genprobe, San Diego, CA, USA),

BDProbeTec-ET (Becton Dickinson Diagnostic Systems, Sparks, MD, USA); (ii) Genotype MTBDR*plus* and Genotype MTBDR*sl*(Hain Lifescience, Nehren, Germany), and INNO LiPARif.TB (Innogenetics, Ghent, Belgium) line probe assays; (iii) Capilia TB-Neo (Taunus, Numazu, Japan) rapid detection and speciation assay; and (iv) Xpert MTB/RIF assay(Cepheid), TruNatMicro RT PCR device.

Amidst the tally of all the commercially NAAT kits, Xpert MTB/RIF (rifampicin) (Cepheid, Sunnyvale, CA), is the most recent one which has been approved by Food and Drug Administration (FDA). It has also been recommended as the initial diagnostic test for children suspected of having multidrug-resistant TB (MDR-TB) or HIV-associated TB in the 2013 WHO policy update (Strategic 2013). This is fully automated *M. tuberculosis* NAAT. The GeneXpert platform is a self-contained cartridge-based system that utilizes microfluidics dynamics for an automated nucleic acid extraction, amplification, detection, and identification of *M. tuberculosis* directly from clinical samples (fresh sputum samples) within two hours. The Xpert MTB/RIF assay uses specific genes to detect *M. tuberculosis* sequences amplified in a real-time PCR assay. The assay is FDA cleared for use on smear-positive and smear-negative respiratory specimens, however, a specific clearance for extra pulmonary specimens is yet to be availed. Apart from *M. tuberculosis* detection, the Xpert MTB/RIF assay can also perform drug susceptibility testing (DST) for RIF resistance. It can detect defined mutations within the core region of the RNA polymerase b (rpoB) gene, and predict RIF resistance of MDR-TB (Theron, Peter et al. 2011).

Xpert MTB/RIF is suggested to hold considerable promise in the diagnosis of TB. This can be attributed to its innate attributes *viz.* accuracy, non-requirement of a dedicated testing facility (the procedure does not generate any aerosolized viable bacilli thus it can be performed in the absence of a biosafety cabinet) (Banada, Sivasubramani et al. 2010, Dheda, Ruhwald et al. 2013). However, Xpert MTB/RIF is more costly than smear microscopy, and its negative predictive value is significantly diminished in HIV-infected patients. Therefore, it is suggested to be useful in combination with other diagnostic tests. Recent work has shown that performing Xpert MTB/RIF only in smear-negative individuals can substantially reduce the overall cost of diagnosis (Vassall, van Kampen et al. 2011).

2.3. Advancements in the Diagnosis of TB from Direct Specimens: Serological detection

The tests, which monitor the humoral antibody immune responses to antigens of *M. tuberculosis* in serum/blood sample of patients is known as serological tests. Most of the bacteria or viruses-borne diseases are currently diagnosed *via* the detection of antibodies or antigens in the blood, but diagnosing TB serologically

remains a challenge. The most commonly used serological tests are rapid immune-chromatographic assays (ICAs) or enzyme-linked immunosorbent assay (ELISA).

Serological tests of TB are based on either detection of antibodies or detection of antigen in serum/blood. For antibody detection, the immune response to specific antigens of *M. tuberculosis* (such as Mpt64, the 6-kDa early secreted antigenic target (Esat6), the 10-kDa culture filtrate protein (Cfp10), and the antigen 85 complex (Ag85)) (Ngeow, Wong et al. 2011) are identified in serum. The immuno-chromatography assays (ICAs) such as lateral flow assay or ELISA-based methods are coated with these antigens, and their corresponding antibodies produced in TB patients are detected.

Mpt64 is a secreted, immunogenic protein that is highly specific for the *M. tuberculosis* complex including *M. tuberculosis*, *M. africanum,* and virulent *M. Bovis.* Most importantly, it is not found in Mycobacteria other than tuberculosis (MOTT) (García, Restrepo et al. 2010). The Ag85 complex of proteins are secreted and can be found in the external layer of the bacterium's cell wall, and the blood (Ronning, Klabunde et al. 2000, Anderson, Harth et al. 2001). In blood, the Ag85 complex binds to plasma fibronectin or immunoglobulin G, and thus alters the host's immune response. This interaction appears to reduce phagocytosis of *M. tuberculosis,* thereby promoting infection (Bentley-Hibbert, Quan et al. 1999, Ronning, Vissa et al. 2004). Cfp10 and Esat6 are early secreted proteins with molecular weights of 10- and 6-kDa, respectively. In culture, they are some of the most abundant *M. tuberculosis* antigens. They are not found in *M. Bovis* BCG or most MOTT (Nguyen, Ma et al. 2012). Since one-third of all TB patients are seronegative for any single antigen mentioned above, therefore combinations of all above proteins, either separately or fused, have been used for diagnosis of TB (Raja, Ranganathan et al. 2008). Commercially available ICAs detect IgG and IgM antibodies against a combination of the above-mentioned TB antigens.

At present, detecting antibodies in blood for TB diagnosis doesn't provide enough sensitivity, and specificity to be used as a first-line screening tool. This can be attributed to the fact that these tests lead to false-positive results since antibodies against environmental Mycobacteria are often present in the general population. Therefore major challenge in the use of serology is the ability to distinguish between infection by *M. tuberculosis*, and infection by Mycobacteria other than tuberculosis. Moreover, serological methods cannot reliably distinguish between active, and latent TB.

Moreover, in 2011, WHO banned the use of serological tests for the detection of antibodies against *M. tuberculosis* infection in TB patients. The experts of WHO analyzed published data indicating specificity, sensitivity, and deciphered that numerous false-positive results were given by serological tests, which ultimately compromised the validity of these assays. Whilst, many scientists criticized this ban,

and suggested that serological diagnosis is a very useful tool that is inexpensive, rapid, and an easy approach to complement diagnosis, and prognosis of *M. tuberculosis* infection (Dowdy, Steingart et al. 2011, Maes 2016).

On the brighter side, antigen detection is still a very useful tool for the diagnosis of TB patients. LAM (lipoarabinomannan) is a 17.3-kDa immunogenic glycolipid component of the mycobacterial cell wall. LAM is released from metabolically active, and degrading mycobacteria. The previous study shows that LAM in urine and pulmonary specimen showed a sensitivity of 93% and a specificity of 95%, respectively (Shah, Martinson et al. 2010). It has been the most extensively studied antigen and offers potential clinical utility in HIV-infected patients with advanced immunosuppression in both inpatient and outpatient (antiretroviral clinic) settings (Dheda, Davids et al. 2010, Minion, Leung et al. 2011).

TB LAM ELISA by Alere had an overall sensitivity of ~50%, which increased to 67% and 85% in HIV-infected patients with CD4 count <50 cells/mL from outpatient and inpatient settings, respectively. The aforementioned assay also depicted an overall specificity of 83–100% (Dheda, Davids et al. 2010, Dheda, Ruhwald et al. 2013). However, apart from LAM, other known antigens could not deliver any promising diagnostic test. So far, TB antigen test sensitivity was as low as 2%, and specificity was suboptimal. Most of the ICAs manufacturing companies detecting antigens of *M. tuberculosis* now use their tests for identification of *M. tuberculosis* in condensation fluid of the solid and/or liquid culture media incubated with patient samples.

2.4 Molecular Techniques for TB Detection

When it comes to the detection of TB using molecular techniques, a majority of these WHO endorsed methods rely upon the detection of bacilli (dead and alive) using Deoxyribose nucleic acid (Oommen and Banaji 2017). These methods have proven to be a souvenir and have inherently revolutionized the field of diagnostic sciences. The reduced cost, sensitivity, specificity are some of the advantageous aspects which have placed these diagnostic methods par apart from the traditional methods *viz.* culture and phenotypic DST (Oommen and Banaji 2017). Diminished contamination is another vital attribute which is offered by these diagnostic methods. Unlike their predecessors, NAATs these methods don't involve the amplification of target DNA, hence probable chances of contamination from amplicons, and inhibitors are circumvented (Parsons, Somoskövi et al. 2011, Oommen and Banaji 2017) thereby resulting in enhanced and accurate detection of TB. Lately, three molecular methods have been devised for TB detection, and they are as follows;

Cartridge Based NAAT (CB-NAAT)

CB-NAAT is a real-time PCR-based nested method that was endorsed by WHO in 2010. This test, in particular, aids in the semi-quantitative detection of MTB, and RIF resistant TB bacilli from clinical specimens (Oommen and Banaji 2017). Amidst, inception, the aforementioned method has been largely employed for the detection of pulmonary and extra pulmonary TB, pediatric TB, and HIV patients with TV. With the advent of time, CB-NAAT has been further utilized for the presumptive diagnosis of drug-resistant-TB (DR-TB) as well.

The method is highly versatile and steady, as it works within an analytical limit of 131 CU/mL within a stipulated period of 2-3 h. Additionally, the corresponding method portrays a respectively high sensitivity of < 99%. All this can be attributed to the self-contained attribute of the cartridges, which play an intricate role in eluding the chances of cross-contamination amidst the given samples(Oommen and Banaji 2017). When it comes to the standard operating protocol, the working comprises of seven stages;

1st stage: Liquefaction of sample (sputum).
2nd stage: Inactivation of the liquefied sample using a sample reagent (killing, and immobilization of TB bacilli).
3rd stage: Transferring of inactivated sample to the cartridge.
4th stage: Insertion of the cartridge into the MTB-RIF test platform.
5th stage: Capturing, followed by washing and lysis of TB bacilli using ultrasonic waves.
6th stage: Amplification of obtained DNA (*rpoB* gene 81 bp) using specific primers, and molecular probes.
7th stage: Detection.

Line Probe Assay (LPA)

For decades, LPA has been endorsed by WHO as a "*gold"* standard method for the diagnosis of TB. This can be attributed to its ability to detect MDR-TB against first-line anti-TB drugs (*viz.* isoniazid (INH), and RIF) (World Health Organization the UNICEF/UNDP/World Bank/WHO Special Programme for Research Training in Tropical Diseases . World Health Organization 2008, MacLean, Kohli et al. 2020). These assays are capable of detecting TB bacilli possessing MDR against fluoroquinolones (FLQs), and second line anti-TB drugs (*viz.* kanamycin, amikacin, and capreomycin) (Diagnostics , Tortoli, Cichero et al. 1999, Tortoli, Benedetti et al. 2002, QING, QIN et al. 2004, Richter, Rüsch-Gerdes et al. 2009, Barnard, Warren

et al. 2012, Organization 2017, MacLean, Kohli et al. 2020). Speaking on a broader term, LPA is basically a hybridization strip assay which allows for a qualitative detection of both the TB DNA, and genetically mutated MDR-TB bacilli in a given sample specimen (Oommen and Banaji 2017). In addition to this, differentiation of variegated type of *Mycobacterium* species is another advantageous perk which is offered by LPA (Oommen and Banaji 2017). Amidst all these, Genotype MTBDRplus (Hain Lifesciences-Bruker, Nehren, Germany), Genotype MTBDRsI version 2.0 (Hain Lifesciences-Bruker, Nehren, Germany), and Nipro NTM+MDRTB II (Osaka, Japan), InnoLiPA assay-Innogenetics are some of the LPA which have been devised on a commercial level (Organization 2016, Maningi, Malinga et al. 2017, Oommen and Banaji 2017, MacLean, Kohli et al. 2020).

A typical LPA assay strip comprises of 27 reaction bands also commonly referred to as reaction zones. Distinctive from these there are six controls and twenty probes. Unlike its predecessor, CB-NAAT, the corresponding assay offers numerous *viz*. reduced time of operation (72 h), enhanced sensitivity (97.9 - 99.7% (RIF resistance)), and specificity, (94.2 – 99.7% (RIF resistance)), respectively (Oommen and Banaji 2017).

Loop-Mediated Isothermal Amplification (LAMP)

On a distinctive note, molecular amplification methods have proven their worth when it comes to the detection of TB. Whilst, owing to factors such as high cost, and complex working protocols the full-scale potential of these expedient techniques is yet to be explored (Oommen and Banaji 2017, Shete, Farr et al. 2019, Phetsuksiri, Rudeeaneksin et al. 2020, Sreedeep, Sethi et al. 2020). In this context, a potential breakthrough was achieved by Eiken Chemical Co., Ltd, Japan (Iwamoto, Sonobe et al. 2003). They devised a highly cost-effective, swift (detection time ((35 (solid medium) - 60 (liquid medium)) and sensitive nucleic acid amplification (5-50 copies) based TB-LAMP assay (MacLean, Kohli et al. 2020).

Speaking in general terms, LAMP is an isothermal PCR amplification-based diagnostic method that aids in the visual identification of TB bacilli. This assay bestows a cutting edge over the traditional diagnostic methodology as it can be performed in a peripheral health care setting, and the requirement for a sophisticated laboratory setup (*viz*. thermocycler, detection system) is circumvented (Organization 2016, Kim, Cho et al. 2018, MacLean, Kohli et al. 2020, Toonkomdang, Phinyo et al. 2020). In addition to this, enhanced diagnostic performance is another technical benefit which is offered by LAMP-based TB assay. Keeping in mind, the variegated advantages presented by the aforementioned assay WHO endorsed this method since 2016 (MacLean, Kohli et al. 2020).

In this method, TB bacillus DNA is amplified using four different types of primers. These primers have an innate tendency to recognize six characteristic regions (*viz. gyrB*, and 16S rRNA) on the target gene. These distinct regions are targeted by carrying out an auto-cycling strand displacement reaction at a constant temperature (65 ^{o}C). Further, an incessant stride has been made in this field which has significantly resulted in the development of a single-step reaction for the amplification and detection of the target genes. Herein, the sample specimen, along with DNA polymerase, strand displacement activity, and substrates are incubated altogether and the reaction process is carried out at a constant temperature. The aforementioned process offers enhanced amplification of target genes (109-1010 times) within a short period (15-60 min) (MacLean, Kohli et al. 2020).

Apart from the aforementioned assays, recently several incessant strides have been made in the field of diagnostic methods. This has ultimately resulted in the innovation of novel diagnostic methods. Some of these methods have been depicted in Table 1, and 2, respectively.

3. CULTURING MYCOBACTERIUM FROM SPECIMEN FOR DIAGNOSIS

Despite many available POC diagnosis methods of TB detection, culturing bacilli, and then identifying it is a suboptimal "*gold standard*". However, a grave predicament is possessed in many TB patients, where an adequate amount of biological specimen is not readily available. Most importantly the sample specimen has a very low concentration of *M. tuberculosis* bacteria, which gives smear-negative results in microscopy. Therefore, WHO has recommended culturing of the specimen in media, which are specific for *M. tuberculosis* growth. This in turn will result in a proper diagnosis of TB disease.

Traditionally, *M. tuberculosis* culture is done on an egg-based solid media, known as Lowenstein-Jensen (LJ) medium or a liquid medium known as Middlebrook 7H9. On a comparative note, Middlebrook 7H9 liquid medium is more commonly used as the bacilli isolated from it is more as compared to LJ media (Pfyffer 2015). The growth of *M. tuberculosis* is very slow, and it may take up to as long as 4–6 weeks to actually visualize bacterial colonies. Nowadays, the traditional culturing methods are replaced by automated detection systems with liquid media such as BACTEC TB-460 (Becton Dickinson, Sparks, MD), BACTEC MGIT 960, or Bact/Allert 3D (bioMerieux, Durham, NC)]. These automated culturing methods reduce the duration for the detection of *M. tuberculosis* by about 1–2 weeks.

3.1. M. Tuberculosis Culturing in Automated Detection System

Since *M. tuberculosis* is slow-growing bacilli, therefore systems in which its growth can be sensed early were developed. The salient feature of these systems is the selection of *M. tuberculosis* from the specimen by its selective media, and then quantifying the rate of its growth based on the consumption of the media ingredients. BACTEC 460 is one such system that was the first of its kind (Kirihara, Hillier et al. 1985). It is a radiometric system for TB identification. In this system, *M. tuberculosis* is inoculated in a Middlebrook 7 H12 broth with carbon 14 (^{14}C) labeled palmitic acid.

The ^{14}C labeled palmitic acid acts as a substrate for *M. tuberculosis* to feed upon. The system estimates $^{14}CO_2$ liberated during the decarboxylation of ^{14}C labeled substrates in the growth medium (Lakshmi, Patil et al. 2006). Although this method is still used in various laboratories, still this system has several drawbacks. The most important drawback of this methodology is the use of radioactive materials in this system. This system requires a labor-intensive workflow and has a potential risk of cross-contamination (Tortoli, Cichero et al. 1999). To overcome the drawbacks of the BACTEC 460 system Becton Dickinson has developed the BACTEC MGIT 960 system. Mycobacteria Growth Indicator Tube (MGIT™), is a non-radiometric method of testing *M. tuberculosis* (Kanchana, Cheke et al. 2000).

It consists of a liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose, and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for the growth of many Mycobacteria, especially those belonging to the *M. tuberculosis* complex. The addition of PANTA is necessary to suppress contamination of other bacterias. The MGIT tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube.

During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With the depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. The growth of bacteria, as well as Mycobacteria, increases the fluorescence (Hanna, Ebrahimzadeh et al. 1999, Somoskövi, Ködmön et al. 2000). Currently, the WHO and the Strategic and Technical Advisory Group on TB recommends phased implementation of these automated liquid culture systems where feasible including low-income countries .

3.2. Differentiation of Mycobacterium

Once the culture has confirmed the presence of *M. tuberculosis* in the specimen, it is verified for the type of Mycobacterium. Classically, these differentiations are done by biochemical tests, which were not very accurate, and were time-consuming (Neonakis, Gitti et al. 2008). At present, various other molecular methods are used for identifying the type of Mycobacterium. PCR-based sequencing is considered the "*gold*" standard for the identification of Mycobacterium. In this method, Mycobacterium specific gene is amplified in an automated thermocycler *via* polymerase chain-based method. There are currently several specific genes of Mycobacterium known to date, however, the most commonly used is the 16S rRNA gene (Kox, Van Leeuwen et al. 1995, Kirschner, Rosenau et al. 1996).

Similarly, DNA probe technology also helps in differentiating variegated clinically important strains of Mycobacterium species *viz. M. tuberculosis complex, M. avium complex, M. avium, M. kansasii,* and *M. gordonae*. The DNA probes are single-stranded DNA oligonucleotides that are labeled with acridinium ester. These probes are complementary to the rRNA of specific Mycobacterium. In this method, the sample specimen is initially sonicated, and later on, the probes are added to the broken Mycobacterial cells, to form a stable DNA-RNA complex. This hybridization is then detected by light emission in a luminometer (Badak, Goksel et al. 1999, Tortoli, Cichero et al. 1999).

Moreover, there are many other molecular techniques available like PCR that can be used for the identification of Mycobacterium from culture. One such method is restriction enzyme analysis. In this method, a 65 kDa heat shock protein is initially amplified, and then it is subsequently cleaved by restriction enzymes. Further, the analysis is carried out on an agarose gel (Telenti, Imboden et al. 1993, Telenti, Marchesi et al. 1993). Following similar footsteps, another novel method by the name of pyrosequencing was innovated. The corresponding method derives its base from the phenomenon of nucleic acid sequencing-by-synthesis. In this method, the pyrophosphate (PPi) released during DNA synthesis is chiefly detected which further gives an affirmation about the target organism (Tuohy, Hall et al. 2005).

4. DRUG SUSCEPTIBILITY TESTING (DST)

Mycobacterium is evolving rapidly and has developed drug resistance. At present, the biggest challenge of TB management is multidrug resistance (MDR) and extensively drug-resistant (XDR) Mycobacterium species. Therefore, after isolation of bacilli in culture for definitive diagnosis, DST determination also becomes a prerequisite.

Moreover, culturing Mycobacterium is also vital, since it helps in developing a plan of action for drug administration.

Lately, two different approaches (*viz.* phenotypic and genotypic methods) have been frequently employed for determining the drug susceptibility for *M. tuberculosis*. Phenotypic methods assess the inhibition of *M. tuberculosis* growth in the presence of antibiotics and define resistance based on the response of the organism when exposed to the drug. Whilst, genotypic methods, on the other hand, are based on the detection of genes or mutations known to be associated with resistance.

Conventional DST utilizes phenotypic methods and depends on a variety of factors. The first of which is the definitive microbiologic diagnosis of *M. tuberculosis* with the isolation of the organism. The classical phenotypic method of DST is done by measuring the growth rate of *M. tuberculosis* in a culture media (LJ or Middlebrook 7 H12 broth) with antibiotics added to it. In phenotypic methods such as commercial liquid culture DST, generally, the WHO-approved radiometric BACTEC 460 TB system and BACTEC MGIT 960 system are extensively utilized (Wallis, Pai et al. 2010). Apart from these, microscopic observation drug susceptibility, colorimetric redox indicator, and nitrate reductase assay are some of the other methods which are also comprehensively used for phenotypic DST (Farnia, Masjedi et al. 2008, Coronel, Roper et al. 2010, Gupta, Singh et al. 2010).

MDR is generally defined as the resistance evoked in Mycobacterium in response to two vital first-line antibiotics *i.e.* RIF and isoniazid (INH). RIF, basically a rifamycin, acts by inhibiting the DNA-dependent RNA polymerase which further hampers the multiplication of Mycobacterium multiplication . RIF resistance is predominantly generated due to the mutations occurring in the RIF resistance determining region (RRDR) of the *rpoB* gene (Telenti, Imboden et al. 1993). Whilst, INH inhibits the pathways of mycolic acid synthesis by ceasing the activity of the Mycobacterial catalase-peroxidase enzyme (Timmins and Deretic 2006). Identification of these altered genes in the Mycobacterium can easily and rapidly identify drug resistance.

Coming onto the genotypic DST, NAAT assays are the invigorated tools of choice. Amidst all the NAAT, LPA is the kind of NAAT that simultaneously detects infection of Mycobacterium and amplifies regions of drug resistance for both RIF and INH, respectively. This system employs DNA strip technology, whereby amplified DNA is applied to strips containing probes specific for *M. tuberculosis* identification and RIF, INH resistance regions. The DNA in the specimen hybridizes with these probes and thus identifies the resistance (Nyendak, Lewinsohn et al. 2009).

An alternative to conventional liquid and solid media culture is the microscopic observation drug susceptibility (MODS) assay. This is a low-cost alternative to the detection of drug resistance. This system involves direct inoculation of a decontaminated sample into the wells of a tissue culture plate containing a liquid growth medium. Some wells include RIF and INH at critical concentrations,

allowing simultaneous detection of drug resistance. Growth is determined by visual inspection using an inverted microscope to detect the presence of Mycobacteria in cords. MODS detection of MDR TB gave a sensitivity and specificity of 95 and 100% respectively when compared with the MGIT 960 system. Moreover, the time for detection was found to be 7 days.

CONCLUSION

Despite the advancements and investments in the field of TB diagnosis, several shortcomings and obstacles are being faced by the scientific community taking into consideration the geographical aspects, cost-related constraints which critically hampers the hope of low-income nations but with a higher burden of TB, the sensitivity of assays and time consumption as well as delivery of results. A quantum leap is still needed in the world to provide an easily accessible-affordable, sensitive, and promising diagnosis. The current scenario demands the need to discover and flourish the novel diagnostic therapies in the otherwise perishable environment.

Table 1. WHO and CFDA endorsed test for TB diagnosis (MacLean, Kohli et al. 2020)

Test	Technique Based Upon	Sensitivity	Specificity	Diagnosis	Ref
Xpert MTB/RIF ultra	qPCR/melting temperature analysis	90-94	96-98	MTB/RIF resistance	(Organization 2020)
GenoType MTBDRsl	PCR/hybridization	86-87	~ 99	FLQ and SLID resistance	(Organization 2016)
MTBC assay	LAMP	~ 78	~ 98	MTB	(Organization 2016)
Truenat MTB plus	Micro RT-PCR	~ 80	~ 96	MTB	(Organization 2020)
Truenat MTB-RIF Dx	Micro RT-PCR	~ 84	~ 97	RIF resistance	(Organization 2020)
EasyNAT	Cross priming amplification	~ 87	~ 97	MTB	(Deng, Sun et al. 2019)
MeltPro TB	PCR/melting curve analysis	64-98	97-99	DST	(Sun, Gao et al. 2019)
GeneChip MDR	PCR/hybridization	79-89	97-98	MDR-TB/ INH and RIF resistance	(Sun, Gao et al. 2019)

RT-PCR: reverse transcriptase PCR; SLID: second-line injectable drugs; CFDA: China Food and Drug Administration.

Table 2. Diagnostic methods for detection of TB

Diagnostic Methods	Instrumentation/ Peripheral/ Staining	Principle/Method	Advantages	Load Required	Detection Limit	Place/ Firm of origin/ Innovator	Ref
Direct Detection (Microscopy)							
(i) Smear microscopy	Acid fast staining (carbol fuschin, fluorochrome dye (auramine, and rhodamine))		(a) Rapid (b) Inexpensive (c) Specific	10,000 bacilli/ ml	20-80%		(Behr, Warren et al. 1999, Mase, Ramsay et al. 2007, Organization 2008, Parsons, Somoskövi et al. 2011)
(ii) Fluorescent microscopy (LED)	Royal blue colored LED		a) Swift (b) Robust (c) User friendly (d) Sustainable (e) Cost effective (f) No hi-tech infrastructure/ instrumentation required (g) Sensitivity/ specificity				(Minion, Shenai et al. 2011, Organization 2011, Shenai, Minion et al. 2011, UNITAID 2012)
(iii) Sodium hypochlorite (Bleach) microscopy	Mathare sodium hypochlorite (MaSH) method		Detection of TB bacilli was increased by 15%			Medecins Sans Frontieres (Mathare, Nairobi)	(Organization 2008, Schramm, Hewison et al. 2012)
(iv) Vital fluorescent staining	Fluorescent viability marker (fluorescein diacetate (FDA))		Detection of live bacteria				(Al-Moamary, Black et al. 1999, Schramm, Hewison et al. 2012)
Automated microscopic technology coupled with CellScope (portable digital FM)		(a) Automated loading, and high-resolution digital imaging of stained slides (b) Swift analysis (200 slides can be loaded at the same time)				TBDx (Signature Mapping Medical Sciences, USA)	(Lewis, Chihota et al. 2012, Tapley, Switz et al. 2013, Oommen and Banaji 2017)
Indirect Detection							
(v) Tuberculin skin testing (Mantoux test)		Initially, the purified protein derivative (PPD) of MTB is administered intradermally in the forearm. Thereafter, the inference is made after 48-72 h of administration (Oommen and Banaji 2017).	Aids in the screening of infection (detection of potential exposure to MTB or latent MTB)(Oommen and Banaji 2017)			Charles Mantoux	(Reichman and Hershfield 2000, Nayak and Acharjya 2012, Oommen and Banaji 2017)

continues on following page

Table 2. Continued

Diagnostic Methods	Instrumentation/ Peripheral/ Staining	Principle/Method	Advantages	Load Required	Detection Limit	Place/ Firm of origin/ Innovator	Ref
Modified tuberculin skin test							
(a) PPD-S PPD (b) PPD-RT 23 (1-TU, 2-TU) (c)International Standard Tuberculins						(a) Siebert (MTB) (b) BCG Vaccine Laboratory, Guindy, Chennai (c) Laboratory of Biological Standards, Staten, Serum Institute, Copenhagen, Denmark	(Oommen and Banaji 2017)
(vi) Interferon-gamma release (*in vitro*) assay		On encountering TB bacilli antigen (ESAT-6, 10 (CFP-10), and TB 7.7) the T-cells sensitized MTB releases interferon gamma (TH 1 cytokine) which is detected and affirms the presence of TB.	(a) Enhanced sensitivity (b) Escalated specificity (recognize T-cells of TB patient) (c) Swift assay (result can be viewed within 24 h)				(Mazurek 2010)
(a) QuantiFeron-TB Gold (QFT-G), and QuantiFeron-TB Gold in Tube (QFT-GIT)						Cellestis, Australia	
(b) T-SPOT TB						Immunotec, UK	
(vii)Beta-Lactamase detection	LED filter and mobile phone camera.	Detects metabolic signature of MTB *viz.* BlaC (ref online bookmark).	(a) Enhanced specificity (b) Enhanced sensitivity (10 CFU (unprocessed sputum))			Global Biodiagnostics, Temple, TX, USA,	(Xie, Mire et al. 2012)

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Chapter 5
Extra-Pulmonary TB:
Changing Paradigm in Diagnosis

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ABSTRACT

Tuberculosis is considered a fatal respiratory disease commonly seen in developing countries. This chapter includes the global scenario of TB patients and brief description of TB history, its pathogenesis, types, diagnosis tests, emergence of MDR (multi drug resistance) and XDR (extensively drug resistance). The traditional chemotherapy of TB includes first and second line drug therapy. These lines of therapies face many difficulties such as low solubility, low bioavailability, and stability issues. Therefore, some new drugs were introduced in the market that showed effective results to the patients. Nanoparticulate drug delivery gained much focus in recent years due to its advantages and ideal characteristics. Numerous nanoparticles, liposomal formulations, and polymeric micelles were reported by the researchers with significant and considerable results. Inhalable formulations were also prepared by scientists that showed effective and remarkable anti-tuberculosis action on TB patients. Many efforts are awaited to completely eradicate TB from the planet.

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1. INTRODUCTION

1.1 Global Scenario

Tuberculosis (TB) is an infectious respiratory disease caused by the bacteria, *mycobacterium tuberculosis* (MTB). The slow-growing acid-fast bacilli, MTB infect lungs, spleen, brain, kidneys and other organs (Sosnik, Carcaboso, Glisoni, Moretton, & Chiappetta, 2010). TB is mostly detected in people of developing countries and increasing the mortality rate rapidly. TB exists as a dreadful disease and comes in the list of top 10 death causing diseases globally. Understanding the pathophysiology and immune response of TB is an immense challenge to the scientists and medical supervisors (Sandhu, 2011). This disease becomes a burden on public health with the high death toll rate only second to deadly after HIV infections. To combat this disease, it is obligatory to understand the pathogenesis of TB in the hope of better management and treatment. According to World Health Organization (WHO) 28 million cases of TB were reported in India, which was about one fourth of the world. Out of this enormous ratio, about 4,23,000 death occurred due to TB excluding HIV patients and 87,000 cases were of HIV patients. Multi drug resistant (MDR) TB patient's population includes 1,47,000 cases in India as per the global TB statistics data 2017. In 2016, 95% deaths occurred due to TB in middle- and lower-income countries such as India, Pakistan, China, Philippines, South Africa, Indonesia and Nigeria. About 10.4 million TB patients were detected and 7.1 million people were died due to TB. About 53 million lives have been saved through TB diagnosis and treatment since year (WHO TB report 2017). In case of drug resistant TB, more than 0.6 million resistant cases were examined and screened for rifampicin (RIF), the first line drug of TB treatment and 0.49 million cases were of MDR-TB in India, China and Russia. These alarming facts gained the attention of world towards TB treatment and eradication to achieve superior public health. Still, we lack approaches to TB therapy as only 54% TB cases were cured, and this cure rate is not satisfactory. Therefore, it is not wrong to recognize it as a disease without boundaries leaving powerful impact on Indian population, as highest TB patient count was reported in our country. WHO has documented the fact that skillful efforts, newer diagnosis tools and funding can completely abolish TB from our planet by 2035.

Recently, newer advancement in bacteria genomics and molecular determinants are carried out to understand the Immunopathophysiology of this disease. Despite these steps such as knowledge of immunology and pathophysiology and recent discoveries at molecular level, TB exists as a vast and challenging burden to developing countries. The sources gap and lack of funding are the the barriers to new inventions and diagnosis tests in this disease eradication pathway (Philips & Ernst, 2012). This book chapter focuses on TB, extra pulmonary tuberculosis (EPTB), knowledge of

causative agent, pathogenesis, TB chemotherapy and some recent advancement (in terms of treatment) done in tuberculosis treatment.

1.2 History

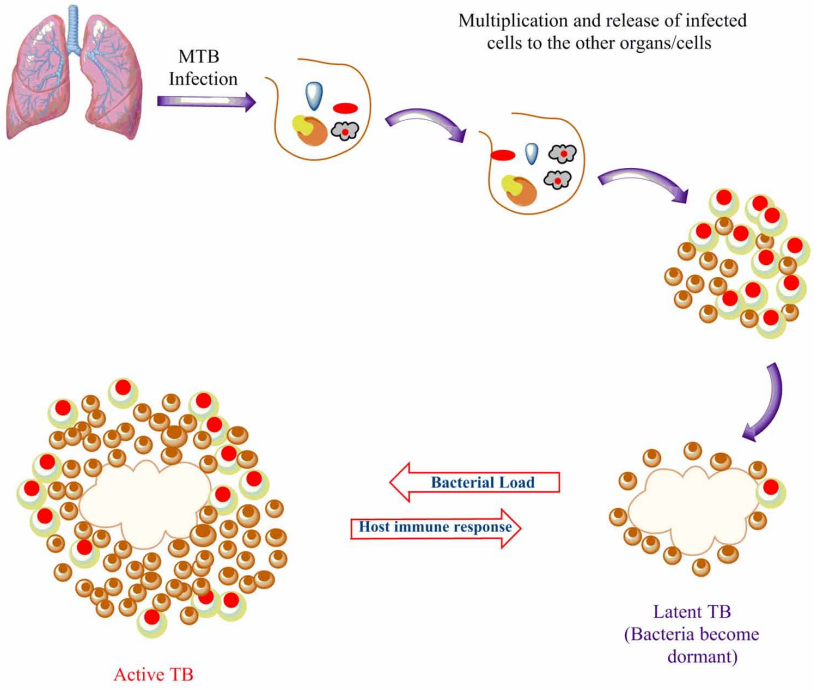
Historically, TB was called, as a "devils' disease" as the diagnosis, cure and treatment for TB was not available. TB was first noticed in the fossil of an extinct bison; (Pleistocene bison) and it was traced by radiocarbon activity as about ten thousand-year-old (Rothschild et al., 2001). In the Eastern Mediterranean part 9000 years ago, first time TB of human origin was identified in mummies of Egypt. In 1689, Dr. Richard Morton first stated that the TB is a disease with miscellaneous symptoms. Prior to 1800's the researchers thought that TB is a not a single disease and named as "tuberculosis" by J. L. Schonlein (Hershkovitz et al., 2008). In 1865, a French surgeon, Jean-Antoine Villemin officially said that TB is a communicable disease. In 1882, a German physiologist Robert Koch discovered the causative agent of TB "*mycobacterium tuberculosis"* and he was awarded the noble prize (physiology or medicine) of 1905 for this discovery (New medical net 2010). Edward Livingston Trudeau, was the first victim of TB who also, become its first survivor in the United States in 1884. An American bacteriologist "Theobald Smith" in 1896 demonstrated that *M. bovis* and some another species also caused *M. tuberculosis*. After 12 years, two scientists Albert Calmette and Camille Guerin both jointly isolated the various form of bovine and grew separately in a culture of ox bile. Later, tuberculin was discovered in 1890 and the Bacillus-Calmette Guerin (BCG) vaccine in 1908. This was an endeavor of 13 years culture laboratory experimentation to develop oral vaccine. ATB victim child was immunized, whose mother died during delivery of baby. Currently known as BCG, the intradermal vaccine is used commonly to combat TB. TB chemotherapy was started in 1960s.

1.3 Pathogenesis of TB

TB is a communicable and air borne disease and the pathogenesis of TB begin with the droplet infection through inhaled air. Other than *mycobacterium tuberculosis, M. bovis, M. africanum. And M. microtia* are also responsible for the TB as causative bacteria. However, these other type of bacteria's are responsible for non-human TB (Prasad et al., 2005). TB affects most of the human body organs, but lungs are the targeted organ for TB bacteria that leads to pulmonary TB. More than 80% of the cases are of pulmonary TB (Pandey & Khuller, 2005). The lungs are the primary and foremost portal for entry of *Mycobacterium tuberculosis* and are main site of disease manifestation, which further spreads to other body organs (Flynn & Chan, 2001).

M. tuberculosis is an intracellular pathogen that targets and inhibits the proficient antigen presenting cells (APC's), the alveolar macrophage (AM) and the dendritic cells during the early stages of infection (Wolf et al., 2007). Whenever, *Mycobacterium* reaches to the alveoli, it starts multiplication intracellularly in macrophage cells and spread through the lymph nodes, and then moves in the bloodstream to infect other organs (Hass 2000). *M. tuberculosis* continuously grows in the lungs for 2 to 12 weeks, and develops an immunocompetent host, cell-mediated immunity (CMI) and can be identified by tuberculin skin test and other diagnosis tests. The mechanism of TB pathogenesis along with its types is depicted in **Figure 1**.

Figure 1. Pathogenesis of TB and its type (active and latent)



1.4 Types of TB

When *M. tuberculosis* multiplies and invades rapidly leading to the condition of illness is known as active TB. Cough, phlegm deposition, chest pain, weakness, weight loss, fever, chills and sweating at night are the main symptoms of active TB. LTBI patients (Latent tuberculosis infection) have *M. tuberculosis* in their bodies

without TB disease condition, but they are unable to spread the TB infections to other people. About 5-10% of total LTBI cases are at risk to progressing from infection to active (primary) TB.

1.4.1 Pulmonary TB (PTB)

TB disease can affect many human body organs such as lungs, brain, uterus, bones etc. When TB bacteria infect mainly lungs, it is considered as pulmonary TB. For example, miliary TB is a type of PTB because the lesions are found predominately in the lungs.

1.4.2 Extra Pulmonary TB (EPTB)

If *M. tuberculosis* affects other organs such as larynx, lymph nodes, brain, pleura, bones, kidneys and joints then it is termed as extra pulmonary TB. TB in thoracic lymphadenitis or pleural effusion, without radiographic abnormalities in the lungs, constitutes a case of EPTB.

1) LYMPHADENTIS TB (LTB)

LTB frequently occurs in children in the peak age of 15-20 years and most commonly noticed in women of America. The disease condition is described with discrete and non-tender lymph nodes and a firm mass of matted nodes were observed that can be clearly seen during examination of patient. Sometimes, severe inflammation may leads to drainage impulsively from patient nodes with sinus tract formation. In anti-tubercular therapy, some new nodes formation may occur that is a sign of an immune response to kill the pathogen bacteria (Shafer, Kim, Weiss, & Quale, 1991).

2) PLEURAL TB

It mainly affects pleural fluid of lungs and characterized by pleuratic chest pain, cough, fever or dyspnea. It is the most frequently occurring TB form about 5% among the TB cases in the United states. In the chest radiography examination, more than 20% patients diagnosed with pulmonary lesions (Valdes et al., 1998). Pleural fluid of this kind of TB patients associated with lymphocyte predominance effusions within two weeks of diagnosis and initially same pattern can be observed in neutrophils.

3) SKELETAL TB

More than 35% of EPTB cases are related to bone and joint. Spine, weight-bearing joints and extra-spinal osteomyelitis are mainly involved in skeletal TB (Lifeso, Weaver, & Harder, 1985). Thoracic spine is the majorly affected part of spine. While in articular TB, arthritis of the hip or knee slowly progressed followed by pain, joint swelling, and reduced mobility. Systemic symptoms are absent usually but in severe cases, sinuses drainage and abscesses can be seen.

4) CENTRAL NERVOUS SYSTEM TB

Tuberculosis meningitis is the most common form of central nervous system TB. After that intracranial tuberculoma, and spinal tuberculous arachnoiditis are other commonly diagnosed forms of EPTB. Meningitis occurs by rupturing of subependymal tubercle into the subarachnoid space at high intensity. Symptoms such as unconsciousness, seizures, and high intracranial pressure are noticed in the patients. Even on the onset of TB manifestation, the patient suffers from headache, fever, or personality change, meningismus, vomiting, confusion, and focusing problems. Sometimes if not treated, mental status worsens and converted into stupor or coma (Kennedy & Fallon, 1979). A young adult is frequently affected by this TB in comparison to older (above 50 years of age).

5) ABDOMINAL TB

Abdominal TB may involve the gastrointestinal tract, peritoneum, mesenteric lymph nodes, or genital-urinary tract. Other organs such as liver, spleen, adrenal glands are the target sites for miliary TB. Swallowing of infected sputum, ingestion of contaminated food are the main reasons for initiation of this TB. Abdominal pain, diarrhea, weight loss, and fever are the symptoms noticed in the patient. Melena, rectal bleeding, and abdominal tenderness also can be present. Anti-TB chemotherapy is recommended for more than six months but in chronic conditions surgery may be advised.

1.5 Emergence of Multi Drug Resistant (MDR) and Extensively Drug Resistant (XDR)

The continuous spread of tuberculosis pathogens become a threat on the global public health. TB exists as a burden due to the failure of response by patients towards drugs.

Despite the immune response pathway understanding and conceptual knowledge, the resistant strain is a challenging task to treat and known as the main reason of MDR and XDR-TB form. TB chemotherapy includes first line drugs (INH, RIF etc.), second line drugs and some fluoroquinolones-based drugs. Patients who are drug susceptible towards chemotherapy are difficult to cure. TB patient who is resistance to INH and RIF, fall in the category of MDR-TB patient (Seung K.J. 2015). MDR-TB is mainly reported in Eastern Europe and Central Asian population and exists as a challenge for physicians to defeat. Early diagnosis is the only suitable way to identify TB patients.

XDR TB term was coined for those MDR TB patients who are resistant towards second line drugs and fluoroquinolones both. WHO have recommended some guidelines for MDR and XDR TB treatment that is included separately here. Both these TB forms are responsible for the increased mortality rate due to TB since last few years. Drug resistance is a biological phenomenon as the bacterial body changes can be observed through clinical and molecular approaches (Seung, Keshavjee, & Rich, 2015). Since the discoveries of RIF in 1947, drug resistance can be controlled in combinatorial approaches with other first line drugs such as INH, PYZ, some injectable drugs and fluoroquinolones. In 1993, DOTS (directly observed therapy, short course) was introduced that become a standard cure for TB even in the limited resources.

1.6 HIV and TB

HIV positive patients are more susceptible to TB infection as, compared to HIV negative person. It is a leading cause of death in HIV patients. According to WHO Indian TB, report more than one quarter of HIV patients with TB died since 2000 (Manosuthi, Chottanapand, Thongyen, Chaovavanich, & Sungkanuparph, 2006). The reason for this is the accelerated progression of HIV disease due to infected TB bacteria in patient body (Seung et al., 2015). HIV and TB exhibited a synergistic and dual direction interaction of infection inside body. The chance of MDR TB development from TB is higher due to co-infection of HIV and TB as the HIV infection favors the resistance TB strains. HIV-AIDS weakens the immune system of patient hence, enhances the probability of getting TB by 30%. This mainly examined with a higher rate in the age range from 15-60 years old. HIV infection drastically changes the nature of cell wall of *mycobacterium tuberculi* and, further the epidemiology that more worsen this disease. In Africa population, HIV positive patients with TB were reported in a large number than other countries in last decade. The diagnosis test sometimes fails to detect TB with HIV positive patients. The diagnosis test such as tuberculin skin, smear-sputum test, atypical X-ray gave negative results and therefore, it is difficult to detect TB. The longer time consumed in detection and

diagnosis can be a reason for resistant TB form (Fatkenheuer, Taelman, Lepage, Schwenk, & Wenzel, 1999). In India, National AIDS Control Organization and the Central TB Division, function as an organization and take some directions and actions for TB treatment.

1.7 Diagnosis of TB

Most people with TB have latent infection, the development of new diagnostic and screening tools and standards has become necessary in order to control the disease (Diel, Loddenkemper, & Nienhaus, 2012). Drug susceptibility testing (DST) is strongly recommended by WHO for previously treated patients at predetermine time intervals.

i. IMMUNOLOGICAL TESTS

Tuberculin skin test (TST) and IFN-γ releasing assay (IGRA) are widely used for diagnosing EPTB, but it has some limitations. IGRA diagnoses LTBI but it is costly, while TST is cost effective test (Thillai, Pollock, Pareek, & Lalvani, 2014). Both, TST and IGRA measures the response of T cells to TB antigens. The results of TST reactivity can be complicated by cross-reactivity with previous bacillus Calmette-Guerin vaccination or latent TB infection in countries where TB is prevalent. Various factors like HIV infection, poor nutritional status, recent viral or bacterial infections, or vaccination with live virus can reduce response to the TST.

ii. TUBERCULIN SKIN TEST (TST)

In this test, a tuberculin purified protein derivative (PPD) mix of proteins is injected into a person under the top layer of skin *via* intradermal route. It causes a type IV delayed hypersensitivity skin reaction to examine whether the individual was either previously exposed to the mycobacterial infection (Thillai et al., 2014). Size of the dermal reaction is measured to determine the TB infection. However, the TST is known to lead to false-positive responses in those who are BCG vaccinated and to false-negative responses in immunosuppressed individuals.

iii. INTERFERON-GAMMA RELEASE ASSAYS (IGRAS)

IGRAs is most reliable, sensitive, and specific TB diagnosis test (Diel et al., 2012) but costly and more technical (Goldman and Schafer 2011). In this test, the release cytokine IFN-g from T cells is detected from the collected blood sample of person and react with antigens that are absent in the BCG vaccine. As per the guidelines in some European countries, IGRAs and the TST are quietly used together to diagnose LTBI, while these tests are not definitive.

iv. T-SPOT TB TEST

This test is performed as described by the manufacturer included in the assay kit. About 10 mL of blood was withdrawn from the subject by veinpuncture and transported to the clinical testing laboratory. After 4 h of venipuncture, the peripheral blood mononuclear cells (PBMs) were isolated through centrifugation. PBMs were washed thrice with serum free culture media and cell number was determined. Then an adequate cell count was adjusted per mL of media, transferred into the wells of 96 well plates with monoclonal IFN-γ antibodies, and seeded in to each well. The positive and negative control is decided and rest will be treated as per designed experiment. The 96-well plate is incubated at 5% CO_2 and 37 ºC temperature was maintained. Washed the plates with phosphate-buffered saline (PBS)and reincubated with 50 μL of alkaline-phosphatase conjugated anti-IFN-γ monoclonal antibody. The results will be evaluated by the expert based on reaction (Meier, Eulenbruch, Wrighton-Smith, Enders, & Regnath, 2005).

v. SPUTUM AND SMEAR TEST

Diagnosis of TB should not be time consuming, as the bacteria grow rapidly. TB can be examined by sputum and smear test in clinical laboratories. This is one of the most frequently over looked test for diagnosis of *mycobacterium tuberculi*. TB suspicious person sputum and smear is collected and tested. The diagnosis yield of TB bacteria is evaluated by the experts (Conde et al., 2003).

vi. PLEURAL FLUID ASSAY

TB patient's pleural fluid level is found to be ≥5 g/dL (Light 2007). In majority of TB patients 50-90% small lymphocytes are present in their pleural fluid. In the

starting phase (less than 2 weeks), pleural fluid of some patient may have neutrophils. Glucose level in pleural fluid of TB patient may decrease sometimes but showed similar serum level. The pleural fluid pH in TB patient is ≥ 7.3 but it may be lower in some cases. Mesothelial cells are rarely observed in TB patients as most of them coated in the visceral and parietal pleura (Levine, Szanto, & Cugell, 1968).

2. CHEMOTHERAPY

After the unsatisfactory results of BCG vaccination, some therapeutics comes in the list of tuberculosis chemotherapy such as INH, RIF, PYZ, antibiotics and become the only hope for TB treatment. The goal of chemotherapy includes a high cure rate without relapse, to decrease mortality rate and to prevent drug resistance. Treatment of active TB should not be possible by a single drug that is why drugs are given in combination.

2.1 WHO Guidelines

WHO recommended TB chemotherapy should include first and second-line drugs. Drugs available for anti-TB therapy can be categorized into two types:

- First line anti-TB drugs: Rifampicin (RIF), Isoniazid (INH), Streptomycin (SM), Pyrazinamide (PZA), Ethambutol (EMB).
- Second line anti-TB drugs: Para Amino Salicylate (PAS), Kanamycin, Cycloserine (CS), Ethionamide (ETA), Amikacin, Capreomycin, Thiacetazone, Fluoroquinolones.

The daily doses are required in different phases of treatment. TB chemotherapy consists of two phases (intensive and continuous). In the intensive phase, INH, RMP, PYZ, and ETB are administered to the patients in different doses, while in the continuous phase, INH and RMP both are given to the patients in combination. INH initially is responsible for killing 95% of TB bacteria, and then, RMP and PYZ replace its role in the intensive phase. When either INH or RMP is not used, then chemotherapy duration is prolonged by 12–18 months. Hence, INH and RMP plays a pivotal role in the TB chemotherapy (Mitchison, 2000). The Most preferred therapeutic regimen for the treatment of pulmonary tuberculosis includes a combination therapy of three drugs, i.e., INH, RMP, and PYZ (Joshi, 2011) (WHO 2000).

However, it is suggested that the addition of Fluoroquinolones along with the appropriate dosage of first-line TB drugs, the total duration of drug-regimen in the case of EPTB may be cut down to 12 from 15 months after sputum-culture

negative confirmation. Certain conditions require special attention before the start of anti-TB therapy: liver and renal disorders, visual and auditory conditions, drug-allergy, and co-administered medications and more caution is also advised in case of administration of second-line anti-TB drugs. Other special considerations for EPTB include identifying best possible therapy regimen for patients with AIDS, particularly noting adverse effects of drug/drug interactions for those receiving anti-retroviral therapy (Iseman, 2000). The severity of the disease is determined by bacillary load, the anatomical site of infection and the extent of disease and resultantly, the appropriate therapy is planned.

TB chemotherapy dose regimen is divided further in to different categories. Category 1 includes four drugs ETB, INH, RIF, PYZ for 2 months and INH, RIF are given for 4 months in combination phase. Category 2 use streptomycin (ST) along with category 1 drugs for a longer period of 8 months and considered for relapse and retreatment cases (WHO Geneva 2008). In category 3, ETB is omitted for children, patients of smear-negative pulmonary or EPTB and for HIV-negative and fully drug-susceptible patients. Category 4, includes combination of second-line drugs and the initial phase five drugs PYZ, kanamycin (Km), ofloxacin (OFX), ethionamide (ETD) and cycloserine (CS) for 6-8 months and use of OFX, ETD and CS in continuous phase for 12 months to treat drug-resistant TB (Caminero, Sotgiu, Zumla, & Migliori, 2010). In case of XDR-TB treatment, capreomycin (CPM), moxifloxacin (MFX), para amino salicylic acid (PAS) +/- cycloserine (CS) are used in addition of two or three agents **(Table 1).**

2.2 Current Drugs used in TB treatment and their Major Issues

At present first line drugs, second line drugs, antibiotics, some injectable and fluoroquinolones are also used for TB therapy. The longer duration of chemotherapy is responsible for some major issues of this chemotherapy in patients. As in case of MDR-TB and XDR-TB patient needs higher amount of chemotherapeutics for treatment and it enhances the chances of toxicity incidences. Some major issues and side effects of these drugs are discussed in this paragraph. INH eradicates most of bacilli in first 2 weeks of TB and it has a great distribution in combination therapy with RIF in longer duration TB therapy. The continuous administration of these drugs in maximum dose as 450 mg for INH and 600 mg for RIF causes many toxic effects in patient's body (Keshavjee and Farmer 2012).

TB chemotherapy causes some major issues related to different organs. Some of them are headache, drowsiness, fatigue, ataxia, dizziness, inability to concentrate, mental confusion, visual disturbances, muscular weakness, pain in extremities and generalized numbness. In case of gastrointestinal disturbances, heartburn, epigastric distress, anorexia, nausea, vomiting, gas, cramps, and diarrhea are common.

Liver dysfunctions, thrombocytopenia, transient leukopenia, hemolytic anaemia, eosinophilia, and decreased hemoglobin can be seen. Thrombocytopenia occurs after administration of RIF and ETB combination for twice weekly.

Table 1. Chemotherapy of TB

Drugs used in TB treatment
Fist line drugs
Isoniazid (INH)
Rifampicin (RIF)
Ethambutol (ETB)
Pyrazinamide (PYZ)
Streptomycin (S)
Second line drugs
Kanamycin
Amikacin
Capreomycin
Ofloxacin
Ciprofloxacin
Levofloxacin
Capreofloxacin
Moxifloxacin
Gatifloxacin
Ethionamide
Prothionamide
Cycloserine
p-aminosalicylic acid
Terizidone
Other drugs
Clofazimine
Linezolid
Thioacetazone
Amoxycillin-clavulanate
Imipenem
Clarithromycin
Thioridazone

3. CHALLENGES IN TB CHEMOTHERAPY

The conventional anti-TB Therapy has some limitations:

- Patient non-compliance due to lengthy therapy duration
- Toxic adverse effects of anti-TB drugs to non-target organs

- Co-existing HIV infections make TB more difficult to treat
- Degradation of active drug molecules before reaching the target-site
- Lower intracellular permeability of drugs to unique structural aspect of mycobacterial cell-wall

Other causes for the failed chemotherapy could be poor water solubility of drug, inadequate bioavailability, unpredictable plasma drug-levels or high dependency upon food (Paramasivan & Venkataraman, 2004).

However, it has become clear recently that the development of new anti-TB drugs alone is not enough to ensure improvement in anti-TB chemotherapy (Gaspar et al., 2008; Kumar, Asthana, Dutta, & Jain, 2006).

Although it may be imperative to search for new drugs and new drug targets to treat TB infection of multi-drug resistant TB (MDRTB), it is also essential to utilize currently available drug-therapy to treat initial mycobacterial infection such that MDRTB does not occur and to overcome the inadequate and failed anti-TB chemotherapy (Pandit and Choudhary, 2006).

However, to minimize drug-induced toxicity and improve compliance of the patient towards the therapy regimen, widespread aggressive efforts are being made to develop drug delivery systems to either target the mycobacterial infection more efficaciously or lessen the drug dosing frequency to improve patient compliance for achieving the therapeutic goal.

3.1 RIF Solubility, Bioavailability Concern

RIF is a water insoluble drug with solubility profile of 0.014 mg/mL. RIF's bioavailability (BA) is the major concern to think for TB therapy. As the drug is required in fixed dose combination (FDC) but due to hydrophobic issues, it is hard to achieve the required BA for RIF.RIF is well absorbed following oral administration. Its absorption from the gut is almost complete but is impaired or delayed by food. About 10 µg/mL peak plasma level is reached within 3 h after a single oral dose of 600 mg and provides effective blood level for more than 8 h. The $t_{1/2}$ is 2 to 5 h, and renal insufficiency does not significantly raise the plasma levels. The particle size and crystalline form of the drug, manufacturing process and the excipients are the factors responsible for the variation of RIF bioavailability (Laing et al. 1999). RIF in combination with INH showed better results, as INH accelerates the degradation rate of RIF in the acidic pH. In presence of INH, RIF undergoes higher decomposition rate as compare to single drug administration (Shishoo, Shah, Rathod, Savale, & Vora, 2001). The longer duration if TB therapy leads to further worsening of patient non-compliance. Drug resistance is also become a major issue to treat as it is a natural tendency of bacterial body and natural phenomenon too. Nanotherapeutic

approach can deal with this and improve the TB chemotherapy via controlled or sustained drug release effect in short time. Therefore, it is an urgency to take some powerful steps to improve the chemotherapy problems for the sake of people life globally. The collaborative affords of scientists and public can resolve this problem in a systematic way and quickly too.

4. NEWER DRUGS INCLUDED IN TB TREATMENT

Unfortunately, the TB chemotherapeutics are still facing some solubility, bioavailability and multi-drug resistant issues. Therefore, the limited sources led clinicians to add some toxic old drugs (PAS, ETH and CS) to improve the patient health and survival rate. New TB drugs with their clinical phases are included in (**Table 2**).

Table 2. List of anti-TB drugs in clinical trials

Newer drugs for TB treatment	Clinical trials phase
Gatifloxacin, Moxifloxacin	III
Rifapentine	II
Bedaquiline (TMC207)	II
Oxazolidinones (linezolid and PNU-100480)	I
Substituted ethylenediamines (SQ 109)	I
Benzothiazinones and Dinitrobenzamides	Preclinical phase
Fluroquinolones	II
Nitroimidazoles (PA-824 and OPC-67683)	II

4.1 Clinical Development of Existing Drug: RIF

Several known drugs and their derivatives are improved, modified, identified, and added in the current TB regimen list. These include rifampicin and fluoroquinolones.

- **Rifapentine, (rifamycin, rifampin):** Rifampin is the most widely used drug for tuberculosis treatment. In clinical studies, it has been seen that higher

dose of rifampin showed higher bactericidal activity, so the high dose of rifampin is included in treatment of TB. Rifapentine, in short time produced higher bactericidal activity against *mycobacterium tuberculi* than rifampin and become an attractive chemotherapeutic for TB regimen. Combination of rifapentine along with INH, PYZ, ETB reduced the time duration of TB treatment according to the previous research studies (Rosenthal et al., 2006; Rosenthal et al., 2007).

- **Fluoroquinolones (FQR):** These are broad-spectrum antimicrobial agents (DNA gyrase inhibition) and have potency against TB bacteria in the *in vitro* and *in vivo* studies. FQR are enlisted in the second line drugs for MDR-TB regimen. The new FQRs methoxy fluoroquinolones, gatifloxacin and moxifloxacin have been shown great potential against *M. tuberculosis* than the previously used, OFX and ciprofloxacin (Gillespie & Kennedy, 1998; Moadebi, Harder, Fitzgerald, Elwood, & Marra, 2007). The newer compounds are in clinical phase trials III and may shorten the TB chemotherapy.

4.2 Newer Anti-TB Regimen

- **Delamanid (OPC-67683) and pretomanid:** These are nitroimidazoles derivatives of antibiotics and shown potential against mycolic acid synthesis inhibitions against drug resistant strains of *M. tuberculi*. Both drugs have shown higher bactericidal activity in case of pulmonary TB. Recently, undergoes in phase clinical trials (Matsumoto et al., 2006).
- **Bedaquiline (TMC207):** It is a diarylquinoline class of antibiotics. The mechanism of action is to target the proton pump of adenosine triphosphate (ATP) synthase, causing inadequate synthesis of ATP, which is required for bacterial metabolism. The minimal inhibitory concentration (MIC) of bedaquiline against *M. tuberculi* is very low, but it has shown more bactericidal activity than INH and RIF. Based on clinical trials (phase II) results, WHO and the US Centers for Disease Control and Prevention suggested that bedaquiline can be prescribed to the patient for2 weeks with 400 mg daily dose and for longer duration the dose should be 200 mg thrice a week for 4.2 months (Diacon et al., 2012).
- **PA-824:** It is anitroimidazole oxazine derivative of metronidazole and act by inhibiting the synthesis of ketomycolates, an essential component of the *mycobacterium tuberculi* cell wall and by providing nitric oxide during enzymatic reduction.
- **Substituted Ethylenediamines:** SQ109 is a 1,2-ethylenediamine ethamubutol. The MOA is to inhibit the cell wall formation by interfering with the function of enzyme named as trehalose monophosphate transferase.

It is free from cross-resistance with ETB. Recently, it is under safety and efficacy studies in humans (Grosset, Singer, & Bishai, 2012).

- **Oxazolidinones:** They act by enzymatic competitive inhibition and causing interference with the ribonucleic acid (RNA) and inhibit translation. Oxazolidinedione, linezolid are given for MDR-TB patients as optimized background observations (OBR). PNU-100480 (sutezolid) has been well tolerated at a dose of 1200 mg by TB patient. AZD-5847 (posizolid) is in phase II trials at 500 mg single or double dose daily, 800 mg twice daily and 1,200 mg in a single dose daily (Swindells 2012).

5. ROLE OF NANOMEDICINE IN EPTB CHEMOTHERAPY

In the last decades, many liposomes, NPs and PM based formulation were developed and investigated for TB treatment. The newer drug delivery therapeutics can be administered via intravenous (*iv*), oral, intradermal and inhalable routes to identify a suitable and effective regimen. These new approaches can reduce the duration therapy ultimately reduce the burden of TB. Currently in TB therapy, there has been extensive progressive efforts in nanotechnology to develop nano-based drug delivery systems to target the *M. tuberculosis* infection and improve therapeutic index resulting in the more positive outcome in the TB therapy (Gelperina, Kisich, Iseman, & Heifets, 2005; Huh & Kwon, 2011; Loudos, Kagadis, & Psimadas, 2011). The high surface-area to volume ratio and unique physicochemical properties have made NPs potential carrier in the novel delivery system.

5.1 Liposomal Drug Delivery

Liposomal systems are self-closed and spherical structures that are created by one or many concentric lipid bilayers as outer shell with an aqueous phase as inner core where the polar head groups interact with the aqueous phase and the fatty acids form the hydrophobic core of the bilayers that are shielded from the aqueous core. The diameter of liposomes may range from 20 nm to several hundreds of nanometers. However, the dual character of liposomes with both hydrophilic and lipophilic regions, their application has expanded from purely bio-membrane mimetic systems to pharmaceutical carriers for both hydrophobic and hydrophilic drugs.

Therefore, the application of liposomes as nano-based drug delivery started in the early 70s (Gregoriadis, 1993) and these liposomal systems became the first type of nanocarriers approved by the US FDA (1995) for application in therapy of Kaposi's sarcoma related with acquired immunodeficiency syndrome (Krown, Northfelt, Osoba, & Stewart, 2004).

Among various nano particles formulations available for design, liposomes have shown enormous potential in the intravenous delivery of therapeutic drugs because of being biodegradable, biocompatible, non-immunogenic in nature and capable of incorporating both hydrophilic and lipophilic drugs and discharging them in a controlled mode (Jia, Joly, & Omri, 2008). Generally, liposomes can be categorised as unilamellar or multilamellar. Unilamellar liposomes are further classified into small size (SUV, 50-100 nm) or large size (LUV, 100-250 nm). SUV and LUV have a single lipid bilayer with a large aqueous central core, apt for loading of hydrophilic drugs; whereas multilamellar liposomes (MLV), generally with a diameter of 1-5 mm, have several lipid bilayers with a limited aqueous space, appropriate for loading of hydrophobic drugs.

Biological properties of liposomes make them widely accepted delivery systems for administration of drug both locally as well as systemically. Some of them are listed below:

- Biocompatible and non-immunogenic
- Amphiphilic carrier of both hydrophilic as well as hydrophobic pharmaceuticals
- Unique opportunity to deliver drug into cell or even inside cellular components
- Their surface properties, size and charge can be easily altered as per specific need
- Reduction in undesirable side-effects to non-target organs when drug is incorporated into these carrier systems. Gaspar and group developed and characterized liposome-based preparations of rifabutin and delivered the formulations via *iv* route. Multilamellar micelles were prepared by a remote loading method using ammonium sulphate. The *in vivo* studies shown great distribution of antibiotic through liposome approach in the liver, spleen and lungs in pathopysiological results up to 24 h after administration as compare to free rifabutin. In the lungs, the concentration of bacteria was lower than free antibiotic. The constituents dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidylglycerol (DPPC:DPPG) were the most effective lipids used in this formulation and exhibited great potency for bactericidal activity (Gaspar et al., 2008). The liposome preparation has shown better bactericidal activity against *mycobacterium tuberculi*. The developed liposomes were effective against EPTB treatment as well. Kaul et al. 2016 developed ^{99m}Tc radiolabeled folate targeted PEGylated liposomes of rifampicin and ofloxacin as a theranostic against tuberculosis. PEGylation enhances the circulation time via surface modification approach and it allowed more interaction between cell receptors and targeted moiety to facilitate internalization. The *in vitro* release profile indicated that rifampicin release and ofloxacin released

28.33% and 30.09% respectively upto 6 h from the liposomal formulation. In contrast the pure rifampicin and ofloxacin released upto 84,2% and 96.1% respectively within 1 h (Kaul et al. 2016).

5.2 Nanoparticles (NPS) Based Drug Delivery

Various polymers such as PLGA, PEG, PLA has been utilized to develop nanoparticulate anti-TB formulations. Different respirable formulation was evaluated in the past for the better cure and treatment of this dreadful disease TB.

5.3 Inhalable or Respiratory Formulations as Nanocarriers

Hwang and colleagues prepared hyaluronan (HN) microspheres of OFX. They delivered the formulation to the lungs and alveolar macrophages via microsphere drug delivery via inhalation route. HN is the sodium salt of hyaluronic acid (HA) and preferred for its great mucoadhesive properties. Microspheres were prepared by co-spray drying method. The *in vitro* studies results revealed that the microspheres showed more AUC concentration in the lungs and plasma. The *in vitro* uptake of OFX from microsphere formulation in the cultured cells line RAW 264.7 was 2.1 and 1.7 folds more than the free OFX and without HN-OFX microsphere formulation (Hwang, Kim, Chung, & Shim, 2008).

5.4 Chitosan and Gelatin NPs as Nanocarriers

A research group formulated and characterized CS and gelatin (G) based PLA-PEG-NPs for controlled drug delivery of RIF for TB treatment. They prepared CS-PLA NPs loaded with RIF (CS-PLA-RIF). RIF release pattern from CS-PLA was more than the CS–PLA–PEG and CS–PLA–PEG–G (gelatin) NPs. In the initial phase, RIF released rapidly and then showed sustained release pattern for few hours. CS–PLA–PEG–G composite exhibited strong electrostatic interaction with drug than CS–PLA and CS–PLA–PEG composite. Conclusively, it was shown that the alkaline environment is more favorable for the release of drug from their respective composites, while acidic environment release the drug rapidly due to the ease of interaction breakage (Rajan & Raj, 2013). CS can be used in combination with PEG and G for controlled and targeted drug delivery system. Saraogi et al. prepared gelatin loaded NPs of RIF by desolvation method and evaluated them for anti-tubercular activity. The spherical shaped NPs were observed in TEM. AFM morphology analysis of NPs. The size of NPs were found to be 264±11.2 nm with a low PDI suggesting the narrow particle size distribution with homogeneity. The cytotoxicity study revealed that the gelatin NPs were safe and non-toxic. Bio-distribution studied gave an idea

about the localization of NPs in lungs and spleen of infected mice. In cultured cell lines studies, the bacterial count of gelatin loaded RIF NPs were less than the blank NPs. Therefore, from the prepared formulation results we can conclude that the NPs can reduce the dose frequency via controlled release effect and helpful in the better management of TB (Saraogi, Gupta, Gupta, Jain, & Agrawal, 2010).

5.5 Polymeric Micelles (Pm) Based Drug Delivery

Gupta and coworkers reported the polymeric micelles of N-2-hydroxypropylmethacrylamide (HPMA) and PLA recently for delivery of anti-tubercular drugs rifampicin (RIF) and isoniazid (INH). Firstly, HPMA was conjugated with a hydrophobic polymer (PLA) and the INH was conjugated as a result, a hydrazone linkage was formed between INH and PLA. The synthesized block conjugates was characterized by thin layer chromatography (TLC), NMR and FT-IR spectroscopy. The CMC value of polymers was determined by Iodine method and UV-Visible spectrophotometric analyzer took measurements of polymers at different concentrations. RIF was physically encapsulated and resultant micelles were prepared by the hydrophobic interaction. The size of PMCs was found to be approximately 87 nm that is sufficing to exclude the renal filtration and but small enough to protect the micelles from macrophages attack (phagocytosis). The anti-microbial/anti-bacterial assays (MABA) were performed to determine the anti-microbial activity. The prepared PMs exhibited a greater anti-bacterial activity than the pure drugs INH, RIF with less minimum inhibitory concentration (MIC). Hence, a combination therapy of both first line drugs was prepared successfully for better efficacy and treatment (Upadhyay et al., 2017). Tyloxapol is a non-ionic polymer consists of alkyl and aryl alcohol chains. It acts as a surfactant and aid to liquefy the mucus and remove the pulmonary secretions or mucus from the respiratory tract. Mehta and Jindal prepared a formulation of mixed micelles that may be use for treatment and better management of EPTB. Lecithin act as a hydrophobic core and tyloxapol is utilized as surfactant. Lecithin and tyloxapol both solubilize the anti-TB drug. The prepared mixed micelles were characterized by surface tension measurement and conductivity both. The interaction between the mixed micelles formulation and drug RIF was synergistic. The morphology analysis suggested that the micelles showed homogeneity with good particle size distribution. The *in vitro* release data revealed that the drug loaded mixed micelles formulation exhibited sustained drug release effect. Kinetic models result also supported the *in vitro* release (Mehta & Jindal, 2013). Recently, Rani and colleagues reported the dual drug delivery favor TB via conjugations of polymers and INH. The author first prepared the polymeric conjugates of PEG-PLA and activated the conjugates. Further, INH was conjugated with activated PEG-PLA conjugate via hydrazine linkage and PEG-PLA-INH

(PPI) conjugate was developed. PM of both PPI conjugate and blank PEG-PLA was prepared. RIF was loaded in prepared polymeric micelles and characterized via size analysis and morphology. The critical micelles concentration of PEG-PLA PM was found to be 8.9 ± 0.96 mg/L with size range of 187.9 ± 2.68 nm. The PM formulation showed less hemolytic toxicity than free RIF. Minimum inhibitory concentration (MIC) of polymeric micelles was 8 times less than the RIF. PPI conjugate's MIC was also less than the pure INH as shown in the MABA results. Microscopic images showed the action of prepared pm formulation on bacterial cell wall. The *in vitro* release studies showed controlled release of RIF from smartly engineered PM. Microplate Alamar blue assay (MABA) assay results revealed that the nano structured PM exhibited better anti-bacterial activity than INH and RIF. Therefore, the PM may be established for *in vivo* studies in future to achieve and confirm better anti-TB therapy (Rani et al., 2018).

6. FUTURE PROSPECTS

Increasing morality rates due to TB rift the different public health organization of developing countries to take some major steps to eradicate this disease. Extra pulmonary TB patient population is causing more harm to patients in different parts of body such as bone, joints, abdominal parts and brain etc. EPTB should be treated with more attention as the last two decades data become an alarming factor for this. Some new diagnostic test must be used at primary basis as sometimes the symptoms are absent in few TB population but after some days the bacterial load increases in the macrophages and become more virulent. WHO threatening data of co-infected TB and HIV patients is terrible. WHO and some health agencies, must join hands together to help these developing countries and make the world population free from TB. The newer treatment strategies with the use of nanotechnology tools can further boost the chemotherapeutic approaches for the betterment of RB as well as EPTB.

However, the development of newer multidrug carrier systems for TB therapy, still poses a great challenge to the global scientific community.

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Section 2
Countermeasures and Strategies for Remediation of AMR

This section comprises of chapters that cover the application of traditional as well as novel therapies in combating antibiotic-resistant bacterial strains. A chapter on computer-aided drug-designing strategies for COVID-19 has also been added.

Chapter 6
Traditional Medicine: Exploring Their Potential in Overcoming Multi-Drug Resistance

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ABSTRACT

Everybody is at risk of being infected by drug-resistant microscopic organisms. Managing with sickness has never been less demanding within the history of our species. At the current rate of antimicrobial resistance (AMR) in microbes, specialists foresee that battling infections tuberculosis, HIV, and intestinal sickness will become more complicated. Antimicrobial resistance is rendering numerous life-saving drugs useless. Antibiotic-resistant microbes, known as "superbugs," are getting to be more various and more harmful, thanks to the proceeding abuse of anti-microbials. Natural medication offers an alternative to these progressively ineffectual drugs. According to the World Health Organization (WHO), traditional medicine is a holistic term enclosing diverse health practices. Concurring to a report by the College of Maryland Therapeutic Center, turmeric's volatile oil serves as a common anti-microbial.

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INTRODUCTION

Multi drug resistance (MDR) is the oversensitivity or resistance of microorganisms such as (bacteria, viruses, fungi and parasites) to the antimicrobial drugs which results in ineffective treatment and spreading of infections. In bacteria it occurs due to the over expression of bacterial genes and accumulation of multiple genes in the bacterial cells which results in multidrug and single drug resistance simultaneously (Nikaido, 2009). MDR with increased morbidity and mortality due to the infecting agents such as bacteria, virus, fungi and parasite is rendered to as "super bugs". Worldwide multidrug resistance is a serious threat to public health in life threatening disease such as tuberculosis, pneumonia, HIV, malaria, yeast infections and other diseases. At whatever point we feel a small beneath the climate, we discover out what's off-base with us and take whatever is endorsed to create our side effects for all intents and purposes vanished. It appears nearly cursory until you think almost the infection-causing bacteria's capacity to adjust. The normal determination can be a perilous amusement. Within the case of disease-causing microscopic organisms, the last mentioned is an unfavorable result of our abuse of anti-microbials. Most major wellbeing specialists fear that drug-resistant microbes, or "superbugs," maybe our following worldwide wellbeing emergency. It's been utilized to treat all sorts of skin and respiratory diseases for thousands of a long time. Lab reports appeared that Guduchi diminished or killed E. coli and upper respiratory disease, concurring to the Indian Journal of Pharmacology 2003. A 2006 study by the College of Madras appeared Triphala restrained development of common bacterial segregates from HIV patients. Risorine having piperine as normal bio-enhancer is utilized in a settled measurements composition with Rifampicin and Isoniazid for the administration of tuberculosis. This diminished the dosage of rifampicin and moved forward its bioavailability. The multidrug resistance can be classified as essential resistance, auxiliary and clinical resistance. Essential resistance is when the medicate of intrigued doesn't stand up to by the specific have micro-organism (Tanwar et al, 2014; Vranakis et al, 2013). Auxiliary resistance happens for the most part after the introduction of the drugs to the living being and classified as: Natural resistance: When a single species of all micro-organisms appeared heartlessness to certain common to begin with line drugs. Broad Resistance: It is the capacity of living beings to stand up to the inhibitory impacts of one or two antimicrobial drugs which are most viable (Lee et al, 2013; Marks et al, 2014). Clinical Resistance: Restraint of contaminating living being by a concentration of an antimicrobial specialist due to helpful disappointment or return of contaminations inside an life form (Tanwar et al, 2014). Earlier studies have shown that for the pain coupled with boils Datura stramonium has been used as a medicinal plant, and also has been used in the treatment of gout, abscesses, rheumatism, and asthma. From the previous reports, it is clear that natural remedies can be considered

as a good alternative to the antibiotics which have shown microbial resistance. From the ancient time, Indian Rishi and Vaidhyas have shown to cure various dangerous diseases and further ayurvedic research is going on in many ayurvedic universities of India. Although the practice of herbal medicine in India is going to be increased, still it requires some therapeutic affectivity in terms of pharmacokinetics data and pharmacodynamic evaluation scientifically. The use of plant by a specific ethnic group in medicine is known as ethnobotanical medicine. Thus the medicinal plant can be defined as any plant having medicinal value or the components present in the plants are used as drugs. According to World Health Organization (WHO) traditional medicine is a holistic term enclosing diverse health practices, plant, animal and mineral based medicines knowledge and beliefs, manual techniques, spiritual therapies and exercises bid to maintain well-being either singularly or in combination and also to diagnose, treat and prevent illness" (Fabricant et al, 2001; Reid et al, 2018). Homegrown pharmaceutical or phytomedicine alludes to the utilisation of plants and herbs for the reason of remedy and moderation of human afflictions. Plants have been utilized for therapeutic purposes by people since long sometime recently recorded history. In spite of the fact that cutting edge pharmaceutical has taken over the lead from homegrown medications within the treatment of illnesses in people, the utilization of herbals has expanded in later a long time around the world, as they are accepted to be more secure than advanced solutions with few or no side effects. The potential of homegrown pharmaceutical in wellbeing care has been built up by different phytochemical and pharmacological studies. Homegrown drugs are commonly managed as an extricate of the total herb, as homegrown tea or new juice. Numerous times, the whole herb is expended either new or within the dried and powdered shape. The adequacy of a medicate can be significantly influenced by the strategy of sedate conveyance. The gradualness within the adequacy of a treatment proposes a growing requirement for a claim to fame approach to the conveyance of drugs to particular targets within the body. Hence a proficient sedate conveyance framework that can deliver an ideal sum of the drug to the location of activity must be created for homegrown pharmaceutical to form a fruitful offered within the treatment of different human afflictions. India is focusing on the idea of synergism for enhancing the safety and therapeutic efficacy of traditional medicines and the drugs used for the treatment of the disease which is also called 'samyoga' in Ayurveda. Synergism is termed as combining the two or more ingredients for achieving the maximum therapeutic efficacy and safety. 'Sarangdhar Samhita' the Ayurvedic literature highlighted the concept of poly-herbalism to achieve higher therapeutic efficacy. Tea tree oil has been used for combating the Mycobacterium tuberculosis infections for eradicating the methicillin-resistant Staphylococcus aureus bacterium from the wounds.

History of Traditional Medicine

There are various medicinal plants which have been used since ancient time for the treatment of skin disease, tuberculosis, mental disorders, cancer, AIDS and other infectious disease. Countries like India, China, South America and Egypt from ancient time are using herbal medicines as treatment remedies for the cure of such diseases ("HST", 2015). The bioactive constituents present in medicinal plants act as a defense system against pathogenic micro-organism. In India the traditional medicines are being practiced for years under (AYUSH) which is Ayurveda, Yoga, Unani, Siddha and Homeopathy (Mukherjee et al, 2017). For centuries, in India Ayurveda has been the mainstream healthcare system for the treatment of various diseases. For enhancing the drug efficacy and safety the traditional medicine of India focusing on the idea of synergism which is also called 'samyoga' in Ayurveda. Synergism is termed as combining the two or more ingredients for achieving the maximum therapeutic efficacy and safety. 'Sarangdhar Samhita' the Ayurvedic literature highlighted the concept of poly-herbalism to achieve higher therapeutic efficacy (Mukherjee et al, 2018). Traditional medicines were sole medicinal system in the African continent approximately 4000 years ago. This also remains dominant in the present with about 80% of the total estimated population of Africa using traditional medicine as a primary source for their health needs. There are ~27 million individuals who use traditional medicine as their health care need in South Africa. In case of plant biodiversity 250,000-500,000 plants species worldwide are found from which South Africa has been richest center comprising 368 families of plants having 24,000 specific and infra-specific taxa and 3000 plants are medicinally recognized. China has been using herbal medicine from last 5000 years for the treatment of diseases (Fair et al, 2014; Mukherjee et al, 2018).

Aloe ferox a South African plant used traditionally as laxative, for conjunctivitis, hypertension eczema and arthritis. Now the plant is commercially used as laxative. *Boophone disticha* also known as 'gifbol bushman poison bulb' or leshoma have used traditionally for septic wounds, postcircumcision wounds and as dressing agents for boils. After mild décoctions the plant provides relief from headaches, eye infections, and abdominal pain while after strong decoctions it is used as hallucinogens and sedatives (Reid et al, 2018). Earlier for the treatment of diabetes *Catharanthus roseus also termed* Madagascar periwinkle or isisushlungu was used, it consist two alkaloids which are in combination with chemotherapy available commercially for the treatment of cancer. Earlier studies shown that for the pain coupled with boils *Datura stramonium* has been used as a medicinal plant, and also has been used in the treatment of gout, abscesses, rheumatism and asthma. The alkaloids present within the plant are commercially available for the treatment of Parkinsonism, motion sickness and as an eye drop. *H. perforatum* was used as antidepressant and

as an antimicrobial agent which was evaluated pharmacologically. *Harpagophytum procumbens* termed as 'thornapple' was used traditionally for the treatment of rheumatism, arthritis and digestion problems. Traditionally *Cinnnamomum camphora* has been reported to be used for the treatment of heart disease, fever, cold, infectious disease, inflammatory disease and respiratory pneumonia. *Mandragora officinarum* known as Mandrake was used as surgical anesthetic in Greek medicine due to the presence of hyoscine alkaloid which steer the use of mandrake as a medicinal plant into traditional medicine practices (Petrovska, 2012).

Tea tree oil (5-10% v/v) has been used for combating the *Mycobacterium tuberculosis* infections before administration of anti-tubercular drugs (ethambutol, isoniazid, pyrazinamide, and rifampin) for eradicating the methicillin resistant staphylococcus aureus bacterium from the wounds (Sherry et al, 2004).

Current Trends in Herbal Medicine for Drug Resistance

Agreeing to World health organization (WHO), 60% of the world's populace depends on home grown pharmaceutical and 80% of the populace in creating nations depends nearly completely on it for their essential wellbeing care needs (Modak et al, 2007). There is 15% growth rate in the trade of medicinal plants and herbal drugs annually according to WHO report. For centuries, in India Ayurveda has been the mainstream healthcare system. From last two decades researchers have intensified their research towards herbal medicines to resist the multidrug resistance. The popularity and acceptability of herbal medicines is increasing due to their safety, easy availability and low cost.

The relentless emergence and increase in resistance of antimicrobial drugs to pathogenic bacteria raised the eyes of the researchers for newer drug formulations approaches to combat the multidrug resistance. Use of traditional medicine can be a valuable approach for combating the multidrug resistance (Mukherjee et al, 2017). Piperine confined from *P Piper longum L.* a normal bio-enhancer utilized in a settled dosage composition with Rifampicin and Isoniazid for the administration of tuberculosis. This combination is promoted beneath the exchange title Risorine which decreased the dosage of rifampicin and made strides its bioavailability (10,16). Piperine moreover has shown synergistic impacts on nimesulide-induced anti-nociception which is dose-dependent. Studies uncovered that co-administration of *Carum carvi L.* which is known as ('Jeera'), when co-administered with a few anti-microbials, antifungals, antivirals, anticancer, anti-ulcers, anti-inflammatory, cardiovascular, anti-leprosy and antihistaminic drugs expanded their bioavailability (25% to 300%) (Qazi et al, 2009). It was found that the bioactivity of rifampicin, tetracycline and ampicillin were improved with the utilize of novel bioactive nitrile

glycosides, Niaziridin and Niazirin individually gotten from *Moringa oleifera Adans*, which prevent tumor promoter-induced Epstein–Barr infection (Khanuja et al, 2005).

There are various bioactive constituent presents in the plants which proved their efficacy against various bacterial and fungal strains such as *Bridelia micrantha*, *Senna occidentalis* and *Clerodendrum myricoides* are the plants which have proven pharmacological activity against malarial infection. *Rauvolfia vomitoria* exhibited a strong anti-plasmodial activity. Phyto-constituents presents in natural terpenoids showed their efficacy against vancomycin resistant enterococci. Plant derived Antimicrobials substance (PDA_m) are the secondary metabolite of plants which had shown profound antibiotic activity against the gram positive and negative strains of bacteria without further causing resistance. PDA_m showed antimicrobial activity by reducing the pH, altering the efflux pumping and reducing membrane permeability of the microbes and found to be effective in the dose range 100 - 1000 $\mu g\ ml^{-1}$ (Srivastava et al, 2014).

Curcuminoids exhibited the anti-chemoresistance activity and can be employed for overcoming the drug resistance induced by adriamycin through the down regulation of p-glycoprotein (pgp) and ABCB1 mRNA. Study reveals that the bioactive presents in the curcumin have low toxicity and the potential to suppress proliferation, metastasis, invasion and angiogenesis in human cancers (Rodrigues, et al 2016).

Network pharmacology is gaining popularity to endorse the synergistic interaction of phyto-constituents present in the botanical drugs and traditional medicines using techniques like protein interaction, genomic expression and mRNA expression data for prediction and validation of the mechanism of action of the drugs to combat the drug resistance. Researchers have explored the connectivity between the phyto-constituents and the target drug molecules using the network pharmacology technique in food plant for hyperlipidemia elucidating the relationships between bio-actives, targets and pathways. *Lagenaria siceraria* ('Lauki') containing bio-active such as p-Coumaric acid, genistin, sinapaldehyde showed high and synergistic activity against hyperlipidemia associated targets (Mukherjee et al, 2017).

Traditional Medicine for Overcoming Drug Resistance

Bhringraj is one of the most potent plants found in various region of India like, Uttar Pradesh, Assam, *Gujrat* etc. This plant has inherent property of antibacterial resistance as it can survive in excessive muddy area and near water canal. Bhringraj has shown its effects since long time according Indian system of medicine and Ayurveda. It has strong effect in treating wound healing specially in case of diabetes and in toothache (Darah et al, 2013). Berberine an isoquilonine alkaloid having broad spectrum antibacterial activity against streptococcal, staphylococcal and enterococcal species found to be 10 times active against gram negative strains of

bacteria (Brown D). Sharma and co-workers suggested that Manuka honey can be efficient in reversing antimicrobial resistance to antibiotics and for the treatment of chronically infected wounds or drug resistance wounds (Sharma et al, 2014). Kudos CM 9 herbal tablets are recently approved for the treatment of cancer. Kudos CM9 tablets could be an alternative approach to treat the cancer in chemo-resistant patients. Figure 1 consists of few herbal medicines with improved drug resistance.

Figure 1.

Table 1 consists of list of traditional medicines which possesses multidrug resistance activity.

FUTURE PERSPECTIVES

In the present era everyone is focusing for a faster treatment of their illness. This may be a reason for higher use of antibiotics; this can lead to the MDR. In India every prescription contains antibiotics. Undoubtedly herbal/traditional drugs still are prominent options to avoid MDR as they never develop MDR. Even repeated therapy of herbal medicine may not cause MDR. The current issue of rising MDR microscopic organisms is posturing a worldwide therapeutic danger and is persistently challenging the logical community. The diminishing viability and expanding harmfulness of engineered drugs is encouraged exasperating the issue. This has

driven analysts to look for plant based antimicrobials for arrangement as they are presently known to play an imperative part within the improvement of compelling therapeutics. Phytoconstituents, either alone or in combination with anti-microbials may be a successful approach to bargain with the worldwide antimicrobial resistance. The viability of herbals in treatment of infections for decades proposes that microbes, organisms and infections may have a diminished capacity to adjust to a plant based antimicrobial administration.

Table 1. Traditional Medicine having Multi drug Resistance activity

Traditional Medicine	Source	Activity	References
Bhringraj (*Eclipta alba*)	Leaves, flowers, stem and root	Antibacterial agent, antifungal, wound healing, sinusitis, hair fall, toothache.	Darah et al, 2013
Berberine (Isoquilonine alkaloid)	Roots stems and barks of golden seal, golden thread, Oregon grape, barberry and tree turmeric	Antibacterial activity against streptococaal, staphylococcal and enterococcal bacteria. Kill *Streptococcus pneumoniae* and *S.aureus* bacteria	Tiwari et al, 2015
Curcumin	*Curcuma longa*	Antichemo-resistance activity, Potential to suppress proliferation, metastasis, invasion and angiogenesis	Rodrigues et al, 2016
Piperine marketed under trade name "Risorsine"	*P Piper Longum L.*	Increase efficacy and decrease tubercular resistance of rifampicin and isoniazid.	Mukherjee et al, 2018
'Triphala' Churna	*Terminilia chebula, Terminilia belerica, Emblica officinalis*	Possess strong antibacterial activity against *Staphlococcus epidermidis* and *S. aureus* and other strains of bacteria.	Sharma et al, 2014
Epigallocatechin-3-gallate	Polyphenols found in green tea	Mild antibiotic activity against gram positive and negative strains of bacteria	Tiwari et al, 2015
Phenolic extracts of essential oil	*Achillea biebersteinii*	Exhibited antifungal and anticandidal activity for treating infectious diseases without conferring resistance.	Baris et al, 2006
'Kutaj'	*Holarrhena antidysenterica*	Strong antibacterial activity against *Salmonella typhi*.	Sharma et al, 2014

CONCLUSION

In India ministry of AYUSH is focusing on development of herbal drugs for MDR. Currently, there's a pressing need for unused commercial models to be created to support improvement of herbal medicine to counter medicate safe organisms as

well as administrative changes so that clinical improvement programs are impartial, attainable, thorough, and clinically important.

CONFLICT OF INTEREST

Author declares no conflict of interest.

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Chapter 7
Natural Products in the Fight Against Multi-Drug-Resistant Bacteria:
Natural Antibiotics and Resistance

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ABSTRACT

It is well recognised that the antimicrobial resistance crisis has approached critical levels, and current treatment options are very limited, especially in the treatment of infections caused by resistant bacteria. Thus, ongoing research is focused on the development of new molecules which have broader antimicrobial activity. However, the advancements in drug development studies using synthetic compounds has led to a lack of success. Also, economic and regulatory issues have formed a challenge as well. Therefore, research has focused again on natural products. A large number of natural products and natural product-derived compounds are still in various stages of clinical development. Here, current research on the potential uses of natural products or their templates as viable sources of new drug candidates have been discussed to construct an understanding towards the goal of development of new antimicrobials to overcome resistant pathogens.

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INTRODUCTION

The life before antibiotics was dark and the discovery of antibiotics has shined upon the darkness of humanity and saved many lives. Average lifespan of a human being has increased significantly after the introduction of antibiotics. But, after a century of gains in the war against infectious diseases, the humanity is losing ground again. Common bacteria, parasites and fungi are developing widespread immunity to our best weapons.

Since its development, the humanity thought that penicillin will be a magic bullet for the infectious diseases. It has been used extensively and irresponsibly together with other following antimicrobials. Since many decades, there is a tremendous effort throughout the world to overcome the global antibiotic resistance. But now, medical authorities are raising concerns that treatment options in certain infectious diseases are narrowing because of alarming global antibiotic resistance problem. As new antimicrobial drugs are developing, the need for newer ones grow as well.

The looming public health crisis of antimicrobial-resistance have been evaluated by Centers for Disease Control and Prevention (CDC) and a report on antimicrobial resistance and its impact on various sectors in United States of America (USA) which highlights the scope of the problem have been published (CDC, 2013). CDC estimates that in the United States, more than two million people are infected every year with antibiotic-resistant bacteria, ending with at least 23,000 deaths as a result. From an economic standpoint, an economic burden of $18,000 -- 29,000 of medical costs per patient in a single year have been reported for USA in the year of 2000 which is similar in many geographies (Thabit et al., 2015). For example, high burden of antimicrobial drug resistance in Asia have been reported for various pathogens, namely; Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *S. aureus* (VISA), Vancomycin resistant *Enterococcus* (VRE), macrolide and penicillin resistant *Streptococcus pneumoniae*, Extended Spectrum Beta- Lactamase Producer *Escherichia coli* and *Klebsiella pneumoniae*, Carbapenem Resistant Enterococci (CRE), Multi drug resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter* spp. are mentioned as superbugs of the 21st century (Lai et al., 2014).

As a result, the emergence of pathogens with different mechanisms of resistance has intensified the challenges associated with infection control and treatment strategies. Therefore, as prudent use of antimicrobials is not at the intended level, development of novel antimicrobial molecules remains as the main strategy to overcome the present narrow pass. In this context, many products have been studied to develop antimicrobials and the major sources of chemical diversity for starting materials were mainly natural products because of their abundant scaffold diversity (Mishra & Tiwari, 2011). Before, the pharmaceutical companies were using crude plant extracts to produce relatively simple therapeutic formulations but nowadays

purified compounds and their chemically modified derivates have been included in many drug formulations. Despite there are plenty of possibilities coming from pure natural compounds, sometimes modification of formulations of natural compounds can be an option in developing antimicrobial molecules too. On the other hand, discovery of antimicrobial effectiveness of a molecule is not enough to use it as a therapeutic agent. For this, a drug approval process has to be carried out. To register a novel molecule as a drug, an Investigational New Drug (IND) application must be submitted to the Food and Drug Administration (FDA) in USA or European Medicines Agency (EMA) in Europe. Afterwards, the process continues with successful clinical trials ending with another application for marketing licenses namely, New Drug Application (NDA) in the USA or a Marketing Authorization Application (MAA) in Europe. This though period rarely ends with the introduction of novel, safe antimicrobials into the market. Nevertheless, many natural products and derived compounds are still in various stages of clinical development. So, the use of natural products or their templates is a viable source of new drug candidates.

Here, various natural products and their applications have been discussed to construct an understanding towards the goal of development of new strategies to overcome infectious diseases caused by antimicrobial resistant bacteria.

HISTORICAL DEVELOPMENT OF NATURAL ANTIMICROBIALS IN DRUG INDUSTRY

The significant history of antimicrobial drug development starts with the fight against African Sleep Sickness which is also called as African trypanosomiasis; a disease spread by infected fly vectors. In 1859, Antoine Bechamp has discovered a drug named as “Atoxyl” with an aim to be used against African Sleep Sickness, which was unsuccessful (Burke, 1925). Despite the discovery of Atoxyl was as unsuccessful attempt, it was a good starting point for Paul Ehrlich and colleagues as it inspired them a lot. In 1909, Ehrlich and colleagues have showed the efficiency of this compound in the treatment of syphilis-infected rabbits. Subsequently, despite its adverse effects, the drug was marketed under the name of “Salvarsan”, which was a great success. A less toxic and more soluble form was also introduced afterwards which was called as “Neosalvarsan”(Aminov, 2017). The need for a drug effective against infectious diseases was so huge in that days. For this reason, Neosalvarsan quickly became the most frequently prescribed drug in the world until its replacement by penicillin in the 1940s (Mahoney et al., 1943).

Many antibiotic molecules have been developed from natural compounds after the “first natural antimicrobial approved for use in humans”, which is Penicillin. Penicillin was discovered in 1928 when Alexander Fleming noticed a halo of

inhibited bacterial growth around mould colonies which led Fleming to conclude that the mould released a substance that repressed the growth of the bacteria (Lax et al., 2005). The discovery of penicillin along with other antibiotics such as sulphonamides or streptomycin have led to a period of 15 years, from 1938 to 1952, with highest rate of decline in infectious disease mortality (a decrease by 8.2% per year in the USA) (G. L. Armstrong et al., 1999). As penicillin was discovered it did not immediately take off as a clinically useful antibiotic. Despite the advantages, penicillin had many drawbacks in the first decade of introduction, such as low yield, instability, purification, and other problems. Actually, the World War 2 has stimulated the development of penicillin into a valuable treatment option for previously untreatable infectious diseases (Aminov, 2017). Afterwards, penicillin has been studied widely and extensively for a better understanding of the molecule structure and action mechanism.

Identification of 6-aminopenicillanic acid as the core of penicillin allowed the synthesis and production of numerous semisynthetic penicillin such as penicillinase-resistant penicillin (namely, methicillin, oxacillin, and nafcillin), the aminopenicillins (namely, ampicillin, amoxicillin, and bacampicillin), the carboxypenicillins (namely, carbenicillin and ticarcillin), and the ureidopenicillins (namely, mezlocillin, azlocillin, and piperacillin) (Aminov, 2017; Batchelor et al., 1959; A. J. Wright, 1999).

Notably, in his Nobel Speech Fleming has warned about the misuse of the drug. He stated that resistant bacteria can be selected evolutionary among the susceptible ones after misuse. Throughout the history, words of Fleming have been approved and in the 21st century, humanity still struggles to find a solution for this problem. Many antibiotic molecules have been developed after Penicillin which can be classified as purely natural, natural derived or synthetic molecules.

TYPES AND CLASSIFICATION OF NATURAL ANTIBIOTIC MOLECULES

Antibiotics can be classified in many ways according to purpose. To summarise, the classifications can occur as; (i) According to mode of action, (ii) According to chemical structure, (iii) According to infections to be used, and (iv) According to sources.

Sources of Antimicrobials

Antibiotics sources are so versatile. For instance, molecules that can have antimicrobial activity can be obtained from marine environments, to fungal agents. Obviously,

the role of microbes in antimicrobial discovery has been always important and it seems like they have a fight by themselves as well.

The majority of antimicrobials have been discovered from prokaryotes, on the other hand, there are many molecules derived from eukaryotic sources such as fungi or plants (Ali et al., 2018). As mentioned before, Penicillin molecule was derived from Penicillium fungi. The other major natural products obtained from fungi are, Cephalosporins from *Acremonium* spp., Gliotoxins from *Aspergillus* spp. and *Trichoderma* spp., Indanonaftol A from *Aureobasidium* spp., Ascochital from *Kirschsteiniothelia maritimais*, Pestalone from *Pestalotia* spp., Pestalachloride D from *Pestalotiopsis* spp., *Aminolipopeptides trichoderins* A, A1, and B from *Trichoderma* spp. (Abraham et al., 1953; Biabani & Laatsch, 1998; Bugni & Ireland, 2004; Cueto et al., 2001; Jones & Hancock, 1988; Masuma et al., 2001; Newton & Abraham, 1955; Okutani, 1977; Pruksakorn et al., 2010; Silber et al., 2016; Wei et al., 2013).

Another eukaryotic group are plants which include some other natural products with antimicrobial activity. Some major natural products obtained from plants are; Sesquiterpenes from terrestrial medicinal plants and marine algae, Halogenated compounds from marine algae *Bonnemaisoniaceae*, Ar-turmerone, turmerone, curlene from turmeric and procyanidins from cranberry (Ahmad et al., 1994; Fenical & Mcconnell, 1977; Negi et al., 1999; Pappas & Schaich, 2009).

Last eukaryotic group of animals are potential sources for natural antimicrobials too. Some major natural products obtained from animals are mainly antimicrobial peptides such as, ll-37 from humans, sulphonamides, furanones and flavanones and some other bioactive molecules from Cockroach and Phenylacetaldehyde from *Musca domestica* (housefly)(Ali et al., 2017; Arora et al., 2011; Bonn, 2000; Srakaew et al., 2014).

The prokaryotic group which are Bacteria, are known to reserve a wide variety of natural antibiotic products which holds the majority of portion in antibiotics industry today. For example, Actinomycin, Streptomycin, Streptothricin, Erythromycin, Tetracyclines, Aminoglycosides and Lincomycin are the antibiotics which are isolated from the genus *Streptomyces* spp. (Darken et al., 1960; Majer et al., 1977; Schatz et al., 1944; Wagman, 1980; Waksman & Woodruff, 1941, 1942; J. L. C. Wright, 1983).

Antibiotics According to their Mode of Action

One of the best approaches to classify antibiotics is to classify them according to their mode of action. As every antibiotic molecule can have its unique mechanism of action, Antibiotics mainly act on three primary targets within bacterial cells. These targets are; the inhibition of (i) cell wall synthesis, (ii) protein synthesis, and (iii) DNA or RNA synthesis (Rossiter et al., 2017).

As one of the most complex and critical organelles in the bacterial cell is the cell wall, most of the antibiotics aim to disrupt the bacterial cell wall structure or to inhibit the synthesis of it. For instance, one of the largest group of antibiotics; Beta-lactams act by disrupting the cell wall synthesis by its active site (beta-lactam ring). Beta-lactams including Penicillin or Cephalosporins inhibit the cross-linking step of peptidoglycan synthesis. A Glycopeptide, Vancomycin targets peptidoglycan synthesis as well but with a different mechanism of action which relies on inhibiting the transpeptidases from cross-linking the terminal D-Ala-D-Ala tail. Lipoglycopeptides such as Oritavancin or Dalbavancin Inhibit bacterial cell wall synthesis similarly but in addition to that, it disrupts the membrane potential, and change cell permeability due to the presence of a lipophilic side chain.

The second group of antibiotics are the group which includes drugs inhibiting the bacterial protein synthesis in the bacterial ribosome. Macrolides such as Clarithromycin or Erithromycin are in this group which targets the larger 50S subunit of bacterial ribosome. The tetracyclines, another group of natural product derived antibiotic, bind to smaller 30S subunit of the ribosome. Natural product derived, Aminoglycosides like Gentamicin, Amikacin and Tobramycin bind both 30S and/or 50S subunit of the bacterial ribosome which leads to death of bacterial cell. Chloramphenicol, Lincosamide and Oxazolidimine are other antibiotics which inhibits the bacterial protein synthesis process.

A third major target for antibiotics is nucleic acid replication and related repair mechanisms. For instance, DNA gyrase which is the enzyme taking role in the unwinding of replicated DNA is a target for the synthetic antibiotic class of quinolones.

Lastly, there are antibiotics which inhibits various steps in folic acid metabolism of bacteria. Some examples of these drugs include Sulphonamides and trimethoprim.

Antibiotics can be classified according to infections that they are used, and this type of classification can be broadened depending on the purpose of use as well, but there will be some consequences. One can classify the antibiotics as “Antibiotics to be used in Urinary Tract Infections” or “Antibiotics to be used in Upper Respiratory Infections” but this type of classification is not reliable and may lead to misuse as every pathogen requires a special attention when determining the treatment regime. For example, a group of antibiotics can be generalised as “Antibiotics to be used in Lower Respiratory Infections” but one of the most severe and hard to treat Lower Respiratory Tract infection which is “Tuberculosis” caused by *Mycobacterium tuberculosis* needs a special attention to be treated successfully.

NATURAL PRODUCTS WITH ANTIMICROBIAL ACTIVITY AND THEIR POTENTIAL FOR NEW DRUGS AND VERSATILE APPLICATIONS

An antibiotic molecule, either natural or synthetic must have many features together and must overcome many procedures to become available for human use. There are many antibiotics discovered or developed for different purposes. Some of them have accomplished to be introduced for human use some are not. So the other antibiotic molecules that have a certain antimicrobial activity but not suitable for systemic human use can be good candidates for many other applications such as food preservation, wound dressing, antibacterial surface coating, and disinfection of pools (Carter & Joll, 2017; Juneja et al., 2012; Kana & Meimandipour, 2017).

Antimicrobial Peptides

An important group which are good candidates for antimicrobial drug development are small antimicrobial peptides. Antimicrobial peptides are the newest group of natural products to have antimicrobial activity and these peptides are produced naturally by organisms internally to contribute the immunity of individual in the fight against infectious diseases. Moreover, they can have different roles throughout the body such as programming apoptosis or stimulating chemokine synthesis. To date, more than 2800, various Antimicrobial peptides have been shown from different organisms such as mammals, amphibians, fishes, plants, insects, echinoderms, crustaceans, fungi, and bacteria. When compared with traditional antibiotics, these Antimicrobial peptides have some advantages. For example, spectrum of activity is wide and at the same time resistance to Antimicrobial peptides is not common. Furthermore, Antimicrobial peptides killing time is quicker than conventional antibiotics where antagonism is not present (Moravej et al., 2018). The Antimicrobial Peptide Database contains 3003 antimicrobial peptides from six kingdoms (335 bacteriocins/peptide antibiotics from bacteria, 4 from archaea, 8 from protists, 14 from fungi, 343 from plants, and 2213 from animals, including some synthetic peptides) with the following activity: Antibacterial peptides; Antibiofilm peptides, Antiviral peptides; Anti-HIV peptides, Antifungal peptides, Antiparasitic peptides; Antimalarial peptides, Anti-protist peptides, Anticancer peptides, Antioxidant peptides, Chemotactic peptides, Insecticidal peptides, Protease inhibitors, Spermicidal peptides, Surface immobilized peptides and Wound healing peptides(Wang et al., 2016). The majority of Antimicrobial peptides such as LL-37 (Human cathelidins), Brilacidin (Human Defensin Analog), PXL01 (Human Lactoferrin Analog) are currently undergoing pre-clinical or clinical trials which is an indicator of their potential as promising antimicrobial candidates for human use in various infections (Crowther et al., 2013;

Malanovic et al., 2015; Maria et al., 2017). In depth detail for the promising studies on Antimicrobial peptides can be found on Moravej *et*. al.`s excellent review and AMP database (Moravej et al., 2018; Wang et al., 2016).

Biopolymers

Biopolymers are a versatile group of natural compounds which have wide range of application capacity and have good antimicrobial activity. Biopolymers are haemostatic and anticoagulant, and they can be used as thickeners, emulsifiers or stabilizers, anti-fouling coating materials, also they can be used in wastewater management. A well-studied natural molecule, Chitosan (derived from Chitin) is a biopolymer that is found in the exoskeletons of crustaceans and arthropods with a variety of applications. Chitosan has good synergy with some other antibiotics and it can be used for a wide range of environmental and/or biomedical applications such as surface coating, tissue engineering, drug delivery and wound healing (Assaad et al., 2015; Devlieghere et al., 2004; Lee et al., 2014; Oryan & Sahvieh, 2017). Action mechanism of chitosan is mainly associated with the positive charge density of the polymer. Where negative charged bacterial cell wall and positive charged chitosan molecule interacts, this interaction leads to the lysis of the bacterial cell. The other important natural biopolymer which have antimicrobial applications is "epsilon-poly-L-lysine" which is produced by *Streptomycetaceae* and ergot fungi species (Nishikawa & Ogawa, 2002). It is an edible, non-toxic polymer that acts by electrostatic interactions between the molecule and the bacterial cell membrane resulting with physiological damage to bacterial cells which leads to death of bacteria.

Others

In the last half-century natural antimicrobial discovery and their subsequent clinical use have peaked mainly after the developments and standardizations in antimicrobial susceptibility testing protocols. Accordingly, many interesting natural products have undergone antimicrobial susceptibility testing to detect at least meaningful antimicrobial activity. One good example for this; Animal venoms have been reviewed recently as rich sources of antimicrobials. Snake, spider, wasp, honey bee venoms have been reported to have antimicrobial activities (Perumal Samy et al., 2017). Similarly, many plant-derived compounds have been showed to possess antimicrobial activity. Flavonoids have good activity against many bacteria and they mostly have a synergistic effect in combined use with other antimicrobials (Savoia, 2012). For instance, Quercetin, a flavonoid which can be found in many fruits and vegetables have shown to possess good antibacterial and antiviral activity (Ganesan et al., 2012; Gopu et al., 2015). Apart from Flavonoids, Alkaloids like Diterpenes

and Berberine had a broad range of antimicrobial effectiveness as well (Atta-ur-Rahman & Choudhary, 1999; S. H. Kim et al., 2002). Several Phenolic compounds which have the function to protect the plants from microbial infections are studied and Tannins, Quinones and Flavones showed good antibacterial activity (Savoia, 2012). Notably, Coumarin class of phenolic compounds have gained an attention for their antimicrobial activity. Furthermore, it has been reported that Coumarins can have inhibitory effect on microbial quorum sensing and biofilm formation as well (Gutierrez-Barranquero et al., 2015).

Lactoferrin, a glycoprotein included in milk is a good example of natural products which have environmental applications. It has been used as a food preservative frequently and recently it had got the approval in USA to be used as a preservative on beef (Del Olmo et al., 2009). A similar natural compound that is used in food industry is Ovotransferrin (a glycoprotein of egg white albumin) which is used as a food additive to increase the lifetime of various food products (J. Kim et al., 2012).

MAJOR NATURAL ANTIBIOTIC DRUG CLASSES

Antibiotics that are drugs are mainly developed in 3 ways; (i)directly from natural products, (ii)by modifying natural products (natural product derived/semi-synthetic molecules) and (iii)by designing synthetic products. In early ages of antibiotic drug development, many of the agents were discovered in fermentation products without further modifications such as aminoglycosides, vancomycin and tetracycline. But the pipeline supplying new drugs from fermentation products has been drained and new antibiotic drugs could not be discovered like at the beginning in the last decade. The only exception is a drug developed for the treatment of *Clostridium difficile* related diarrhoea; Fidaxomicin which got the approval in 2011 (Hardesty & Juang, 2012). The other most common method in the discovery of antibiotics is to modify the known natural products. Many antibiotics such as daptomycin have been developed with this method (Debono et al., 1987). Sulphonamides, Diaminopyrimidines, Fluoroquinolones, Oxazolidinones, Nitroimidazoles are some of the synthetic antibiotics which will not be covered within the scope of this article.

Beta-lactams

Antibiotics which its active site is the Beta-lactam ring are one of the most widely used antibiotic classes as they are efficient and safe. But the resistance is emerging for this group of antibiotics especially because of Extended Spectrum Beta-Lactamase producer microorganisms such as *E. coli* and *K. pneumoniae*. Beta-lactamases are enzymes produced by certain bacterial species which hydrolyse the beta-lactam ring

of the compound which is the active site resulting with the ineffectiveness of the drug. Therefore, biggest challenge in overcoming the beta-lactam resistance is to develop newer beta-lactamase inhibitors (Bush & Bradford, 2016). Combination of beta-lactamase inhibitors are also suggested as a promising strategy to inhibit bacterial beta-lactamases. Ceftazidime-avibactam, Ceftaroline-avibactam, Ceftolozane-tazobactam are some of the promising beta-lactam and beta-lactamase inhibitor combinations (D. T. King et al., 2016).

Table 1. Some antibiotics in clinical use

Group Name	Discovery Year	Major Representative antibiotics	Source	Target
Beta-lactams	1929	Penicillin	N/ND*	Cell wall
		Cephalosporins	N/ND	Cell wall
		Carbapenems	N/ND	Cell wall
Glycopeptides	1953	Vancomycin	N/ND	Cell wall
Macrolides	1950	Erythromycin	N/ND	Protein synthesis
		Clarithromycin	N/ND	Protein synthesis
		Azithromycin	N/ND	Protein synthesis
Lincosamide	1963	Clindamycin	N/ND	Protein synthesis
Aminoglycosides	1943	Gentamicin	N/ND	Protein synthesis
		Tobramycin	N/ND	Protein synthesis
		Amikacin	N/ND	Protein synthesis
Streptogramin	1953	Quinupristin/Dalfopristin	N/ND	Protein synthesis
Tetracycline	1945	Doxycycline	N/ND	Protein synthesis
		Minocycline	N/ND	Protein synthesis
Rifamycin (Anzamycins)	1957	Rifampin	N/ND	DNA/RNA synthesis
Lipopeptide	1947	Daptomycin	N/ND	Cell wall
		Colistin	N/ND	Cell wall
Amphenicols	1947	Chloroamphenicol	NS***	Protein synthesis
Sulfonamides	1935	Sulfomethoxazole	S**	Folate biosynthesis
Diaminoprymidines		Trimethoprim	S	Folate biosynthesis
Quinolones	1962	Ciprofloxacin	S	DNA/RNA synthesis
		Levofloxacin	S	DNA/RNA synthesis
		Moxifloxacin	S	DNA/RNA synthesis
Oxazolidinone	1952	Linezolid	S	Protein synthesis
Nitroimidazole	1955	Metronidazole	S	DNA/RNA synthesis

*N: Natural/Natural derived Product, **Synthetic Product, *** Naturally discovered but produced synthetically

Glycopeptides

Glycopeptides are natural antibiotics isolated from *Amycolatopsis orientalis* a soil bacterium. Their main action mechanism is the inhibition of cell wall synthesis. Glycopeptides are thought to be a last option in the treatment of severe, complicated infections, but global resistance problem has affected this class of antibiotics as well. First antibiotic resistant bacteria was reported in a Vancomycin resistant Enterococci strain in 1988 (Uttley et al., 1988). Afterwards, the resistance started to disseminate. Resistance is mainly related with the ability of bacteria to produce cell wall precursors that have low affinity to vancomycin due to genetic modifications of *van* genes. Nowadays, Vancomycin resistant Enterococci and Vancomycin resistant *S. aureus* strains are globally threating the healthcare facilities. In addition to misuse, use of Avoparcin (which is a growth promoting agricultural agent) is reported to be responsible from the increased resistance rates (Bager et al., 1997).

Studies working on modifications on vancomycin molecule mainly aim to increase the affinity of the molecule to the cell wall of the bacterial cell. Crowley and Boger have suggested a re-engineered vancomycin molecule which can bind to an additional cell wall target (Crowley & Boger, 2006). On the other hand, resistance levels to other Glycopeptide antibiotics are still in lower levels in contrast to Vancomycin.

Glycopeptides other than Vancomycin are Teicoplanin, Telavancin, Oritavancin and Dalbavancin. Teicoplanin is a drug which can be used against infections caused by Vancomycin resistant strains excluding VanA resistant strains (Aminov, 2017). Comparing to first generation Glycopeptides (which are Vancomycin and Teicoplanin), Telavancin, Oritavancin and Dalbavancin have better affinity features, and more than one mode of action (Klinker & Borgert, 2015; Van Bambeke, 2006; G G Zhanel et al., 2010).

Macrolides

Azithromycin, Clarithromycin, Erythromycin, Fidaxomicin and Telithromycin are the antibiotics of Macrolides class which get the Federal Drug Administration approval. First of the macrolides, Erythromycin was isolated from an actinomycete bacteria; *Saccharopolyspora erythraea*. This group of antibiotics serve as the second mostly prescribed antibiotic group after beta-lactams (or in conditions when beta-lactams are not applicable) and mainly make action by inhibiting the protein synthesis.

Current studies aiming to modify macrolides focus mainly to improve the affinity of the molecule to bind the ribosome better and/or to find different binding sites for the molecule. There are 2 molecules currently being developed which are Ketolides (Telithromycin) and Flouroketholides (Solithromycin). Despite Solithromycin is still

in the last phase of clinical trials it can be concluded that both new modifications have fulfilled the purposes as they possess a better affinity and a wider spectrum.

Lincosamides

Clindamycine is the drug of choice among Lincosamides, whereas the original formulation; Lincomycin is not used commonly due to its low level of spectrum and serious adverse effects. Clindamycin which is a far better choice has been developed by simply adding a Chlorine atom with the inversion of chirality of the older Lincomycin molecule. The main action mechanism of lincosamides is to stop the protein synthesis thus bacteria can gain resistance by modifying the binding site (namely through modifying the *erm* gene).

Aminoglycosides

Aminoglycosides are a wide group of antibiotics which are mainly active against gram negative bacteria and tuberculosis bacilli. Aminoglycosides which act by inhibiting the protein synthesis, are active against most gram-negative aerobic and facultative anaerobic bacilli but not active against gram-negative anaerobes and most gram-positive bacteria. There are more than 10 modifications of aminoglycosides namely, Kanamycin A, Amikacin, Tobramycin, Dibekacin, Gentamicin, Sisomicin, Netilmicin, Neomycins B, Neomycin C, Neomycin E (paromomycin) and Streptomycin.

After the discovery of the first Aminoglycoside, Streptomycin there has been a dramatic deadline especially in Tuberculosis cases. Good activity of Streptomycin in tuberculosis cases has put the drug in one of the most life-changing drugs ever as mortality of tuberculosis has decreased by 47% between 1990 and 2015 (Aminov, 2017). But there are resistant *M. tuberculosis* strains being reported from different regions in the world in the last decades. After multi drug resistant strains, extremely drug resistance strains started to appear during 2000s and in 2009 a totally drug resistant strain has been reported as well (Migliori et al., 2007; Plikaytis et al., 1994; Velayati et al., 2009). Apart from Streptomycin, Gentamicin and Amikacin have gained good popularity in clinical use as well especially in severe intensive care infections. Synergism studies exploring the interactions between Aminoglycosides and other natural antibiotics could result in newer combinations in the future.

Streptogramins

Streptogramins which are produced by many strains of *Streptomyces* spp. are have good activity against a wide range of bacteria especially to Methycillin Resistant *S. aureus* and Vancomycin Resistant Enterococci, therefore they are thought to be

one of the last-resort antibiotics. Characteristically, two different types of chemical substances; the group A streptogramins, (which are polyunsaturated macrolactones) and the group B streptogramins are combined into one drug (Mast & Wohlleben, 2014). Main action mechanism is the inhibition of protein synthesis. One of the well-known Streptogramins is Virginiamycin. The common adverse effects has prevented Virginiamycin for human use, but it has been used as a food animal growth promoting agent very popularly for a long time (Yates & Schaible, 1962). New considerable Streptogramins which are eligible for human use are Pristinamycin and Quinupristin/Dalfopristin which were introduced in 2001 (Allington & Rivey, 2001; Hamilton-miller, 1991).

The similar action mechanism of Streptogramins, Macrolides and Lincosamides lead to a resistance overlap. Once a bacterium develops resistance upon drug contact it become resistant to other two as well (Tenson et al., 2003). Future studies investigating the development of chimeric streptogramin-tyrocidine combinations can improve understanding regarding to streptogramin resistance (Mukhtar et al., 2005).

Tetracyclines

Tetracycline group of antibiotics were firstly introduced mainly as growth promoting agents for food animals. A study reported that, Tetracyclines were most common antibiotics sold for food producing animal industry (Administration & Services, 2015).

The first Tetracycline, which was chlortetracycline, was discovered in 1945 and afterwards Aureomycin was introduced to market. Source of Aureomycin was a soil bacterium named *Streptomyces aureofaciens* (Aminov, 2017). After a long period of usage in food producing animals industry, chemical structure of natural Tetracycline molecule has been solved and this progress has led to development of new Tetracycline molecules with the aim of fighting growing antimicrobial resistance problem (Nelson & Levy, 2011). These molecules were minocycline and doxycycline which were discovered in 1966 and 1967 respectively. However, bacteria quickly have developed resistance to these antibiotics too. Therefore, studies focusing on the development of new molecules have been in progress until 2005, that a third generation Tetracycline has appeared with the name of Tigecycline (Rose & Rybak, 2006).

Introduction of Tetracycline was an important development for the drug and infectious disease societies as it has a good activity on resistant pathogens especially Multi drug resistant *Enterobacteriaceae* strains. Luckily, the resistance rates are still low. Major challenge in this class of antibiotics is to identify and elucidate further possible resistance mechanisms of bacteria. For instance, discovery of *tetX* gene has provided a good understanding of Tetracycline resistance (Aminov, 2013).

Rifamycins

Rifamycins which are also named as Ansamycins are natural antibiotics isolated from soil bacteria *Nocardia mediterrenai* (formerly named as *Streptomyces mediterrenai*) (Margalith & Beretta, 1960). There are many types of different molecules in this class but the one which has got most of the attention is the Rifampin. Rifampins importance comes from its effectiveness in the treatment of mycobacterial infections such as tuberculosis or leprosy (Sepkowitz et al., 1995). Other modified molecules of this class include the drugs such as Rifabutin, Rifapentin and Rifaximin where the usage is not as common as Rifampisin due to poor activity and side effects. The resistance for Rifamycins is growing as similar with other classes and it needs attention especially for Multi drug resistant Tuberculosis cases (Floss & Yu, 2005).

Lipopeptides

Lipopeptides comprise an important group of antibiotics which are highly effective against multi drug resistant strains especially. In this group, the most important antibiotics are Colistin (Polymixin E) and Daptomycin which are isolated from *Paenibacillus polymyxa*, *Streptomyces roseosporus* respectively (Storm et al., 1977; Tally & DeBruin, 2000). Another group of molecules which have good antifungal activity are Echinocandins.

Despite their toxicity in various systems, lipopeptides are life saver in many severe infections caused by multi drug resistant bacteria including gram negatives such as *P. aeruginosa, K. pneumoniae, A. baumannii* or gram positives like Vancomycin resistant Enterococci and Methicillin resistant *S. aureus*. Being active mostly in gram negative infections, Colistin can be useful in treating the most life threating infections caused by Metallo-beta lactamase producer *Enterobacteriaceae* strains as well (Kumarasamy et al., 2010). On the other hand, Daptomycin is the drug of choice in infections caused by gram positive bacteria. However, despite the reported rate of resistant strains is rare Lipopeptide resistance has started to emerge similar with other antibiotics which requires a special attention (Aminov, 2017). Further studies elaborating the possible mechanisms of resistance to this group of antibiotics and studies which will evaluate possible modifications to decrease the toxicity of lipopeptides would be useful.

Polypeptides

This group of antibiotics did not gain much popularity due to their limited usage capacity in human infections. The main drugs in this class are Tyrocidines and Gramicidines. Tyramicidines and Gramicidines are isolated from a soil bacterium

namely, *Brevibacillus brevis* (Gause & Brazhnikova, 1944). Main limitation in this class is their adverse effects and these drugs can be extremely toxic limiting their use to a topical level. However, in some extraordinary cases systemic use can be thought when run out of options. A drug which is commonly used in the differentiation of Streptococcus species, Bacitracin is a polypeptide as well (Hallen et al., 2007).

NOVEL NATURAL ANTIMICROBIALS IN THE FIGHT AGAINST RESISTANT BACTERIA

Development of new antibiotic drugs is the main strategy to overcome superbug infections. New drugs can be developed by the modification of older ones or from new source molecules. On the other hand, rediscovery of older drugs for drug resistant infections is becoming a popular strategy for the management of these infections (Poulakou et al., 2014). Sometimes, modification of the dosage regimens or trying new combinations can be effective as well (Daikos et al., 2014; Qureshi et al., 2012; Tumbarello et al., 2015)..

Older drugs such as Colistin, Fosfomycin and Tigecycline can be effective in antibiotic resistant gram-negative bacterial infections. But there are many adverse effects and toxicity issues to tackle. For example, sensitivity to colistin's have been reported in such carbapenem resistant gram negative bacterial infections and despite the neurotoxicity and nephrotoxicity of colistin is known, it can be a last resort of option to be used in such severe infections (Giamarellou & Poulakou, 2009; Tumbarello et al., 2015). Another older drug which is effective in resistant bacterial infections is Fosfomycin. In addition to its effectiveness in gram negatives, Fosfomycin can be effective in gram positive bacterial infections as well. Like colistin, Fosfomycin must be used with care due to its side effects (Raz & Unit, 2011). Tigecycline, an old Tetracycline have a wide range of spectrum and it can be used in certain Extended Spectrum Beta-Lactamase producer infections with the consideration of adverse effects (Pournaras et al., 2011).

Apart from older drugs, new drugs or combinational regimes are alternative strategies for drug resistant gram-negative bacterial infections. In the last decade, some new beta-lactamase inhibitors have been introduced into the market and new combinations have emerged (Sfeir et al., 2018). Ceftazidime/Avibactam, one of the new combinations has reached to 100% susceptibility in Carbapenem resistant *Enterobacteriaceae* infections. Overall research suggests Ceftazidime/Avibactam as a better alternative than older ones such as colistin (M. King et al., 2017). Ceftolozane/Tazobactam is the other natural combination of cephalosporins and beta lactamase inhibitors. This combination has been reported to be very useful in the management

of urinary tract infections caused by Extended Spectrum Beta-Lactamase producer *Enterobacteriaceae* and especially *P. aeruginosa* (E. S. Armstrong et al., 2016).

Carbapenemase inhibitors developed in last 2 decades are becoming very helpful in the management of infections caused by Carbapenemase producers. Use of carbapenems together with these inhibitors reactivates the action of carbapenems (Garau et al., 2005). For instance, Imipenem/Relebactam and Meropenem/Vaborbactam combinations have been showed to be very effective against Carbapenem Resistant *Enterobacteriaceae* and to lower the Minimum Inhibitory Concentrations to carbapenems in vitro (Hackel et al., 2018; Livermore et al., 2013).

A new generation Aminoglycoside, Plazomicin is currently being developed. First step in vitro studies have demonstrated good activity against Multi Drug Resistant *Enterobacteriaceae* including Aminoglycoside resistant isolates (Walkty et al., 2014). Another new drug in development, Cefederecol is a new cephalosporin with promising activity against Carbapenem Resistant *Enterobacteriaceae* (Ito et al., 2016). Tetracycline derived new antibiotic molecules; Omadacycline and Eravacycline are the latest antibiotic molecules currently being developed with promising first step results against resistant bacteria groups (Pfaller et al., 2018; George G Zhanel et al., 2016).

Fifth generation cephalosporins, Ceftaroline and Ceftobirole are the beta-lactam antibiotics which have good activity against MRSA. To compare, Ceftaroline has a broader activity in contrast to Ceftobirole (Kelley et al., 2015; Long et al., 2014; Nicholson et al., 2012). However, Ceftaroline which reported to be very effective against MRSA infections reported to be ineffective in some parts of the world where the drug never used before (Mendes et al., 2012).

Dalbavancin and Oritavancin are Lipoglycopeptides which have been introduced to market recently. FDA and EMA recently approved Dalbavancin for the treatment of skin infections. Both of the new Lipoglycopeptides possess a good activity against Resistant Gram positive pathogens with low toxicity (Klinker & Borgert, 2015; G G Zhanel et al., 2010).

Another group of novel natural derived antibiotics are from Tetracycline class namely, Omadacyclines. Omadacyclines are currently received fast track development status from FDA and they are active against a large portion of pathogens including gram positives and negatives (Villano et al., 2016).

Lastly, Lefamulin a novel semi-synthetic drug of Pleuromutilin class has been developed to inhibit 50S subunit of bacterial ribosome. Lefamulin is strongly active against MRSA, VRE and Penicillin resistant *S. pneumoniae* infections (Paukner et al., 2013). Tedizolid, Delafloxacin, Nemonoxacin and Zabofloxacin are new synthetic drugs under development for the fight against gram positive resistant bacteria which are not covered here (Abbas et al., 2017).

Table 2. Promising natural antibiotic drugs in the fight against resistant bacteria

Target infections	Antibiotics
Gram negative bacterial infections	Colistin, Ceftazidime/Avibactam, Fosfomycin, Ceftolozone/Tazobactam, Tigecycline, Imipenem/Relebactam, Meropenem/Vaborbactam
Gram positive bacterial infections	Ceftaroline, Oritavancin, Ceftobirole, Lefamulin, Dalbavancin, Omadacycline

CONCLUSION

Currently a global antibiotic crisis is ongoing. Many known pathogens are developing resistance against many known antibiotics and new resistant pathogens are emerging at the same time. Mortality and morbidity rates due to infections caused by these pathogens are increasing to an alarming level. Therefore, immediate actions combining several parallel approaches are needed in the drug development industry. These actions are focused mainly on the modifications of existing natural products/ scaffolds aiming known bacterial targets. In this regard, *in-silico* studies focusing on new targets such as energy metabolism could be a promising approach for new drug discoveries.

New discoveries of natural products can also generate new insights in the fight against antibiotic resistant bacteria. Simultaneously, the experiments focusing on the optimisation of the usage of older natural antimicrobials alone or enlightening potential synergistic interactions can still be useful. Moreover, development of synthetic molecules is another field that needs attention.

Putting it all together, the current strategy should mainly focus on the preservation of existing drugs by proper usage. The large number of natural products in the current industry and natural product derived compounds in various stages of clinical development are all indicators of their potential as candidates for the new antibiotic drug development.

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KEY TERMS AND DEFINITIONS

Adverse Effects: When medical drugs, like antibiotics, have harmful or distracting effects on person being used or applied.

Antibacterial: Class of substances that can kill or inhibit the growth of bacteria.

Antibiotic: Class of substances that can kill or inhibit the growth of some groups of microorganisms. Despite being a wide term, herein the word antibiotic resembles a drug that kills or stops the growth of bacteria particularly. Examples include penicillin and streptomycin.

Antifungal: Class of substances that can kill or inhibit the growth of fungi.

Antimicrobial: Any substance (including an antibiotic) used to kill or inhibit the growth of bacteria, viruses, fungi, or parasites. This term is a non-specific one which is a wider term than antibiotic.

Antimicrobial Resistance: The ability of a microbe to survive and continue to multiply during and after the encounter with a certain antimicrobial agent. Antimicrobial resistance includes antibiotic, antibacterial, antifungal, and antiviral resistance.

Infection: An invasion of an organism by a pathogen such as bacteria or viruses, often causing an immune response from the host. Some infections lead to disease.

Natural Product: Substances, compounds or molecules isolated from natural sources.

Pathogens: Bacteria, viruses, parasites, or fungi that can cause disease.

Chapter 8
Alternative Therapies: Toolbox to Combat Antibiotic-Resistant Bugs

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ABSTRACT

Antibiotic resistance is one of the leading public health concerns across the globe. Antibiotics are losing their effectiveness, leading to uncertainty in available treatment options to clinicians. Resistance to antibiotics is at an all-time high, and there is a pressing demand to look for alternative antimicrobial candidates other than antibiotics. Alternative therapies include use of bacteriophages, lytic proteins, nanoparticles, phytochemicals, quorum quenchers, and other antibacterial or antivirulent agents that can eradicate bacterial infection alone or in conjunction with antibiotics. Alternative therapies can replace or lower the effective antibiotic dose, which can help to tackle antibiotic resistance as well as counter its side effects. For sustainable development of antimicrobials against drug resistant bugs, novel alternative strategies need to be explored in the near future. Alternative therapies can help researchers to construct a toolbox containing a variety of antimicrobial agents, which can be used alone, in combination with other agents, or in rotation.

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ANTIMICROBIAL RESISTANCE

Antibiotic resistance is a state when microorganisms show resistance to an antibiotic upon exposure. Antibiotic resistance is a global problem which can cross international boundaries and spread between continents in recent times. Various forms of resistance spread with astonishing speed which could not be paralleled by the development of novel antimicrobial products. Researchers have described antibiotic resistant microorganisms as "nightmare bacteria" that can "pose a catastrophic threat" to global population. The problem is so serious that majority of pathogenic bacteria such as *Streptococcus pneumoniae, Mycobacterium tuberculosis, Acinetobacter baumannii, Neisseria gonorrhoeae, Klebsiella pneumoniae, Enterococcus faecium, Staphylococcus aureus, Pseudomonas aeruginosa*, and species of *Enterobacter, Shigella* and *Salmonella* are now resistant to most of the available antibiotics (Nathan, 2014). Perhaps the single biggest public health threat today is antibiotic resistance (Laxminaraya et al, 2013). For instance gonorrhea, was treatable with penicillin in the 1970s but now has become resistant even to third generation oral cephalosporins (Fernandes et al, 2017). Drug resistance causes 25 000 deaths per year ("ECDC/ EMEA Joint Technical Report", 2009). Similarly, in same epoch in the USA and China, resistance caused 100 000 and 80 000 deaths, respectively (Prestinaci et al, 2015). Antibiotic resistance can lead to escalating costs and deterioration of health care systems. Patients suffering from drug resistant nosocomial (hospital acquired) bloodstream infections or infection following consumption of food contaminated with antibiotic resistant pathogens experience longer recovery and a higher occurrence of septicemia and mortality (Angulo et al,2004).

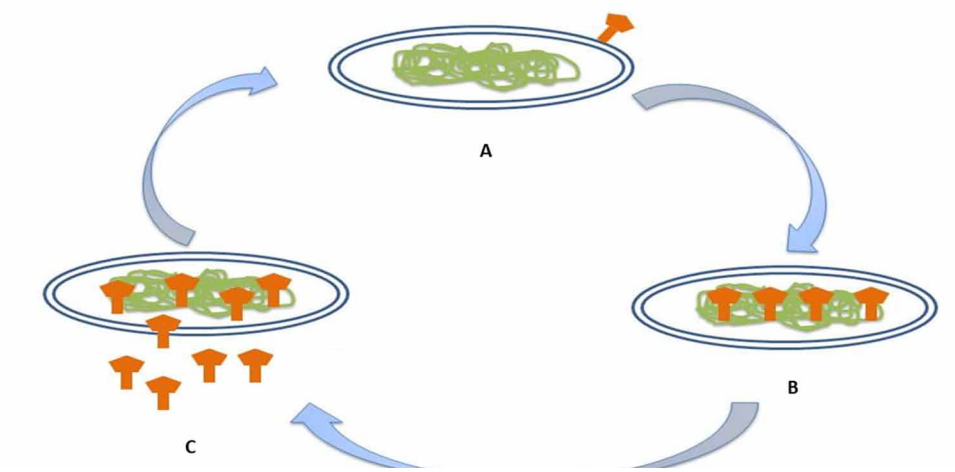
There are several reasons that contribute towards antimicrobial resistance crisis. These includes overuse of antibiotics, inappropriate prescription, extensive non-clinical use, and slow development of newer antibiotics. The overuse and misuse of antibiotics in clinical practice clearly leads the evolution of antibiotic resistance. A number of epidemiological studies have established a direct correlation between antibiotic utilization and emergence of drug resistant bacterial strains. In bacteria, genes which confer antibiotic resistance can be inherited from closely related or from nonrelated on the mobile genetic elements which primarily includes plasmids (Reed et al, 2014). Antibiotics are poorly regulated and easily available over the counter without a proper prescription (Michael et al, 2014). Poor regulatory system results in antibiotic misuse due to their easy availability and low cost. Faultily prescribed antibiotics have dubious therapeutic effects and may expose patients to the possible complications of antibiotic treatment (Murthy et al, 2015). Subinhibitory antibiotic concentrations can also promote the development of antibiotic resistance by supporting genetic alterations (Viswanathan et al, 2014). The antibiotics used in farm animals are ingested by humans when they consume such foods (Golkar et al, 2014). Even

environmental use of antibiotics also affects the microbiome (Bartlett et al, 2013). Antibacterial products used frequently for hygienic and cleaning purposes, also contribute to the current problem, as they may lead to development of low level of immunity to these environmental pathogens. The advancement in developing new antibiotics, an approach that had been successful at tackling bacterial pathogens in the past decades, had essentially slowed down due to economic and regulatory barriers (Bartlett et al, 2013). The emergence of antibiotic resistance has become a critical problem in modern medicine and because of this mankind is reentering the "pre-antibiotics" era.

Alternative Therapies, a New Tool from the Old Books

Increasing antibiotic resistance has prompted clinicians to prescribe fewer antibiotics, which, in turn has led to search for alternatives to antibiotics. In post antibiotic era, treatment of drug resistant bacterial pathogens is cumbersome and there is a pressing need to consider alternative options. Alternative therapies has gained attention in antibiotic resistance era especially lytic bacteriophages, endolysins, antibacterial nanoparticles, plant extracts, quorum quenching and other non-antibiotic antimicrobial products. These have been acquired the highest priority and possible options in the modern health care system. Alternative therapies contain the potential to reduce the antibiotic usage, tend to be safer upon usage and increases the immune potential of the host to counter pathogen.

Figure 1. Image showing potential alternative therapeutic agents to antibiotics



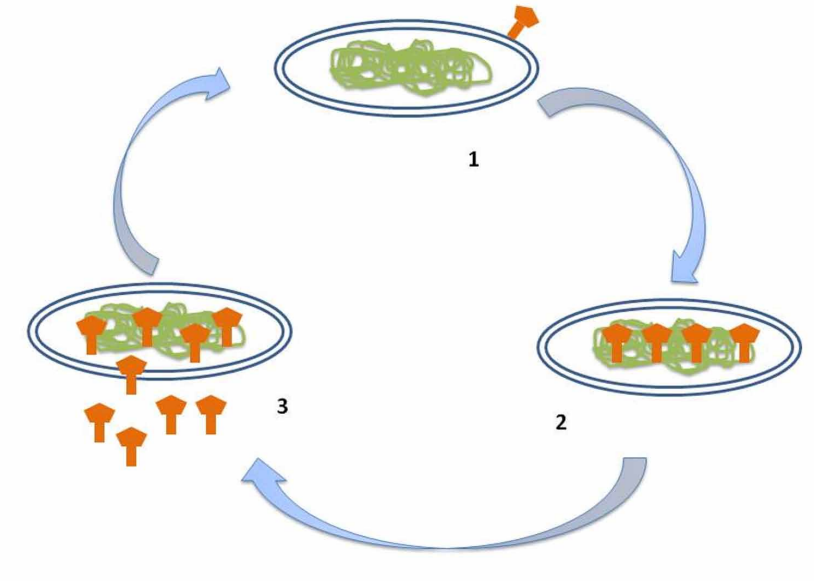
Lytic Bacteriophages

Fredrick Twort and Felix D'Herelle proposed the use of bacteriophages/phages (viruses that infect bacteria) to treat bacterial infections. The first reported bacteriophage based clinical trial was performed by Richard Bruynoghe and Joseph Maisin in 1921 ("BACTERIOPHAGE THERAPY", n.d.). In their clinical trails, they used phages to treat Staphylococcal skin disease by injecting the phages into/around surgically opened lesions. However, following the discovery of antibiotics, attention on bacteriophages as potential antimicrobial tools for controlling bacterial infections decreased swiftly (Sulakvelidze et al, 2001). With the emergence of antibiotic resistance, application of bacteriophages or their products in eradicating infections caused by *E. coli, P. aeruginosa*, *K. pneumoniae, S. aureus* and proteus strains in human and animal models has increased (Barrow et al, 1997). Phage therapy is beneficial over antibiotic therapy because of the high specificity of phages whereby they have an effect on the target bacteria only and are non-toxic to normal microflora. Phages are self-replicating, self-limiting and are successful against multidrug resistant pathogens. Phage production is uncomplicated, fast and relatively economical (Azeredo et al, 2008). Phages can be used in concoction with other antibacterial agents including other phages (phage cocktails), the lytic range of phage products can be much wider than the range of activity of monophage (Liu et al, 2020; Yang et al, 2020). Phage cocktail loaded liposomes also lead to fast resolution of the infection process as compared to non-liposomal free phage cocktail. Phages showed antibody mediated inactivation when used without delivery system, but liposomal delivery system provided 100% immune protection from anti-phage antibodies as constant phage titre was seen and can be used further to evade antibody mediated phage inactivation (Singla et al, 2016). Use of model organisms to treat cumbersome pathogens such as *Mycobacterium tuberculosis* using phage therapy has also been suggested in literature (Gondil et al, 2008). A recent clinical trial of intravesical application of bacteriophages for treatment of urinary tract infections showed non-inferiority to standard antibiotic treatment but opened a new prospective of well-designed clinical trials for therapeutic application of phages (Leitner et al, 2020).

Endolysins

Endolysins are phage encoded peptidoglycans hydrolases (PGHs) that are employed by most lytic phages to degrade the peptidoglycan layer of the bacterial host at the end of their lytic-multiplication cycle (Young et al, 1992). Peptidoglycans, as we know, is a most important structural component of bacterial cells supporting the internal structures inside the cell. This membrane also helps in maintaining the turgor

Figure 2. Phage mediated bacterial lysis (a) attachment (b) multiplication (c) lysis (d) image showing clear plaques after phage infection



pressure of 20-50 atmosphere in case of gram-positive organisms (Whatmore et al, 1990). Breaching of this peptidoglycan layer leads to changes in turgor pressure and lysis of bacterial cell that results in liberation of virion progeny along with other bacterial cell components in the outer environment. This disruption of bacterial host cells is time dependent as the liberation of phage progeny occurs with the help of another protein called 'Holin' which is produced during the release of double-stranded bacteriophage exactly at the end of lytic cycle (Wang et al, 2000). Holin forms pores in host's cell membrane that helps endolysins to reach and degrade peptidoglycans. Cpl-1, a lytic enzyme produced by phage Cp-1, exclusively and rapidly lyses several serotypes of *S. pneumoniae*, including antibiotic susceptible and antibiotic resistant strains, and exerts synergistic antibacterial activity with well-known antibiotics (for example penicillin and gentamicin) *in vitro* (Loeffler et al, 2003). Cpl-1 was shown to be beneficial in animal models of pneumococcal diseases such as sepsis, endocarditis and meningitis (Entenza et al, 2005). Other than Cpl-1 several potent anti-streptococcal phage lysins have also been reported in literature which mainly includes PlyC, ClyV, ClyR, Cpl-7, ClyJ, and Cpl-711 (40-45). Recently a chitosan based Cpl-1 lysin delivery system has been reported to increase its *in-vivo* antibacterial potential (Gondil et al, 2021). Anti-staphylococcal phage lysins have been also extensively studied in literature as various endolysins (lysK, ClyF, $CHAP_{K,}$ Ply187, LysGH15 and MR10) has been reported so far (Gondil et

al, 2020). *S. aureus* specific lysin MV-L derived from phage φMR11 rescued mice from MRSA fatal infection. MV-L also showed the activity against vancomycin-resistant strains when used in combination with vancomycin resulting in the increase of efficacy of a non-effective antibiotic (Rashel et al, 2007). ClyS, an endolysin bioengineered construct from Twort phage lysin and phiNM3, has also showed synergy with oxacillin to treat murine oxacillin resistant MRSA infections. It opened an avenue for use of discontinued antibiotics due to resistance issues (Daniel et al, 2010). Chopra *et al* also showed efficacy of MR-10 endolysin alone and along with antibiotic as a therapeutic agent to control MRSA induced burn wounds (Chopra et al, 2016). The exogenous use of endolysins in case of gram-negative pathogens is limited because of the outer membrane which acts as a permeability barrier for entry of endolysins into the peptidoglycan layer. However, some endolysins are known to permeate the outer membrane as LysAB2 derived from phage φAB2 can disrupt *A. baumannii* by facilitating the formation of transmembrane pores by its amphipathic α-helix (Lai et al, 2011). LysPA26, an endolysin against *P. aeruginosa* showed its antibacterial activity without any pretreatment for destabilizing the outer cell membrane. LysPA26 showed its potent antibacterial activity as it can reduce 4 log units of *P. aeruginosa* in 30 minutes time interval (Guo et al, 2017). In a recent study, LysAB54 also exhibited high degree of antibacterial activity against number of gram-negative pathogens (Khan VSG, et al, 2021). Bioengineered Artilysins such as Art-175 and Art-240 have also shown their potential in controlling gram-negative pathogens (Briers et al, 2015) . The spectrum of gram negative endolysins is expanding markedly with advancement of molecular engineering techniques.

Antibacterial-Nanoparticles

Silver in it salt form has been used for treatment of burn wounds and chronic wounds. Carl S.F Creed‘s in 1881 treated ophthalmia neonatrum, and cured it as the solution used for eye drop was silver nitrate. Recently, due to emergence of drug resistant strains, antibiotic usage is limited (Chopra et al, 2007). These factors renewed the interest of scientific community in silver salts and led to exploitation of silver salts to give better and effective alternative therapy. Nanoparticles exhibit a size range of 1-1000 nm. The use of metallic nanoparticles is increasing as potent antibacterial agent because of their large surface area to volume ratio (56). Nanotechnology is emerging as a rapidly growing field along with its applications. Many types of metallic antibacterial nanoparticles like zinc, silver, copper, titanium, magnesium, and gold have been reported but silver nanoparticles are known to have significant antimicrobial activity (Schabes-Retchkiman et al, 2006). In recent studies, Histidine capped silver nanoparticles, sodium acetate capped silver nanoparticles, glycolic acid functionalized silver nanoparticles, sodium benzoate capped silver nanoparticles

and Seabuckthorn capped silver nanoparticles showed significant activity against drug resistant and food-borne pathogens (Kumar et al, 2017). Silver nanoparticles can also be used as disinfectant, however, at high concentrations it may be toxic to mammalian tissues. The recent studies support that silver nanoparticles can be exploited in medicine for burn, dental treatments and coating material used in hospital so that biofilm formation on instruments can be reduced (Abbasi et al, 2016). Moreover, silver nanoparticles can be used to make water potable for drinking. It can also be used as a sunscreen agent in various cosmetic preparations. Wound dressings containing silver as an antimicrobial agent are very popular (Castellano et al, 2007). Silver nanoparticle diffusion is limited within biofilms matrix because of the presence of meshes of exopolysaccharides (EPS) network and environmental DNA (eDNA). Peulen *et al* showed the diffusion coefficient of 2 nm silver nanoparticles in a biofilm, that remained at 86% of its actual value (Peulen et al, 2011). In nanometric form, antibacterial activity of silver is accentuated. As it can easily enter the cells, binds to various metabolic enzymes, inhibit respiratory chain, alter DNA synthesis and induce oxidative stress to bacteria resulting in degradation of bacterium.

Copper in metallic micrometric size do not exhibit any antibacterial activity as compared to copper nanoparticles. As copper nanoparticles can oxidize easily and soluble ions can confer toxicity to bacterial cells. Copper nanoparticles in polylactic matrix have been shown to inhibit the growth of *Pseudomonas* spp (Longano et al, 2012). Ananth *et al* showed the antibacterial activity of copper nanoparticles against a range of gram positive bacteria *Streptococcus parauberis, Streptococcus iniae* and gram negative bacteria *E. coli and Vibrio anguillarum* (Ananth et al, 2015). Titanium is always a metal of choice for making implants during past few decades (Chouirfa et al, 2019). Nowadays titanium nanoparticles are being investigated for their antibacterial efficacy. Titanium also showed potent antibacterial activity against gram positive strains such as *S. aureus* and *S. epidermidis*. Antibacterial activity of titanium resulted from photocatalytic reaction under solar light or exposure to light, producing OH that can distrupt bacterial cell wall (Vimbela et al, 2017). Zinc has also been used in biomedical applications due to its abundance and low toxicity (Jiang et al, 2018). Zinc oxide nanoparticles confer antibacterial effects which possibly result from reactive oxygen species generation and Zn^{2+} release. Oxidative stress resulting from superoxide anions, hydrogen peroxide and hydroxide, may damage lipids and proteins, whereas zinc ions can possibly disrupt important metabolic machinery and pathways. Zinc oxide nanoparticles showed potent antibacterial activity against *E. coli*, *Salmonella*, *L. monocytogenes*, and *S. aureus* and minimal cytotoxicity on mammalian cells (Jones et al, 2018). Magnesium is biocompatible and biodegradable metal which can inhibit bacterial growth by generating oxidative stress. Magnesium oxide nanoparticles also exhibit antibacterial, anti-inflammatory, antioxidant as

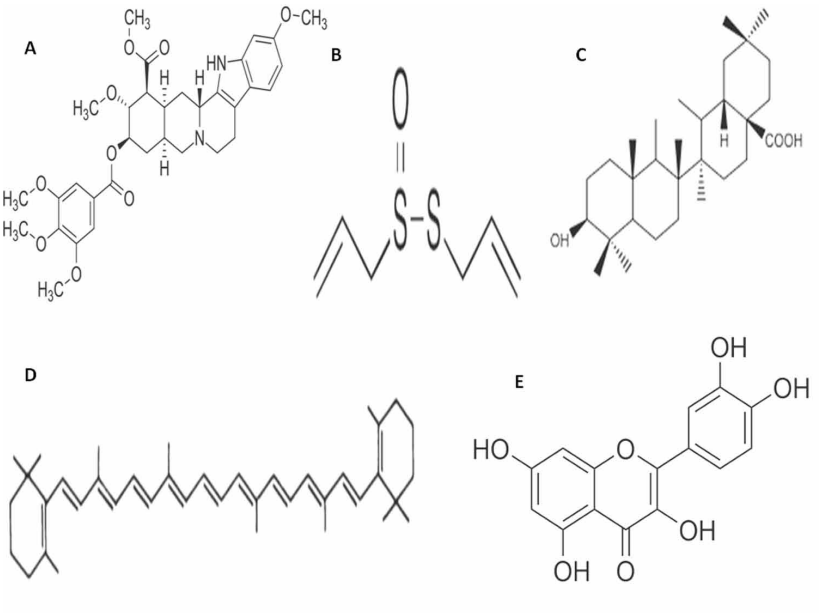
well as bone regeneration capability which makes them suitable candidate for use in bone and connective tissue infections (Sushma et al, 2016).

Plant Extracts

Plant extracts or phytochemicals constitute a range of chemical compounds of natural origin that confers color, flavor, aroma and texture. These compounds are widely distributed in fruits, vegetables, legumes, seeds and herbs. Phytochemicals are classified as alkaloids, sulphur containing groups, terpenoids, carotenoids and flavanoids according to the biosynthetic grouping. Alkaloids are organic nitrogenous bases with their variable chemical structure, produced by a variety of organisms such as plants, bacteria, fungi. These have been used in medicine since ancient times. Various alkaloids possess antibacterial and antiviral activity. Magesh showed that berberine, an alkaloid inhibits the biofilm formation in *K. pneumoniae* (Magesh et al, 2013) Another alkaloid reserpine, isolated from Rauwolfia (Apocynaceae) showed inhibitory activity against *K. pnuemoniae* biofilm. Sulfur-containing compounds such as allicin, ajoene and isothiocyanates have shown promising antibacterial activity against both gram-positive and gram-negative bacteria by interfering with biofilm formation and quorum sensing process. Allicin found in garlic inhibited *P. aeroginosa* PAO1 biofilm adhesion and also reduced the expression of quorum sensing (QS) regulated virulence factors (Militz et al, 2014). Terpenoids represent the large and most diverse class of chemicals derived from plants. The major function of these is in growth and development whereas some have a specialized function like protection from biotic and abiotic factors (82). Different types of terpenes like monoterpenes, limonoids, and triterpenes have been evaluated for their anti-biofilm and anti-QS activities. Cunha evaluated the antibacterial activity of oleanolic acid and ursolic acid (triterpenes) extracted from *Miconia ligustroides* against *K. pneumoniae* (Cunha et al, 2010). Carotenoids (tetraterpenoids) are well known to have nutritional properties and health benefits. Several studies have evaluated their biological and antibacterial activity against gram-positive and gram-negative bacteria (Honda et al, 2019). Polyphenols constitutes a diverse range of phytochemicals found abundantly in vegetables, fruits, and its products. In recent years, polyphenols have been labeled as good therapeutic and chemopreventive agents because of their direct antimicrobial and antibiotic modulation activity (Stermitz et al, 2009). Cheng and Huang studied 4 different flavonoids (galangin, kaempferol, quercetin and myricetin) and these inhibited the *K. pneumoniae* growth by inhibiting DNA B helicase with dNTPs at 10μM concentration (Chen et al, 2011). Quercetin mainly found in the diet (onion and propolis) is most extensively studied flavonoid due to its anti-cancer, anti-inflammatory, and antibacterial activities (Harborne et al,2000). Epigallocatechin (EGCG) (flavonoids with catechin) shows stronger

effects on gram positive bacteria than against gram negative bacteria as latter posses lipopolysaccharide layer. Cho showed weak and moderate activity of ECGC against imipenem-resistant *K. pneumoniae* (89). Flavonoids also show great antibacterial activity in synergy with antibiotics. Gopu et al has shown the synergistic effect of phytochemicals present in *Syzygium cumini*, an Indian blackberry with antibiotics against *K. pneumoniae* (Gopu et al, 2015).

Figure 3. Image showing representative chemical structures of each class of phytochemicals A:Resperine (Alkaloid), B:Allicin (sulphur containing compound), C:oleanolic acid (Terpenoid), D:β-carotene (carotenoid) and E:Quercetin (flavonoid)

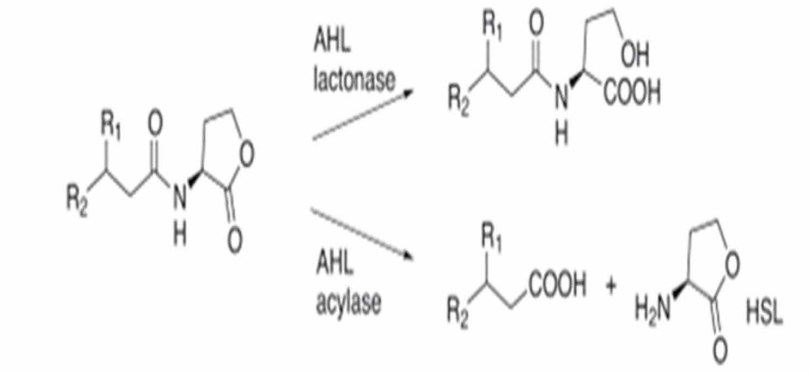


Quorum Quenching

Majority of the bacterial population is capable of monitoring their population density and gene expression through a specific controlling mechanism known as quorum sensing (QS), which helps to keep a check on the multicellular activities (growth and development of biofilm), horizontal gene transfer, host-microbe (symbiosis and pathogenesis), microbe-microbe interactions and switching 'on-off' of a gene that has direct effect on virulence factors of the microorganisms (Fuqua WC et al, 1994). This control is performed by synthesis, exchange, and perception of bacterial compounds, known as autoinducers (e.g. N-acyl homoserine lactone). N-acyl homoserine lactone

(AHL) molecules are highly conserved in different organisms as they contain the basic homoserine lactone ring, but differ in the length and substitution of acyl chain. Inhibition of bacterial quorum sensing system by disrupting or blocking quorum sensing molecules can be an attractive way of combating bacterial infection because it can think to exert a reduced pressure to select resistant bacterial strains from the population. This is known as quorum quenching and is mediated either through structural mimics of quorum sensing molecules or through inhibition of enzymes involved in the synthesis of quorum sensing molecules. Enzymes like AHL-lactonase and AHL-acylase can degrade quorum sensing molecules by hydrolyzing lactone ring or by liberating free homoserine lactone along with fatty acid (Czajkowski et al, 2009). The major advantage of using quorum sensing inhibitors is that as it does not enforce selection pressure, it restricts the evolution of drug-resistant strain. Both virulence and biofilm formation require cell to cell communication via quorum sensing mechanism. Inhibition of the same enhances efficacy of antibiotics that can be considered as an extra advantage (Kalia et al, 2014). Gupta *et al* showed that a combination of lactonase and antibiotic can potentially attenuate the virulence of *P. aeruginosa* in murine burn wound model (Gupta et al, 2016).

Figure 4. Image showing activity of AHL-lactonase and AHL-acylase.



Other Antimicrobial Products

Non-antibiotic antimicrobial products which include killing factors, ant microbial peptides, capsular depolymerases and pigments can be used to treat antibiotic resistant infections in near future. Killing factors is the term used to define the factors released by bacterial cells to kill the sibling cells during starvation. The nutrients released from the lysed cells are utilized by killer cells for their survival and spore formation.

Antimicrobial peptides are short antibacterial positively charged ribosomal or non-ribosomal peptides such as esculentin and bacteriocins. Esculentin-1a showed potent anti-bacterial activity against pseudomonas as well as pro wound healing activity (Grazia et al, 2015). Bacteriocin is generally defined as small ribosomally synthesized peptides of 12-70 amino acid long that are secreted by bacteria and inhibit the growth of closely related species and decrease the competition in the surroundings. Lactic acid bacteria are mostly considered as bacteriocin producers, which can infiltrate the outer membrane of gram-negative bacteria and control their growth. Bacteriocins can limit a growth of number of food spoilers/pathogens such as *S. aureus, E. coli, S. enterica*, *B. cereus* (de la Fuente-Salcido N, et al, 2008). Nisin, commercially used bacteriocin is currently used in more than 50 countries as an antibacterial agent (Liu et al, 2005). Pompilio showed reduction in biofilm formation of multidrug-resistant (MDR) *P. aeroginosa* strains isolated from a cystic fibrosis patient treated with bacteriocin (Pompilio et al, 2011). In a recent study, bacteriocins also showed potent antibiofilm activity against *P. aeruginosa* biofilms (Sharma et al, 2018). Capsular depolymerases disrupt the integrity of capsular matrix of bacterium and increases the susceptibility of pathogen to antibiotics. Treatment with capsular depolymerase increased the susceptibility to gentamicin and other antibiotics to *K. pneumoniae in-vivo and in-vitro* (Bansal et al, 2015). Microbial pigments also showed significant anticancer and antibacterial activites. Lee *et al*, Priya *et al* and Gondil *et al* showed an antibacterial activity of prodigiosin, a pigment from Serratia spp against several potent pathogenic microorganisms (Leet et al, 2014). The antibacterial activity of pigments is attributed to their ability to pass the bacterial cell membrane cause inhibition of cellular enzymes, induces ROS generation and DNA fragmentation (Darshan et al, 2016).

CONCLUSION

Alternative therapies present themselves as an attracting option to combat drug resistant superbugs, which are exceedingly difficult to eradicate with established drug therapies. Alternative therapies have gained success in their initial attempts such as *in-vitro* models, *ex-vivo* as well as *in-vivo* animal models but the studies are extremely limited in terms of human healthcare system. Some therapies such as phytochemicals, silver nanoparticles lack exact elucidation of their mechanism which makes treatment process trickier. On other hand personalized therapy with bacteriophages have gained attention of scientists in developed countries. Alternative therapies can help in combating antibiotic resistance and assist sustainable use of accessible antibiotics but their validation in human healthcare system should be encouraged and supported.

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Chapter 9
Ancient Pediocin to Innovative Antimicrobial

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ABSTRACT

Multi-drug resistance among patients suffering from infectious diseases has reached such proportions as to render them ineffective. WHO has to put out advisories time and again as to regulate their use. The presently available antibiotics are targeted at inhibiting vital biochemical pathways of pathogens, like nucleotide, protein, or cell wall synthesis in a very specific manner. Antibiotics have been rendered ineffective due to chemical modification, gene mutation, or transport mechanisms employed by pathogens. The novel approach to this problem can be naturally occurring antimicrobial peptides like bacteriocins produced by food grade bacteria. Pediocins produced by pediococcal strains have been found to inhibit a broad spectrum of pathogens by mechanisms that are robust enough to withstand development of resistance. Thus, these pediocins are attractive molecular precursors to develop novel antimicrobials. However, their application as such poses challenges that can be overcome with developing innovative technologies of chemical modifications and delivery strategies.

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INTRODUCTION

The emergence of Multi Drug Resistant pathogens in last few decades is posing a real threat to the world community. With emerging new pathogenic diseases, the repertoire of drugs suddenly seems to be woefully inadequate. This scenario becomes scarier when we factor in lack of reported novel antimicrobials in the pharmaceutical research pipelines. A cause of concern is also an increasing population living in closely packed clusters in cities and increased mobility, creating conditions conducive to quick spread of infectious agents as well as their antimicrobial resistance.

Such is the extent of the problem that in 2017, World Health Organization (WHO) published its first ever list of antibiotic-resistant "priority pathogens" –basically cataloguing 12 families of bacteria which were identified as greatest threat to human health. So much so that WHO further prioritized them into critical, high and medium based on the urgency to develop novel antibiotics specially for pathogens spreading in hospitals, threatening the medical fraternity itself (Table 1). ESKAPE is the acronym of six such antibiotic resistant species of pathogenic bacteria, the six species are ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter.*

Table 1. WHO prioritization of antibiotic resistant pathogens (WHO, 2017)

Priority 1: CRITICAL	Priority 2: HIGH	Priority 3: MEDIUM
Acinetobacter baumannii, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant *Enterobacteriaceae*, carbapenem-resistant, extended spectrum β-lactamase (ESBL) producing	*Enterococcus faecium*, vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant *Helicobacter pylori*, clarithromycin-resistant *Campylobacter* spp., fluoroquinolone-resistant *Salmonellae*, fluoroquinolone-resistant *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant	*Streptococcus pneumoniae*, penicillin-non-susceptible *Haemophilus influenzae*, ampicillin-resistant *Shigella* spp., fluoroquinolone-resistant

BACKGROUND

In order to curb this spread, a few restrictions were imposed on widespread use of antibiotics and only the permitted antibiotics and other chemicals to be used in feed supplements were allowed. However, the non-compliance with these regulations at

country and regional level is being encountered all over the world, thus, compromising the health and overall wellbeing of mankind. To cite an example the recently released WHO (2018) guidelines for treatment of MDR/RR-TB, recommended with drawing kanamycin and capreomycin; from long term treatment regime, keeping in view the spread of resistance to the same. Thus both consumer and manufacturer are searching and looking forward to newer and safer alternatives to antibiotics.

The mechanisms of action of presently available antimicrobials involve inhibiting a pathway essential for pathogen survival like synthesis of nucleotides, cell wall and proteins. Most of these interventions are inhibitors of proteins involved in the above mentioned pathways as depicted in Figure 1.

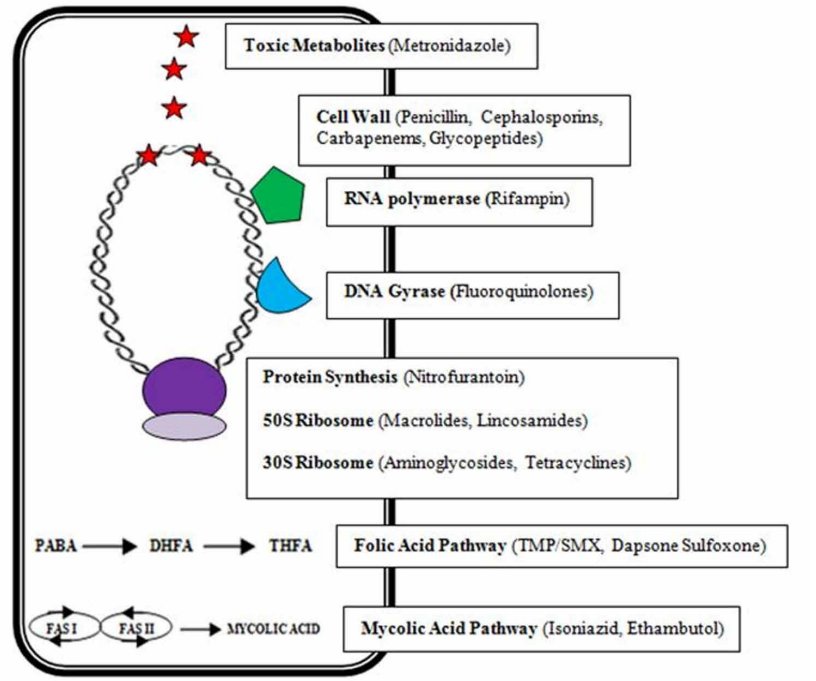
A functional mutation in such proteins as mentioned above, that influences their activity or expression, can result in the survival and proliferation of the microorganism in presence of such antimicrobials resulting in propagation of antibiotic resistance phenomenon. The functional mutation may influence either the activity or the expression level of the target protein; thus the occurrence and evolution of antibiotic resistance basically involves mutagenesis of particular target protein. This then acts as the main source of multi drug resistance (MDR) which spreads with the exchange of genetic material in nature via spread of extrachromosomal plasmids, transposons and acquired genetic elements among microbial populations. Apart from these mechanisms the overexpression of efflux pumps capable of pumping out variety of antibiotics from the bacterial cells, also result in MDR ; which once established provides the pathogen a selective advantage and helps in its spread.

Various mechanisms have been discovered that underlie antibiotic resistance. Inactivation of the drug, its modification or alteration of its binding site, change in bacterial cell's membrane permeability and biofilm formation are some such mechanisms that render the pathogen resistant to the drug. Bacterial production of enzymes that can modify the antibiotic molecule and lead to its inactivation has been observed to be the mechanism underlying carbapenem, aminoglycosidic antibiotic and chloramphenicol resistance by producing β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases respectively.

Another mechanism involves mutation in the gene encoding for drug binding protein like the penicillin-binding proteins (PBPs), leading to penicillin resistance. Bacterial membrane permeability determines the level of antibiotic within a bacterial cell. The membrane permeability is dependent on the presence of porins in the membranes of Gram-negative bacteria, which form channels for passage of hydrophilic antibiotics. A reduction in porin protein OprD in *P. aeroginosa* results in less drug entering bacterial cell rendering it to be imipenem resistance (Papp-Wallace et al., 2011). Similar multidrug resistance mechanisms underlie loss of outer membrane proteins known as OmpK35 and OmpK36 along with production of β-lactamase enzymes in K. *pneumonia* (Sugawara et al., 2016). The intracellular

level of antibiotic is also dependent on the efflux pumps present in bacterial cell membranes. An increase in such pumps in some bacteria render them to be multi drug resistant. Efficient secretion of biofilm forming exopolysaccharides also render many bacterial strains resistant to antibiotics.

Figure 1. Popularly used antibiotics and their target protein pathways



Hence for development of novel antibiotics, such moieties need to be identified as are based on newer mechanisms of action other than protein inhibition. Presently scientists are investigating bacteriophages, lytic enzymes of phages, probiotics, bacteriocins and antimicrobial peptides from various organisms; as potential antibiotics. The main premise underlying these biologics is the robustness of these systems to evolution of resistance. Biologists are also looking at other common traits of pathogens like quorum sensing and siderophores, though such approaches are very limited in their inhibitory potential presently and cannot be used solely as standalone antibiotics (Allen et al., 2014; Ross-Gillespie et al., 2014; Czaplewski et al., 2016). An example of bacteriophage use for prophylaxis of respiratory tract infections like tonsillitis among Russian military personnel was reported recently (Akimkin et al.,

2016). Since the mechanism underlying phage lysis is known even lytic enzymes of phages when tested, gave positive result in a mouse model in gastrointestinal and oral infections (Lood et al., 2014). More recently, bacteriophage derived lysins (endolysins and holins) have been investigated for their antimicrobial properties. These lyase-based antibacterial drugs kill the bacteria by destruction of the bacterial cell wall structure. The endolysins act on the amide bond between peptidoglycans, and holin being a transmembrane protein acts by creating holes in the bacterial cell membrane (Gondil et al., 2020). Although the phage based lysins are mainly known for their activity against Gram-positive bacteria. A recent study has reported the use of engineered lysin complexes called "artilysins" that are capable of penetrating the lipopolysaccharide-rich cell wall of Gram-negative pathogenic bacteria such as *Helicobacter pylori* with bacteriolytic effect against the pathogen (Xu et al., 2021).

Antimicrobial peptides (AMPs) are another class of naturally occurring molecules found in organisms ranging from prokaryotes to humans. These peptides are active against a broad spectrum of bacteria, yeasts, fungi, viruses and cancerous cells. These properties form the basis for development and use of AMPs as antibiotics to overcome the challenge of drug resistance (Zasloff, 2002; Haney and Hancock, 2013; Zhang & Gallo, 2016; Boparai & Sharma, 2020). AMPs expressed in insects and plants protect them against pathogenic microorganisms and microbial cells produce these peptides to safeguard their ecological niche from other fellow microorganisms (Hoffmann & Hetru, 1992; Boman, 2003; Lemaitre et al., 1996). AMPs are known to play a wide array of roles including immunomodulatory properties, immune homeostasis. More than 60 peptide drugs have already reached the market and several hundreds of novel therapeutic peptides are in preclinical and clinical development. Rational designing can be used further to modify the chemical and physical properties of existing peptides. The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) contains more than 2,500 entries for AMPs at present (Wang, 2020). There are open access resources available with comprehensive research data and machine learning prediction tools. Some examples include collection of antimicrobial peptides or CAMP (www.camp.bicnirrh.res.in), CAMPSign, and ClassAMP that have been developed to enhance research on AMPs (Waghu et al., 2016). Table 2 provides a comprehensive list of naturally occurring AMPs along with their antimicrobial activity.

Probiotic Lactic Acid Bacteria (LAB) produce potent antimicrobial metabolites and peptides that are considered to be suitable for exploration as antimicrobials since their mode of action is different from the antibiotics mentioned above. Probiotics are commonly defined as viable microorganisms (bacteria or yeasts) capable of exerting a beneficial effect on the health of the host upon ingestion (FAO/WHO,

2002; ICMR, 2011). These probiotics produce antimicrobial like bacteriocins, organic acids (lactic and acetic acid), hydrogen peroxide, and antifungal peptides making them efficient at eliminating related and not so related strains of bacteria and fungi from their surroundings. An extensive research on exploring this antibiotic potential of different probiotic LAB strains is being carried out worldwide.

Table 2. Comprehensive list of naturally occurring antimicrobial peptides

Source	AMPs	Antimicrobial activity	Reference
Insects	Acaloleptin	G^+, G^-	Vogel et al., 2014; Mylonakis et al., 2016
	Andropin	G^+	McCaskey et al., 2016; Abry et al., 2017
	Apidaecin IA	G^-	Mylonakis et al., 2016; Farouk et al., 2017
	Cecropin	G^-	Lee & Lee, 2015; Mylonakis et al., 2016
	Defensin- α	G^+, G^-	Price et al., 2015; Mylonakis et al., 2016
	Drosomycin	F	Mylonakis et al., 2016; Allocca et al., 2018
	Holotricin	G^+, G^-	Mylonakis et al., 2016; Thiyonila et al., 2018
	Sapecin- α	G^+, G^-	Lee et al., 2014; Manabe & Kawasaki,017
	Tenicin 1	G^+, G^-	Yang et al., 2017
	Thanatin	G^+, G^-	Duwadi et al., 2018; Ma et al., 2019
Humans	Cathelicidins	F, G^-, G^+	Sheehan et al., 2018
	A Defensins	F, G^-, G^+	Candille et al., 2007; Schaal et al., 2018
	Human Histatin 8	F, G^-, G^+	Khurshid et al., 2017; Sun et al., 2020
	LL37	F, G^-, G^+	Singh et al., 2013; Baxter et al., 2017
Animals	Androctonin	F, G^-, G^+	Hetru et al., 2000; Panteleev et al., 2017
	Bactenecin	G^-, G^+	Young-Speirs et al., 2018
	Brevinin	G^-, G^+	Savelyeva et al., 2014; Timmons et al., 2019
	Buforin II	F, G^-, G^+	Lee et al., 2008; Sun et al., 2015
	Cupiennin	G^-, G^+	Upadhyay, 2018
	Dermaseptin S1	G^-, G^+	Belmadani et al., 2018
	Lycotoxin	G^-, G^+	Tahir et al., 2018
	Tachyplesins	G^-	Kuzmin et al., 2017
Plants	Hevein	F	Rojas et al., 2001; Coulen et al., 2017
	Purothionins	G^+, G^-	Thao et al., 2017

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Table 2. Continued

Source	AMPs	Antimicrobial activity	Reference
Microorganisms	Nisin	G^+	Gharsallaoui et al., 2016; Mills et al., 2017
	Alamethicin	G^+	Su et al., 2018
	Enterocin	G^+, G^-	Braiek et al., 2018
	Hominicin	G^+, G^-	Kim et al., 2010; Ebrahimipour et al., 2014
	Ericin S	G^+	Sharma et al., 2018
	Plantaricin A	G^+, G^-	Jiang et al., 2018
	Carnobacteriocin B2	G^+, G^-	Hammi et al., 2016
	Leucocin A, C	G^+, G^-	Chen et al., 2018; Li et al., 2021
	Subtilin	G^+	Singh et al., 2017
	Pyrularia thionin	G^+, G^-	Guzman-Rodriguez et al., 2015
	Microcin J25	G^-	Zhao et al., 2016
	Gramicidin A	G^+, G^-	Muhammad et al., 2016
	Pediocin PA-1/ AcH	G^+	Araujo et al., 2016
	Mesentericin	G^+	Arakawa et al., 2016
	Carnobacteriocin BM1, B2	G^+, G^-	McCormick et al., 1996; Tulini et al., 2014
	Streptin 1	G^+	Bosma et al., 2011
	Planosporicin	G^+, G^-	Gajalakshmi, 2017
	Gassericin A	G^+, G^-	Pandey et al., 2013; Maldonadi-Barragan et al., 2016
	Circularin A	G^+, G^-	Perez et al., 2016
	Divercin V41	G^+	Brillet-Viel et al., 2016
	Listeriocin 743A	G^+	Kalmokoff et al., 2001; Wan et al., 2013
	Plantaricin C19	G^+	Wang et al., 2018
	Enterocin P	G^+	Le et al., 2014; Ben Braïek et al., 2017
	Subtilosin A	G^+, G^-	Thennarasu et al., 2005; Venturina et al., 2016
	Plantaricin ASM1	G^+	Bhat et al., 2018
	Lichenin	G^+, G^-	Hollmann et al., 2018

ANTIMICROBIALS FROM PROBIOTIC LAB (MAIN FOCUS OF THE CHAPTER)

Lactic acid bacteria have been extensively exploited in Food industry to produce various food products, pointing to their nutritional value and safety status on consumption. Further, these are being prescribed along with antibiotic therapy as additives, to overcome the mass extermination of microbes in human system caused by antibiotic therapy (FAO/WHO, 2002). These bacteria include a diverse group of Gram-positive, heterotrophic and relatively fastidious bacteria, which produce lactic acid as an end product of carbohydrate fermentation. Genus *Lactococcus, Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* form the microbial core are of the family Lactobacillaceae. The ubiquitous occurrence of LAB in natural food products, along with their Generally Recognized as Safe (GRAS) status and ability to exert beneficial health effects beyond basic nutrition have led to their exploration for other applications. Especially in therapeutics as a recent study pointed to use of probiotic strains of Lactobacillus showing antimicrobial effect against MDR strains isolated from urinary tract infections (Naderi et al., 2014). LAB produce various antimicrobial compounds, such as lactic acid, hydrogen peroxide, carbon dioxide, diacetyl and uncharacterized compounds, which are of low molecular mass and other high molecular mass compounds like bacteriocins (Ammor et al., 2006; Mobolaji & Wuraola, 2011; Sanlibaba & Gucer, 2015).

Some of these attributes are discussed in detail below:

1. Lactic Acid

The primary antimicrobial effect exerted by LAB species is attributed to the production of organic acid such as lactic acid, as a result of fermentation (Mobolaji & Wuraola, 2011). These bacteria produce lactic acid in L- or D-isomeric forms. L-lactic acid is desirable for food and pharmaceutical applications and is also used as starting material for biopolymer production. D-lactic acid is not associated with any applications due to its toxicity in human hosts. Due to this reason, metabolic engineering studies have been aimed at the production of pure L-lactic acid by homofermentative LAB. Furthermore, both the stereoisomeric forms of lactic acid display variations towards their antimicrobial activity, with L-lactic acid being more potent inhibitor than the D-form (Papagianni, 2012). The mechanism of antimicrobial action exerted by lactic acid is through interference with the maintenance of cell membrane potential. The inhibition of active transport across cell membrane leads to a reduction in intracellular pH and inhibition of metabolic functions (Rattanachaikunsopon & Phumkhachorn, 2010). Lactic acid production and lowering of pH has been found to be affected by type of bacterial strain or species, culture composition and growth conditions (Olaoye

& Onilude, 2011). The acid production inhibits the growth of Gram-positive and Gram-negative bacteria, yeast and moulds (Rattanachaikunsopon & Phumkhachorn, 2010). Most of the lactic acid is in undissociated form at low pH, with toxicity towards several bacterial, fungal and yeast strains. Due to differential sensitivity of different microorganisms towards lactic acid, it was found to be toxic in case of spore-forming bacteria whereas non-toxic against yeasts and moulds (Yang, 2000).

2. Diacetyl

Diacetyl is a flavor compound in dairy products produced as a result of co-fermentation of citrate and lactose. Strains belonging to genera *Lactococcus lactis*, *Leuconostoc* and *Weissella* are commonly used as diacetyl producers (Kleerebezem et al., 2000). Citrate-utilizing LAB produce diacetyl during fermentation of milk for the production of butter, buttermilk and various types of cheese. Diacetyl is responsible for the typical buttery aroma in these dairy products. These properties have lead to development of metabolic engineering strategies for efficient production of diacetyl from lactose instead of citrate. Gram-negative bacteria are much more sensitive to diacetyl as compared to Gram-positive bacteria. Diacetyl affects the arginine utilization pathway by interaction with arginine-binding protein leading to growth inhibition of Gram-negative bacteria. Strains of *Listeria, Salmonella, Yersinia, Esherichia coli*, and *Aeromonas* are sensitive to diacetyl at a concentration of 350µg/ml (Yang, 2000; Ammor et al., 2006; Papagianni, 2012).

3. Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) has been widely used in food and pharmaceutical products, dental products, textiles, environmental protection. It is also involved in advanced oxidation and biochemical processes (Abbas et al., 2010). H_2O_2 produced by LAB strains under aerobic conditions, leads to denaturation of several enzymes by oxidation of their sulfhydryl groups. Further, it also has a strong oxidizing effect on membrane lipids and cellular proteins. It increases membrane permeability by peroxidation of membrane lipids, and generates bactericidal free radicals such as superoxide (O^{-2}) and hydroxyl (OH^-) radicals which can damage DNA (Yang, 2000; Ammor et al., 2006, Sunil & Narayana, 2008; Rattanachaikunsopon & Phumkhachorn, 2010). It can inhibit the growth of pathogenic microorganisms such as *Aeromonas hydrophila, Listeria monocytogenes, Yersinia enterocolitica,* and *Clostridium botulinum* type E (Yang, 2000; Ito et al., 2003; Zalan et al., 2005; Abbas et al., 2010).

4. Reuterin

Reuterin is a glycerol-derived antimicrobial compound produced under anaerobic conditions by a number of lactobacilli. Its production has been found to enhance in the presence of glycerol. It is capable of inhibiting the growth of fungal strains of *Aspergillus* and *Fusarium*, and plays an important role in neutralizing mycotoxins. It has potential antimicrobial activity against Gram-positive and Gram-negative bacteria, including enteropathogens, yeast, fungi, protozoa, as well as viruses (Nes et al., 2012). Many pathogenic species of *Candida*, *Clostridium, Listeria, Salmonella*, *Shigella, Staphylococcus,* and *Trypanosoma* are sensitive to reuterin (Yang, 2000).

5. Enzymes

Probiotics also inhibit pathogens by producing enzymes that either block toxin mediated effects or modify the toxin receptors, for example, degradation of *C. difficile* toxin receptors by *S .boulardii* enzymes in rabbit ileum (Pouthoulakis et al., 1993). Probiotics have also been reported to improve immunity of intestinal mucosa as shown by Kaila et al. (1995). Majamaa et al. (1995) pointed to adjuvant like effects on intestinal and systemic immune response by oral intake of probiotics and a special stimulation of Immunoglobulin A was observed in response to viruses. Probiotics are observed to enhance phagocytosis of intracellular pathogens.

6. Bacteriocins

The above discussed antimicrobial compounds are important when the live probiotics produce them and are used as whole cells, whereas bacteriocins have recently attracted attention as antimicrobial peptides as antimicrobial drug precursors for exploration as alternative therapy. Bacteriocins are ribosomally synthesized antimicrobial peptides with activity against other bacteria of the same or different genus and species (Soomro et al., 2002; Yang et al., 2012). Some LAB species also produce antimicrobial bacteriocins and bacteriocin-like compounds (Mobolaji & Wuraola, 2011). These are small thermostable or large thermolabile proteins or protein complexes with antimicrobial effect against other Gram-positive bacteria. The bacteriocin-producers are immune against their own bacteriocins and antimicrobial action is often displayed against closely related members (Zacharof & Lovitt, 2012). Bacteriocin production is affected by pH, nutrient sources and incubation temperature (Todorov and Dicks, 2005c; de Vuyst & Leroy, 2007). On the basis of biochemical and genetic characterization, LAB bacteriocins have been classified

into four distinct classes, namely Class I lantibiotics, Class 2 comprises of small, heat-stable nonlanthionine peptides, Class 3 includes large heat-labile proteins and Class 4 with complex bacteriocins having lipid and carbohydrate chemical moieties (Hernandez et al., 2005).

Bacteriocins harbor several attractive features which render them suitable for use as therapeutics. Their proteinaceous nature proves detrimental to microbes but harmless to humans. The non-toxicity of bacteriocins has already been proven in case of laboratory animals. These are generally non-immunogenic and thermo resistant. Some bacteriocins have broad bactericidal activity affecting majority of Gram-positive bacteria, while others affect pathogenic Gram-negative bacteria with compromised outer membrane integrity after osmotic shock or low pH or detergent treatment. The genetic determinants of bacteriocins are generally located on plasmids. These attributes facilitate genetic manipulation in order to increase the variety and efficacy of natural peptide analogues with desirable traits (Juodeikiene et al., 2012).

Nisin is the most thoroughly studied bacteriocin till date with commercial applications in food as biopreservation. It is produced by *Lactococcus lactis* and is the only FDA approved bacteriocin to date with activity against a wide range of Gram-positive and Gram-negative pathogenic microorganisms. It is most effective when applied at high concentration or on target cells pre-treated with EDTA (Todorov & Dicks, 2005c). The solubility and stability of nisin increases substantially with increasing levels of acidity. Nisin is stable at a low pH of 2 and can be autoclaved at 121ºC. Under alkaline conditions, the antimicrobial activity is reduced substantially, with complete inactivation at 63ºC and pH 11 after 30 min (Jozala et al., 2005). Studies have reported the production of various bacteriocins like acidophilin, alctocidin, lactolin, acidolin, lactobrevin and lactobacillin by strains of lactobacilli (Zacharof & Lovitt, 2012; Mokoena, 2017) *Lactobacillus rhamnosus* produces an antimicrobial compound inhibitory to the growth of *Bacteroides fragilis, Clostridium difficile, Escherichia coli*, *Streptococci* sp.*,* and *Salmonella* sp. (Banna et al., 2017). Several studies have advocated the therapeutic utility of bacteriocins (Evangelin et al., 2015; Mokoena, 2017).

ANTIMICROBIAL PEDIOCINS FROM PEDIOCOCCAL SP.

One genera belonging to Lactic acid bacteria is Pediococcus, recognized as a probiotic (FAO/WHO, 2002; ICMR, 2011) These are Gram-positive, highly fastidious, non-motile, non-sporulating, catalase-negative, homofermentative facultative anaerobes of family lactobacillaceae (Kawai et al., 2004). They produce lactic acid as a result of sugar fermentation and can survive at very low pH and high temperature as well (Gonzalez & Kunka, 1987). These were first isolated and characterized from plants by

Mundt et al. (1969). Apart from plants, these are commonly found in dairy products, and in alcoholic beverages (Galvez et al., 2007) and are used in the food industry and as silage additives (Nes et al., 1996; Kumar et al., 2012). They have also been found in human saliva and faeces (Jimenez–Diaz et al., 1993; Jong et al., 2006). The genus Pediococcus is comprised of *P. acidilactici, P. cellicola, P. claussenii, P. damnosus, P. dextrinicus, P. ethanolidurans, P. halophilus, P. inopinatus, P. parvulus, P. pentosaceus,* and *P. stilesii* representative species. Among genus *Pediococcus*, two species namely *P. acidilactici* and *P.pentosaceus* (Golledge et al., 1990; Mastro et al., 1990; Sire et al., 1992; Facklam & Elliot, 1995) are mostly studied and are commonly used in the fermentation of vegetables (Pederson, 1949) and meats (Smith & Palumbo, 1983; Kumar et al., 2012). *P. acidilactici* include food grade strains which secrete bacteriocin, pediocin PA-1.

Pediococci are capable of producing antimicrobial bacteriocins termed as 'Pediocins' which form an integral component of natural bacterial defense against other microorganisms. A number of pediocins have been identified and characterized from these prokaryotic organisms. These peptides are recently gaining attention due to their remarkable heat stability, activity and stability over a wide pH range, broad antimicrobial spectrum; high specificity and efficacy even in very low concentrations (Jack et al., 1995; Lavermicocca et al., 2000; Galvez et al., 2007; de Vuyst & Leroy, 2007; Kumar et al., 2012; Anu & Singh, 2018; Balandin et al., 2019).

Pediococcal strains have also been shown to fulfil the mandatory criterion for use as a probiotic. These acid and bile tolerant bacteria exert beneficial effects on host by efficiently adhering to intestinal epithelial cells (Kumar et al., 2012; Balgir et al., 2013). A study by Balgir et al. (2014) reported improved iron bioavailability and haemoglobin status of anemic subjects after oral consumption of a strain of *Pediococcus acidilactici* MTCC 5101 mainly due to production of acid and pediocin leading to killing of iron-leaching pathogens. Like other LAB, the antimicrobial activity in members of Genus Pediococcus is partly due to production of organic acids such as lactic acid and H_2O_2 and majorly attributable tothe presence of gene cluster for production and expression of pediocins (Ray & Daeschel, 1994; Jack et al., 1995; Galvez et al., 2007; de Vuyst & Leroy, 2007; Kaur et al., 2019). A large number of pediocins have been isolated and characterized from different Pediococcal sp. (Table 3).

Table 3. Pediocin producers and antimicrobial range of pediocins produced

Bacteriocin	Producer strain	Antibacterial range	Reference
PediocinAcH	*P. acidilactici* H, E, F, M	*Aeromonashydrophila, Bacillus cereus, Brochothrix, Clostridium perfringens, C. botulinum, Enterococcus faecalis, E. faecium, E. hirae, Escherichia, Listeria monocytogenes, L. innocua, L. seeligeri, Lactococcuslactis, Leuconostocmesenteroides, Micrococcus sedentarius, Pediococcusacidilactici, P. pentosaceus, Pseudomonas putida, Salmonella, Staphylococcus aureus, S. xylosus, Yersinia*	Bhunia et al., 1987, 1988, 1990; Ennahar et al., 1996
Pediocin PA-1	*P. acidilactici* PAC1.0 NRRL-5627	*B. cereus, L. bifermentans, L. brevis, L. plantarum, L. lactis, L. dextranicum, L. mesenteroides, L. monocytogenes, P. acidilactici, P. pentosaceus*	Pucci et al., 1988; Marugg et al., 1992
Pediocin PO2	*P. acidilactici* PO2	*B. coagulans, E. faecalis, L. curvatus, L. monocytogenes, L. mesenteroides, S. aureus, Streptococcus faecalis*	Hoover *et al*., 1988; Liao *et al*., 1993;
Pediocin JD	*P. acidilactici* JD-123	*L. monocytogenes*	Berry *et al*., 1990; Berry *et al*., 1991
Pediocin PC	*P. acidilactici* PC	*C. perfringens, Listeria, Leuconostoc, Pediococcus*	Jager & Harlander, 1992
Pediocin SJ-1	*P. acidilactici* SJ-1	*C. perfringens, L. brevis, L. plantarum, L. leichmanni, L. monocy-togenes*	Schved et al., 1993
Pediocin L50	*P. acidilactici* L50	*B. cereus, C. botulinum, C. perfringens, E. faecalis, L. monocytogenes, S. aureus, L. brevis, L. plantarum, L. sake* 148, *L. innocua, L. lactis, L. mesenteroides, P. acidilactici, P. pentosaceus*	Cintas et al., 1995
PediocinAcM	*P. acidilactici* M	*A. hydrophila, B. coagulans, B. cereus, C. perfringens, L. monocytogenes, S. aureus*	Elegado et al., 1997
Pediocin F	*P. acidilactici* F	-	Osmanagaoglou et al., 1998; Osmanagaoglou et al., 2000
Pediocin CP2	*P. acidilactici* MTCC 5101	*Aspergillus flavus, C. sporogenes, E. faecalis, L. brevis, L. bulgaricus, L. mesenteroides, L. monocytogenes, Micrococcus flavus, Neisseria mucosa, P. acidilactici, P. pentosaceus, Pseudomonas putida, P. aeruginosa, Staphylococcus albus, S. aureus, Streptococcus mutans, S. pyogenes*	Kaur & Balgir, 2004; Kaur & Balgir, 2006; Kaur et al., 2009
Pediocin SA-1	*P. acidilactici* NRRL B5627	*B. cereus, C. sporogenes, C. thiaminolyticum, E. faecalis, L. brevis, L. bulgaricus, L. casei, L. curvatus, L. jensenii, L. plantarum, L. sakei, L. lactis, L. monocytogenes, L. innocua, L. mesenteroides, M. flavus, M. luteus, P. acidilactici, P. pentosaceus, S. carnosus*	Anastasiadou et al., 2008
Pediocin A	*P. pentosaceus*ATCC 43200, ATCC 43201	*B. cereus, C. botulinum, C. perfringens, C. sporogenes, C. tyrobutyricum, E. faecalis, E. coli, L. monocytogenes, L. innocua, L. sake, L. brevis, L. plantarum, L. lactis, L. mesenteroides, P. acidilactici, P. pentosaceus, Salmonella typhimurium, S. aureus*	Ray & Daeschel (1994), Daeschel & Klaenhammer (1985), Grahm & McKay (1985)

continues on following page

Table 3. Continued

Bacteriocin	Producer strain	Antibacterial range	Reference
Pediocin N5p	*P. pentosaceus*	*Lactobacillus hilgardii, Leuconostocoenos, P. pentosaceus*E5p	Strasser de Saad & Manca de Nadra (1993), Strasser de Saad & Manca de Nadra (1995)
Pediocin PD-1	*P. damnosus* NCFB-1832	*Oenococcusoeni*, several food spoilage and pathogenic bacteria	Green et al., 1997
Pediocin ISK-1 (nukacin ISK-1)	Pediococcus sp. ISK-1	*Bacillus subtilis, L. casei ssp. casei, L. lactis, M. luteus, P. acidilactici*	Kimura et al., 1997, Sashihara et al., 2000
Pediocin K1	Pediococcus sp. KCA1303-10	*E. faecalis, E. faecium, L. monocytogenes*	Kim et al., 2000
Pentocin L	*P. pentosaceus* L	Broad inhibition spectrum, *B. subtilis, B. cereus*	Yin et al., 2003
Pentocin S	*P. pentosaceus* S	Broad inhibition spectrum, *B. subtilis, B. cereus*	Yin et al., 2003
Pediocin ACCEL	*P. pentosaceus* ACCEL	*B. subtilis, B. cereus, C. perfringens, L. helveticus, L. plantarum, L. monocytogenes L. lactis, P. pentosaceus, S. faecalis, S. epidermidis*	Wu et al., 2004
Pediocin ST18	*P. pentosaceus* ST18	*L. innocua, L. plantarum, Pediococcus* spp.	Todorov & Dicks, 2005a
Pediocin SM-1	*P. pentosaceus* SM-1	*C. thiaminolyticum, C. sporogenes, L. monocytogenes, L. innocua, Pediococcus* spp., several LAB species	Anastasiadou et al., 2008
Pediocin pK23-2	*P. pentosaceus* K23-2	Gram-positive bacteria, especially *L. monocytogenes*	Shin et al., 2008
Pediocin 05-10	*P. pentosaceus* 05-10	*Enterococcus, Lactobacillus, Leuconostoc, Listeria, Pediococcus, Streptococcus*	Huang et al., 2009
Bacteriocin ST44AM	*P. pentosaceus* ST44AM	*E. coli, Klebsiella pneumoniae, L. monocytogenes, L. innocua, L. ivanovii subsp. ivanovii, P. aeruginosa,* other LAB	Todorov & Dicks, 2005b
pediocin 2292	*Pediococcus acidilactici NCIM 2292*	*st Listeria monocytogenes, Staphylococcus aureus and Bacillus subtilis.*	Mandal et al., 2014

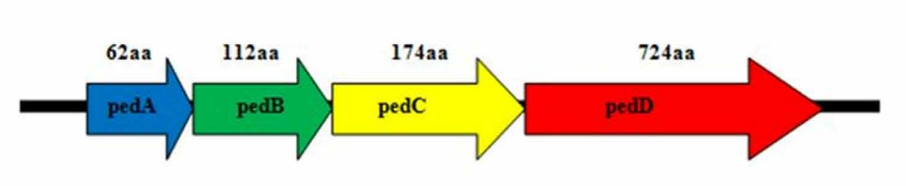
Pediocins are attractive antimicrobial peptides for developing alternative antibiotic therapies as: (i) they are safe for consumption, as they are completely digested in the gastrointestinal tract of humans and animals, (ii) being 10^3 to 10^6 times more potent than conventional antibiotics, (iii) being resistant to common thermal treatments for pasteurization or even sterilization (Abriouel et al., 2010).They display a broad antimicrobial range and have been found specially inhibitory to *Listeria monocytogenes*, a pathogen that causes listeriosis, fatal in as many as 20–30% of cases, and has been involved in neonatal deaths, miscarriages, severe meningitis and sepsis (Jackson et al., 2010). Another commercially produced bacteriocin is pediocin PA-1 produced by *Pediococcus acidilactici* and marketed as ATTATM 2431 (Yang et al., 2012; Sanlibaba & Gucer, 2015). Khalaf et al. (2016) reported the antimicrobial activity of bacteriocins against periodontal pathogens. A recent study has compared the antimicrobial activity of pediocins for root canal disinfection with conventional agents such as chlorhexidine (CHX) and calcium hydroxide (Ca(OH)2) and found it active against *Enterococcus faecalis* and *Staphylococcus epidermidis* biofilms (Ooi et al., 2019).Pediocin is an attractive antimicrobial agent against many pathogenic bacteria. Certain strains of pediococci also act as potent probiotics in modulating gut microbiota and aids in lowering cholesterol along with antidiabetic and antihypertensive properties. All these properties of bacteriocin-producing pediococci highlight their use as antimicrobials in food and health sector for various applications. Further research investigations need to be carried out in future in order to provide valuable insights into mechanism and mode of action behind probiotic and antimicrobial properties displayed by members of this not-so-famous LAB genera.

1. Genetic Organization and Structure of Pediocin Operon

The genetic organization of plasmid-linked pediocin operon in pediococcal strains has been studied by site-specific mutation and deletion analysis. The results indicate the presence of a cluster of four genes with common promoter and terminator sequences (Marugg et al., 1992; Bukhtiyarova et al., 1994; Motlagh et al., 1994; Kaur & Balgir, 2006). Gene *pedA* encodes a 62 amino acid long precursor prepediocin, from which 44 amino acid long mature pediocin is formed by cleavage of 18 residue long leader sequence at N-terminal of pre-pediocin during processing and translocation of pediocin through cell membrane (Henderson et al., 1992; Miller et al., 1998). Gene *pedB* following *pedA* is responsible for providing immunity to producer organism by production of a 112 amino acid protein PedB. Gene *pedC* encodes a 174 amino acid long amphiphilic protein PedC, which regulates trans-membrane export of prepediocin along with PedD protein. Gene *pedD* specifies a 724 amino acid residues polypeptide, showing homology to ATP dependent transport proteins and is essential for secretion of pediocin (Marugg et al., 1992). *P. acidilactici* PAC1.0

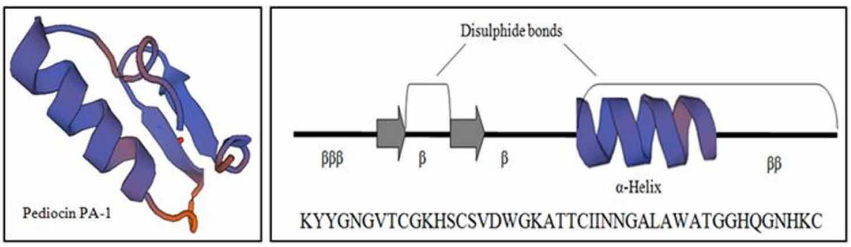
contains *pedABCD* operon which expresses Pediocin PA-1 (Venema et al., 1995) and *P. acidilactici* H produces Pediocin AcH linked to *papABCD* operon (Bukhtiyarova et al., 1994). The sizes and organization of the various pediocin-encoding plasmids from different pediococcal strains are similar (Ray et al., 1992; Bhunia et al., 1994; Rodriguez et al., 1997; Cui et al., 2012). Structure, immunity and secretion system genes are linked together in the operons, with similar promoter sequences. Figure 2 depicts genetic organization of pediocin operon.

Figure 2. Genetic organization of the pediocin gene cluster found in different plasmids of Pediococcal sp.



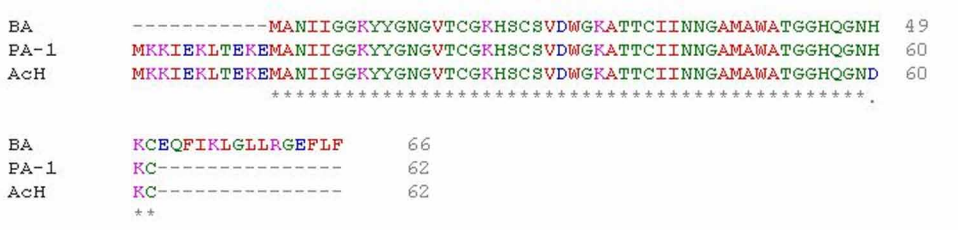
Pediocin PA-1 is a class IIa bacteriocin produced and secreted by *P. acidilactici* PA-1. It is a 62aa protein encoded by gene *pedA* of pediocin operon. The protein is synthesized as a propeptide which is 18 amino acid in length (position 1 – 18) and a 44 amino acid residue long peptide chain (position 19 – 62). Pediocin PA-1 peptide contains two disulfide bonds between Cys9–Cys14 and Cys24–Cys44 (Figure 3). The Cys9–Cys14 bond is conserved among the class IIa bacteriocins, as is the N-terminal YGNGV sequence, both structures being signatures of this family (Bedard et al., 2018).

Figure 3. Secondary structure of Pediocin PA-1 peptide predicted using Swiss Model



The sequence of hydrophobic peptide chain is conserved among pediocins derived from Genus Pediococcus (Figure 4).

Figure 4. Multiple sequence alignment of PA-1 with other pediocins using Clustal omega

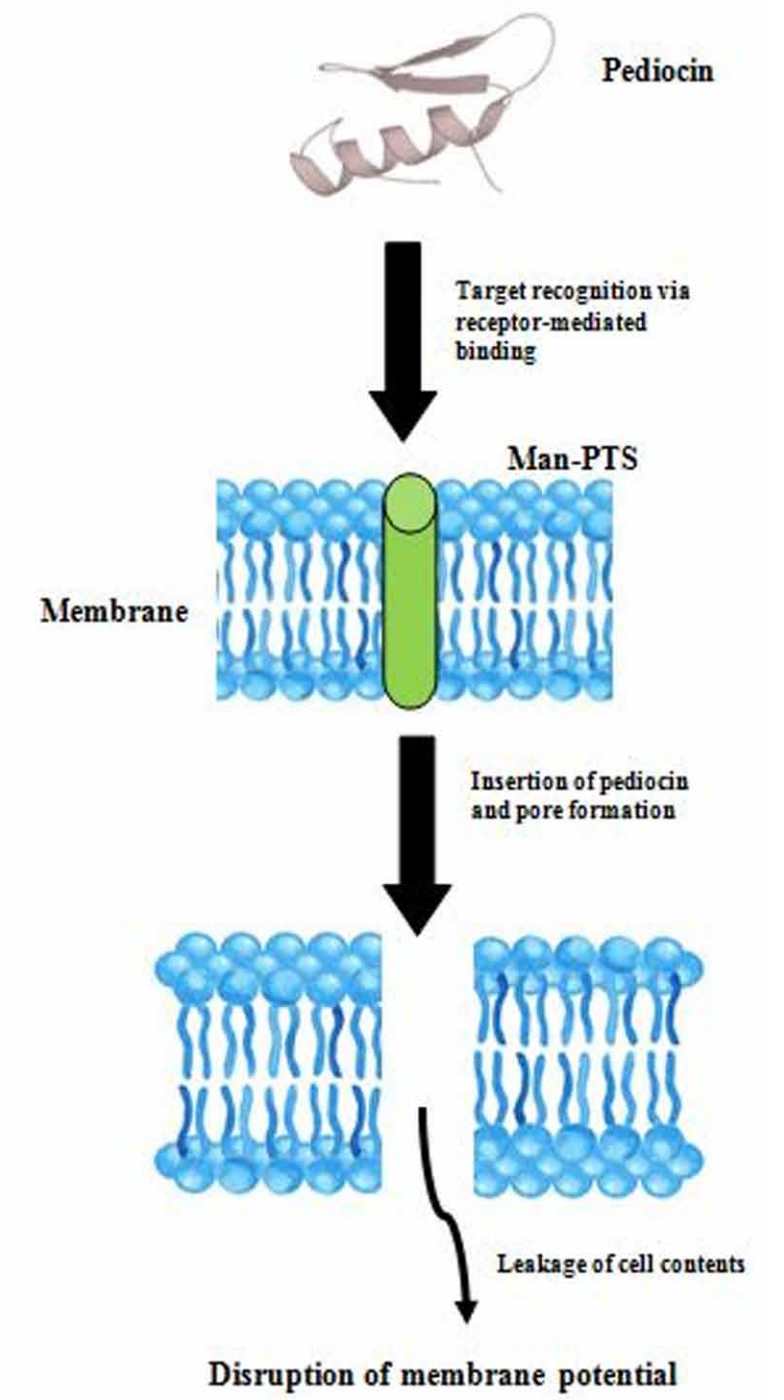


2. Mechanism of Pediocin Action

Antimicrobial peptides are rich in cationic amino acid residues which interact strongly with anionic bacterial cell membranes. The sensitive bacteria are killed due to creation of pores in their cell membranes, which disrupts the trans-membrane potential leading to imbalance between microorganisms and their environment (Chikindas et al., 1993). Liposome-mediated delivery of pediocin PA-1 into membrane vesicles in both sensitive and immune cells initiates efflux of small ions in a concentration dependent manner (Chikindas et al., 1993). Pediocin is capable of effectively releasing high molecular weight compounds at a high concentration. These peptides are amphipathic in nature with properly segregated polar and non-polar residues, which aids in more peptide internalization and membrane perturbation. Trans-membrane potential in bacteria, acts as a potential driving force for insertion and internalization of these peptides promoting peptide interaction (Manuel et al., 2009). Pediocin monomers accumulate on the bacterial surface forming circular patterns, and adopt an orientation parallel to the lipid bilayer. Then they reorient and penetrate into the membrane with their hydrophobic surfaces touching the lipid and hydrophilic moieties oriented towards the center forming a barrel shaped pore (Figure 5).

Pediocin PA-1 is known to exert bactericidal or bacteriolytic effect depending on the type and sensitivity of species (Bhunia et al., 1991). Pediocin ST18 and Pediocin CP2 act in a bacteriostatic manner inhibiting the proliferation of sensitive cells with antifungal and spore-inhibitory acitivity against *A. niger*i solates (Kumar et al., 2012). There are fundamental differences in structure of membrane of bacterial and mammalian cells which makes them resistant to antimicrobial pediocin. The interaction of pediocin with bacterial cells is electrostatic in nature whereas in case of mammalian cells, a relatively weak hydrophobic interaction has been reported. This implies that any therapeutics based on pediocins will be safe for animal and human consumption.

Figure 5. Mechanism of pediocin action



PRESENT SCENARIO (SOLUTIONS AND RECOMMENDATIONS)

Current research is focused on using antimicrobial peptides as an alternative line of treatment for curing bacterial infections. A study has reported the effectiveness and efficiency of using bacteriocin 'nisin' as a therapeutic for the treatment of Staphylococcal mastitis (Falagas et al., 2006; McFarland, 2007; Fernandez et al., 2008; Kaur et al., 2010; Bastos et al., 2010). *In vitro* and *in vivo* studies performed during another study reported the successful application of a class III bacteriocin 'lysostaphin' alone or in combination with other antibacterial agents in prevention or treatment of staphylococcal infections (Bastos et al., 2010). Further, purified bacteriocins such as pyocin, colicin, pediocin, and microcin are available now-a-days which are known to possess anti-neoplastic activity (Zacharof & Lovitt, 2012; Mokoena, 2017). The effectiveness of modified bacteriocins has also been proven in a glioblastoma xenograft mouse model (Gilbert et al., 2008).

Pediocin like other bacteriocins have stability issues as far as its systemic application is concerned, since it is prone to protease activity. Studies suggest introduction of D-amino acids which will not only resist action of proteases but also prohibit aggregation of bacteriocin monomers and reduce their toxicity. Bacteriocins are also reported to be protease resistant when modified at their terminal regions by amidation, acetylation, or other hydrophobic tags. Substituting Tryptophan and pegylation are other ways to improve stability of bacteriocins in presence of proteases (Carmona-Ribeiro & de Melo Carrasco, 2014; Kumar et al., 2018).

Naturally occurring antimicrobial peptides like pediocin to be used as antimicrobial therapeutics pose a number of challenges (Jenssen et al., 2006; Vaara, 2009):

- The susceptibility of degradation by proteases in the digestive tract if given orally or in the blood if i.v. or intra muscular route is chosen.
- Rapid clearance by kidneys
- Toxicity observed with systemic application

To overcome these challenges chemically modifying these molecules to improve their activity as well as biocompatibility is an option selected in number of studies (Gentilucci et al., 2010; Berthold et al., 2013). Various strategies to protect peptides against protease activity include incorporation of D-amino acids, cyclization of the peptide, and addition of acetyl moiety at N-terminal as well as designing peptidomimetic backbone in such molecules; have been investigated. Though non-natural D-amino acids protect the molecules from protease activity, presently it is a very costly option, thus alternatives are needed, keeping in view the economics of the application.

Apart from chemical modifications half life of peptides can also be improved by the delivery systems used. These systems may include covalent linkage to delivery molecule or non-covalent encapsulation in various materials (Reinhardt & Neundorf, 2016; Nordstrom & Malmsten, 2017). These authors have listed silica, metal nanoparticles, including titanium, gold and silver, quantum dots, graphene and carbon nanotubes for the purpose. Graphene oxide nanotubes with covalently attach nisin were found to enhance its antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) (Kanchanapally et al., 2015). Micelles, liposomes and micro-emulsions have been employed to deliver antimicrobials as in case of nisin against *S. aureus* (Sadiq et al., 2016). Polymer based gels, fibres, multilayers hydrogels and conjugates are other options available for loading the antimicrobials. Polyethylene glycol conjugation or PEGylation is known to protect proteins from degradation by proteases. Other polymers used include chitosan, hyaluronic acid, hyperbranched polyglycerol, all increased the therapeutic index of drugs loaded onto them (Kumar et al., 2017).

Lam et al. (2016) have reported developing star shaped structurally nano-engineered antimicrobial peptide polymers (SNAPPs) S16 and S32 as antimicrobials effective in killing of Gram-negative bacteria, such as clinical MDR isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. A peptide synthesis approach termed ring-opening polymerization to α-amino acid N-carboxyanhydrides was applied. The antimicrobial activity was found to involve disruption of *E. coli* cell membrane as well as trigger an apoptotsis like process that killed the gram negative cells. Further no drug resistance was detected even after 600 generations of *A. baumannii* growth in the presence of low S16 concentrations. The SNAPPs have been reported to display low toxicity to human RBCs.

The therapeutic application of Pediocin has faced the challenge of low level production both from native strains as well as recombinant, which recently has been tackled by chemical synthesis using a solid and solution based technology, which also provides with the handle of making changes to its sequence and structure (Bedard et al., 2018). They produced a number of Pediocin PA1 linear analogues eliminating disulfide bonds, which displayed enhanced antimicrobial activity against listeria as well as *Clostridium perfringens.*

CONCLUSION (FUTURE RESEARCH DIRECTIONS)

Bacteriocin 'Pediocin' from one of LAB Genus Pediococcus is an attractive antimicrobial agent against many pathogenic bacteria. Certain strains of pediococci also act as potent probiotics in modulating gut microbiota and aid in lowering cholesterol along with antidiabetic and antihypertensive properties. All these

properties of bacteriocin-producing pediococci highlight their use as aids for health improvement. Pediocins as antimicrobials for application in health sector for various applications are attractive potential drug molecules. Further investigations need to be carried out in future in order to provide valuable insights into mechanism and mode of action behind probiotic and antimicrobial properties displayed by members of this LAB genera in order to widen the horizons of strain specific applications of probiotics in general and Pediococcus in particular.

Since FDA is yet to approve of a bacteriocin for antibiotic use, at current level of research a combination therapy of bacteriocin along with antibiotic can be useful in preventing the development of antibiotic resistance as well as reducing the amount of antibiotic drug used. The strategy of using bacteriocin or bacteriocin producer probiotic synergistically with the antibiotic certainly helps in dose reduction as it facilitates the entry of antibiotic into the pathogen. Such dose reduction will reduce the side effects associated with antibiotics affecting the whole microbiome instead of killing the pathogen alone. For future, a number of paths for improving the druggability of the bacteriocins are being researched, ranging from synthetic peptides with substituted D-amino acids, modified non natural amino acids, end modified and encapsulated or immobilized antimicrobial bacteriocin molecules. Using a range of molecular engineering strategies, novel and more potent antimicrobial peptides can be produced. The engineered antimicrobial peptides are increasingly gaining attention as therapeutic agent due to robust action mechanisms and low production costs.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Chapter 10
Nanomaterials to Overcome Emergence and Re-Emergence of Superbugs:
Nanoarsenals for Superbugs

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ABSTRACT

Antimicrobial resistance remains a substantial global health concern, invigorating the critical need for alternate therapeutic options to combat chronic intracellular infections and biofilms so as to shorten the hospital stays, and hence mortality. Nanomaterials have been developed as delivery carriers for antibiotics to improve their penetration through these biofilms. Nanoformulations of existing antibiotics has led to enhanced bioavailability and site specificity. Moreover, diagnosis of infections using efficient nanosensors or probes may speed up the treatment process at earlier stages of infection.

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1. INTRODUCTION

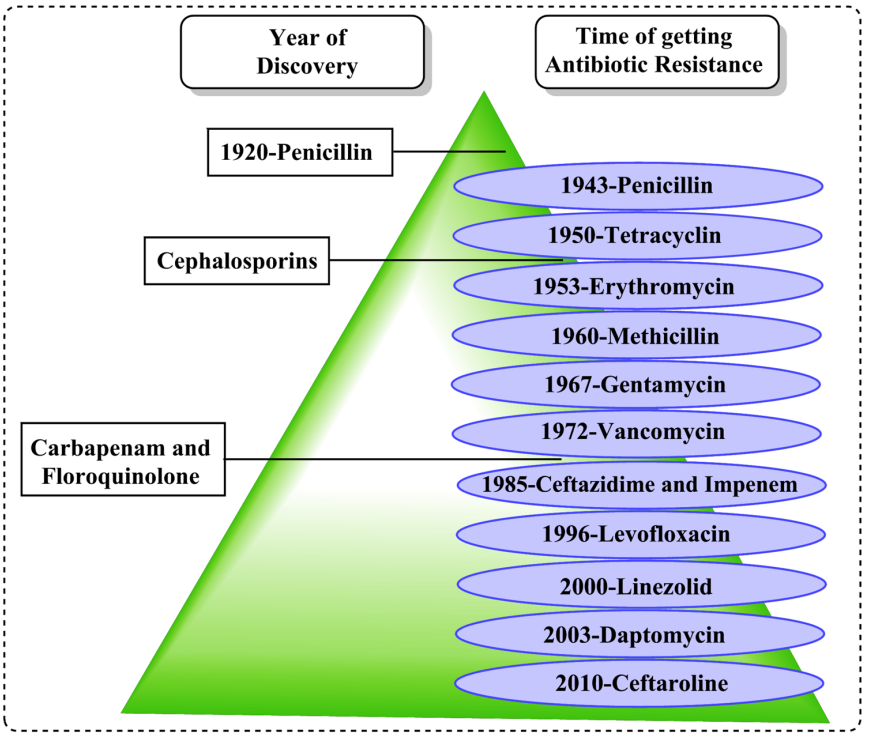
1.1 Concept of Emergence and Re-Emergence of Superbugs

Approximately 70 years ago, antibiotics were introduced to cure infectious diseases. These are the drug molecules used to kill bacteria and they reduce the risk associated with various infections. Antibiotics exhibited their action by inhibiting the proliferation process of bacteria. Overtime, antibiotics started showing resistance to different bacteria's called superbugs (Drug-resistant microbes). Antibiotic resistance is the tolerance developed by bacteria to overcome the effects of antibiotics (https://www.davolterra.com/content/antibiotic-resistance-wonder-drugs-facing-rise-superbugs). Antibiotic resistance increases the duration of infection, cost of treatment and decreased success of treatment which finally led to economic loss (Zaman *et al*., 2017). Drug resistance does not just prevail in healthcare environments but it is also increasing amongst community-acquired pathogens (Padhy *et al*., 2016). For example, penicillin was the first antibiotic developed and was successfully used to control infections. Just after four years, it causes resistance to major microbe *i.e. Staphylococcus aureus* (https://www.statnews.com/2016/09/12/superbug-antibiotic-resistance-history/, Ventola, 2015). Also, *Klebsiella pneumonia* causes fatal and untreatable infections in a healthy population. It may cause a danger to life of individual (Gu *et al.*, 2018). Some other examples of antibacterial discovery with their resistance are given in Figure 1.

World Health Organization (WHO) has already warned about the emergence of infectious diseases at a faster rate which has never seen before (https://www.who.int/newsroom/factsheets/detail/antibiotic-resistance). Several human activities have led to the emergence and spread of new diseases such as moving into wildlife habitats, improvisation in agriculture techniques, modern transport and misuse of antibiotics (Bloom *et al*., 2017). Re-emerging infectious diseases are those that once were foremost health problems across the globe and declined considerably, but they again became health problems for a significant population (https://www.sciencedaily.com/releases/2017/08/170831101508.html). Some of the re-emerging diseases with a causative agent are given in Table 1. The re-occurrence of old infections along with emergence of new infectious diseases and constant persistence of various intractable infections is a major challenge to the researchers.

One of the reasons for antibiotic resistance is a development of biofilms. Most of the bacterial strains occur in the form of a biofilm which are especially microbial aggregates that rely on a solid surface and extracellular products such as extracellular polymeric substances (EPS's). Bacteria tend to move reversibly on the surface, but the expression of EPSs makes this attachment irreversible. After settlement of bacteria, the synthesis of bacterial flagellum is suppressed and rapid multiplication

Figure 1. History of antibiotic discovery



of bacteria results in the formation of a mature biofilm. They all are in aggregates at this stage and forms a barrier for antibiotic action which led to systemic chronic infections. Bacteria within biofilms develop superantigens that tend to evade the immune system. Thus, the formation of biofilms is a serious threat to human health and makes infection control more challenging (Frieri *et al.,* 2017, Von Wintersdorff *et al*., 2016). These biofilms are embedded into extracellular polymeric substances (EPS) matrix. The EPS is composed of phospholipids, nucleic acids, teichoic acid, exopolysaccharides and extracellular proteins. In addition, mineral crystals, silt, milk residues and blood parts or dirt may be present in EPS matrix, depending upon the location and conditions under which biofilm is formed (Ramos *et al*., 2018). The biofilm tends to adhere irreversibly to organic phenomenon or biotic surfaces and 80% of pathogenic infections remain persistent due to this biofilm. For example, *P. aeruginosa* (linked to cystic fibrosis) and *Staphylococcus aeruginosa* (accountable for wound infections) are traditional examples of persistent pathogens that lead to formation of biofilm (Algburi *et al*., 2016). The formation of micron size biofilms occurs around solid surfaces in contact with water, like living tissues and water bodies/systems (Clatworthy *et al*., 2007). The development of a biofilm involves

various steps i.e. adhesion, growth, and production of EPS matrix. A cycle indicating the 5 stages is discussed below (Ramos *et al*., 2018) (Figure 2) and strategies for prevention of biofilm is shown in Figure 3.

Stage 1: This involves deposition of bacterial cells when the microbes arrive to site of adhesion and macromolecules present on that site act as the substrate for these microbial cells which lead to formation of biofilm (Myszka *et al*., 2011).

Stage 2: A reversible adhesion of bacterial cells is followed by communication between the cells which are associated with cells of next stage. The EPS matrix is formed during this stage (Wilkins *et al*., 2014).

Stage 3: This stage involves enhanced EPS production that ends up in a rise of bacterial cells. The matrix formed act as a reservoir for genetic material to alter gene transfer, to supply nutrients and to protect against adverse conditions like exposure to biocides, antibiotics, drying, oxidation, certain metallic cations, ultraviolet (UV) radiations, *etc*. The assembly of the matrix represents the formation of biofilm communities, propagation and survival of bacterial cells in their native environment (O'toole *et al*., 2000).

Stage 4: At this stage, biofilm gets matured by forming characteristic "mushroom" structures due to polysaccharides.

Stage 5: Finally some cells start to detach and the biofilm will disperse (https://www.immunology.org/public-information/bitesized-immunology/pathogens-and-disease/biofilms-and-their-role-in).

Figure 2. Various stages of biofilm formation

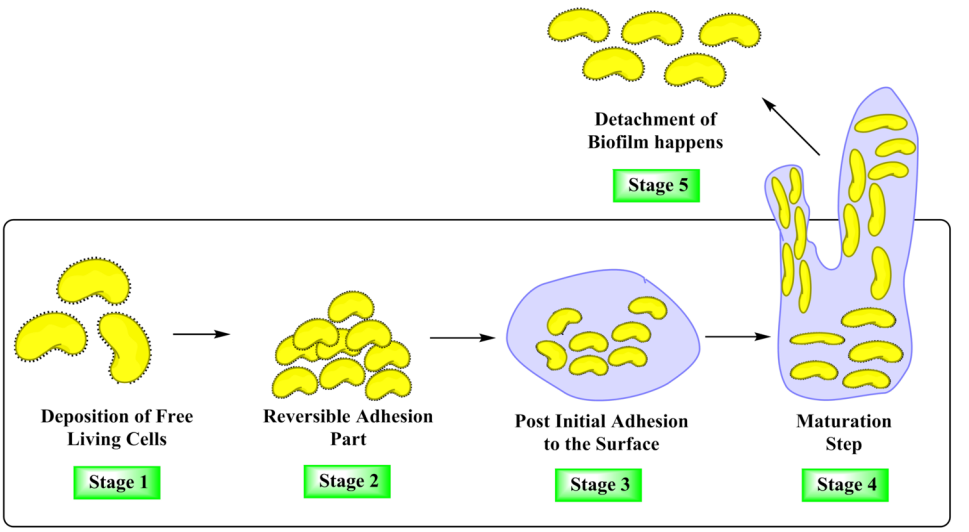


Figure 3. Strategies for the prevention of biofilm

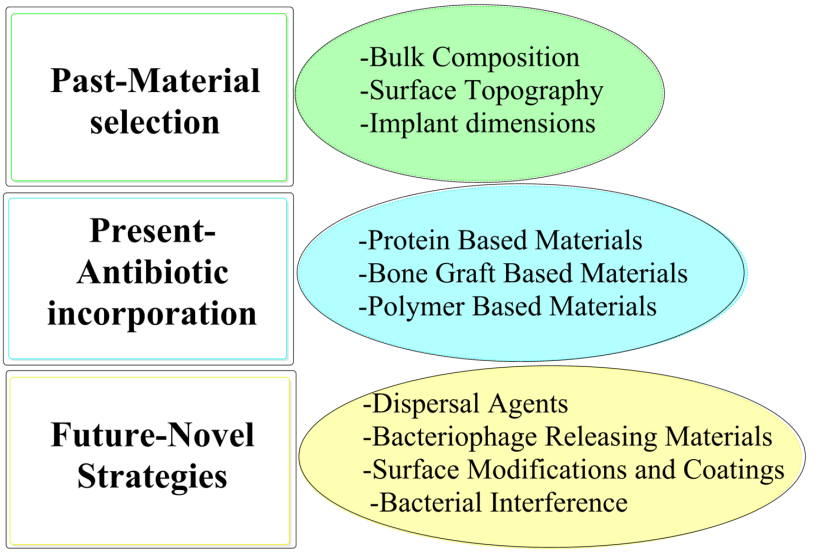


Table 1. Various Re-emerging diseases with causative agent

Disease	Infectious agent
Cryptosporidiosis	*Cryptosporidium pavum* (protozoa)
Malaria	*Plasmodium* species (protozoon)
Diphtheria	*Cornebacterium diptheriae* (bacteria)

1.2 History of Various Infectious Diseases

Influenza (flu) is an example of widely spreading disorder that is due to natural and human factor both. The influenza virus is known for its ability to alternate its genetic information. The possibility of huge genetic changes (which are going on and surpassed into people) is increased when human beings are in close contact with agricultural animals such as chickens, pigs and ducks. These animals act as permanent host to influenza virus and can create a new form of influenza virus that has no existence before. Large modifications in influenza virus can be the reason for pandemics since human immune system is not expected to recognize and protect against the new variant strain. **Avian H5N1 influenza (or chicken flu)**, has been confined to rare situations of contamination in humans who came into direct contact with contaminated birds. It is a deadly virus and may cause more than half deaths from the number of infected people. In contrast, **H1N1 influenza virus** (Swine flu)

spreads from swine (pigs) and gets transmitted easily. The spread of H1N1 virus in human across the world is quicker than any other viruses. But fortunately, it is less deadly than the H5N1 virus. **Severe acute respiratory syndrome (SARS)** and **Middle East respiratory syndrome (MERS)** represents another example of infectious diseases that can spread across the globe. SARS appeared in China in 2002 and spread rapidly in various nations. Occurrence of MERS in 2012 is due to a related strain in Arabian Peninsula. Humans are supposed to get contaminated with **HIV** through close contact with chimpanzees during hunting in isolated areas of Africa. The intravenous administration, sexual transmission and transfer of blood led to a fast and huge spread of HIV. **Chikungunya disease** is triggered via the chikungunya virus, similar to dengue virus. It was transmitted *via* the tiger mosquito. It was restrained to tropical regions around the Indian Ocean in the past. The virus arrived in the United States in the summer season of 2014, although therefore, the local transmission of chikungunya virus has been constrained to Florida and Texas. **Zika virus** emerged in America and is related to a birth defect identified as microcephaly. The **Ebola virus** pandemic emerged in 2014 in West Africa region. At initial stage, the virus affected only small population (hundreds) but fortunately there was a rapid increase of virus which affected a large population (thousands). Worst outbreak of Ebola in world which had never seen before was due to several factors including high population, closer contact with wild animals, increased travel and weak health care systems. (https://www.bcm.edu/departments/molecularvirologyandmicrobiology/emerginginfectionsandbiodefense/emerginginfectious-diseases). Coronavirus disease (COVID-19) is an infectious disease caused by a newly discovered coronavirus. People infected with COVID-19 virus will experience mild to moderate respiratory illness and recover without requiring special treatment. Older people and those who are suffering from medical problems such as cardiovascular disease, diabetes, chronic respiratory disease and cancer are more likely to develop serious illness. This virus spreads primarily through droplets of saliva or discharge from the nose when an infected person coughs or sneezes (https://www.who.int/health-topics/coronavirus#tab=tab_1). Table 2 summarizes various emerging infectious diseases with their causative agents.

1.3 Challenges for Antibacterial Therapy

Superbugs are the biggest threat these days due to development of antibiotic resistance caused by formation of biofilm. Globally, the situation is getting worse as approximately seven lakh deaths occur annually from these drug-resistant infections (Zaman *et al*., 2017). Therefore, there is an urgent need to control the emergence and re-emergence of superbugs. The alarming situation can be improved by use of antibiotics/anti-microbial, early diagnosis of superbugs, improved sanitation and

Table 2. Various emerging infectious diseases with infectious agent

Year recognized	Disease	Infectious agent
1967	Murburg hemorrhagic	Marburg virus
Prior to1976	Salmonellosis	*S. entertidis*
1976	Ebola hemorrhagic fever	Ebola virus
1983	AIDS, gastric ulcers	HIV, *Helicobacter pylori*
1989	Hepatitis C	Hepatitis C virus(HCV)
1998	Nipah encephalitis	*Nipah encephalitis*
2002	VRSA infection	Vancomycin resistant *S. aureus*
2003	SARS (severe acute respiratory syndrome)	SARS-associated coronavirus
2015	Zika	*Zika* virus

hygiene, reduce pollution and increase global surveillance of superbugs (Chen, 2013). The proper use of vaccines and the development of new antibiotics can also reduce antibiotic resistance by preventing infections in its first stage. Some regulations and policies must be practised in every country to stop unnecessary drug promotions. Some measures need to be taken to follow WHO guidelines for minimizing the dual use of antimicrobials related to human beings and food products (Padhy *et al*., 2016). There are some diagnostic techniques and devices that can identify the presence of superbugs at its earlier stage (Frieri, 2017). Development of nanomaterials for diagnostic and therapeutic application in antibacterial therapy has come out with a wide area of research. Various nanomaterials for diagnostic and preventive applications are discussed in the following sections.

1.3.1 Nanomaterials for Diagnosing Superbugs

Nanomaterials are the new tools to fight against superbugs. Various nanosystems have been worked out to control and prevent various infections (Blecher *et al*., 2011). Diagnosis of infections *via* economical nanosensors or probes fastens the treatment at early stages (Okeke *et al*., 2011, Zhu *et al*., 2014). Researchers have developed diagnostic techniques to examine bacterial infections and even other superbugs such as tissue culture, microscopy, lateral flora immunoassays (called immune chromatographic test (ICT), enzyme-linked immunosorbent assay (ELISA) and various biochemical tests (Qasim *et al*., 2014, Zhu *et al*., 2014). These techniques are costly and time- consuming. These are mainly utilized in developed countries but are often poorly fitted to developing countries in which infectious diseases are chief causes of death (Cars *et al*., 2011, Torres *et al.*, 2016). Figure 3 depicts various nanotechnology-based diagnostic techniques used for the early detection

of superbugs. A faster diagnostic test *i.e.* In-Dex has been developed to detect dangerous bacteria's like *E. coli, Staphylococcus* and other superbugs. Results may be produced in two hours using a blood sample, spit, wound, urine, stool samples or cerebral spine fluid samples etc. (https://www.hindustantimes.com/health-and-fitness/scientists-develop-faster-method-to-detect-harmful-bacteria-likesuperbugs/storyILaxy8D14lhzuxKt0COcEP.html). Solution- based circuit chip (SSC) for synchronous detection of resistant and non- resistant *E.coli* strains responsible for urinary tract infections has been designed as an another approach. These SSCs were designed by applying a sequence of lithographic steps followed by electrochemical deposition that further create 3-D nanoscopic morphology on microsensors. Further, these microsensors were functionalized via peptide nucleic acid (PNA) probe to target a specific region of pathogen. This multiplexed technique is able to distinguish between bacterial strains such as *E.coli* and *S. aureus* (Lam *et al*., 2013). Similarly, for the diagnosis of MDR-TB, a novel NanoELIwell device has been developed. The principle of the technique is a combination of *mycobacteria* antigen immunoassay and microwell technology. It is capable of performing quick identification and detection of antigens released by resistant *mycobacteria* (Nguyen *et al*., 2012). Also, the nanoparticles with better fluorescent property led to improved sensitivity in diagnostic molecular bioimaging technique. Fluorescent silica nanoparticles (FSNPs) are developed to detect *Mycobacterium tuberculosis* (MTB). FSNPs entrap two metallic fluorescent organic dyes *i.e.* Tris (2,2-bipyridyl) osmium bis(hexafluorophosphate) (OsBpy) and Tris (bipyridine) ruthenium(II) dichloride (RuBpy), that are excited by a single wavelength. These FSNPs displayed photostability and high signal amplification. MTB is detected using an anti-MTB primary antibody and a second antibody labelled with FSNPs for better detection of anti-MTB antibody. Using this system, MTB can be detected in a mixture of bacteria and sputum with high sensitivity and in a short time of four hours (Qin *et al*., 2007). Europium [Eu (III)] polymeric nanoparticles are designed for easy detection of anthrax antibodies by applying fluorescence enzyme- linked immunosorbent assay (ELISA) (Qin *et al*., 2007). Table 4 depicts various diagnostic tests to detect infectious diseases along with their limitations.

1.3.2 Use of Nanomaterials for Prevention Against Superbugs

After diagnosing the specific superbug, the next approach is to reduce the effects of the superbug in the body. Various nanoparticulate carriers of anti-microbial/anti-bacterial compounds have been developed and being used to fight against superbugs or to overcome antibacterial- resistance. The nanoparticulate system has proved it more fatal for microbes and has gained interest due to enhanced bioavailability, penetration ability, effectiveness and site-specific targeting.

Figure 4. Nanotechnology based approaches for early detection of superbugs

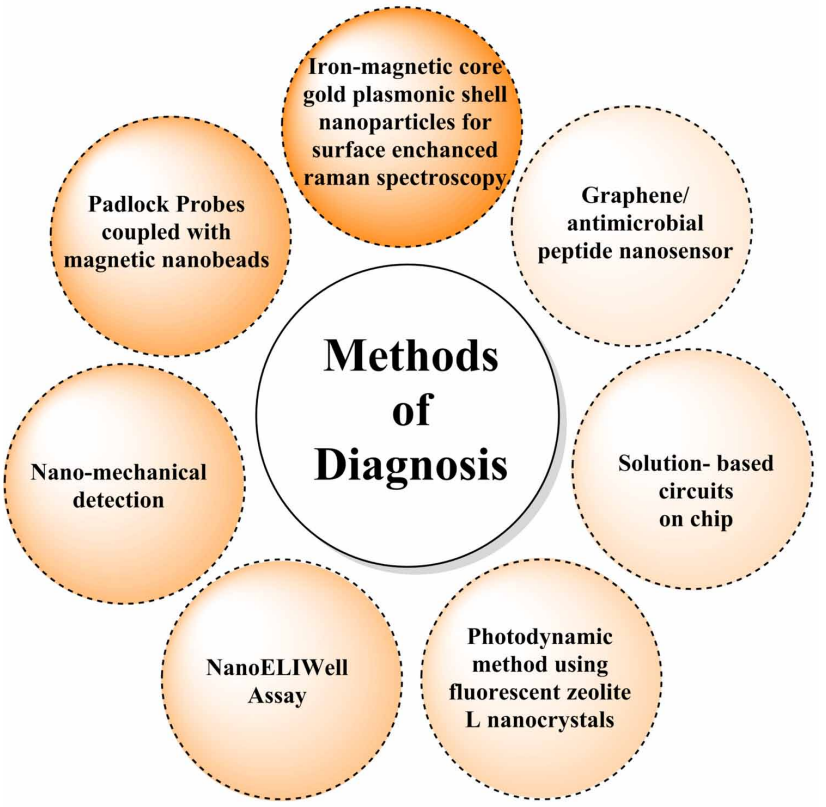


Table 3. Available diagnostic tests for infectious diseases and their limitations

Disease	Diagnostic test	Limitations
Acute respiratory infection (ARIs)	Blood and sputum culture	• High cost and time consuming method, dependence of results on stringent transport conditions to maintain specimen. • Require sufficient reagents, equipment and well trained staff.
Acquired immunodeficiency syndrome (AIDS)	Serology; detection by PCR amplification of LTR region of gag gene, env gene and pol gene	• High rate of false positives and negatives. • PCR cannot be done during HIV incubation period. • Nucleic acid amplification technologies (NAAT) such as PCR are costly and lead to false positives because of contamination.
Diarrheal diseases	Microscopy stool culture	• Requirement of trained technologists. • Expensive and time consuming method.
Malaria	Blood film, detection of antigens (dipstick) and detection of antibodies (ELISA)	• High false positive rates.
Tuberculosis (TB)	Sputum microscopy; tuber skin test ; pathogen solid and liquid culture ; PCR- based Gene Xpert	• Need of expert staff • Occurrence of false positives during antibody tests, tuber skin test (tuberculin) because of allergic reactions.
Visceral leishmaniasis	Serological field test; Direct agglutination test (DAT); microscopy / culture of spleen or bone marrow cells.	• Bone marrow sampling is painful and invasive.

The Penetrating mechanism of nanomaterials is divided into two parts-

Diffusion- Nanomaterials generate reactive oxygen species (ROS) into bacteria by using diffusion mechanism. ROS persists for a sufficient and long time to diffuse into bacterial cells. Generation of hydroxyl radicals and their diffusion into bacterial cells is responsible for maximum antibacterial activity against methicillin resistance *Staphylococcus aureus* (MRSA). Hydroxyl radicals may penetrate the cell membrane easily then super oxide radicals.

Absorption- The metallic ions of nanoparticles tend to release into the outer media and bind with the negatively charged functional group of the bacterial cell membrane, such as carboxyl and phosphate groups (biosorption). Different metal ions have different binding abilities for their targets for example, zinc ions bind with high affinity to the –SH group of proteins. The inherent structure of cell membrane gets destroyed which further lead to bacterial cell death (Wang *et al*., 2017).

There are various approaches to transport drug-loaded nanoparticles to diseases sites such as passive targeting, active targeting and physical targeting. The mechanism of passive mode of targeting is to enhance permeability-retention (EPR) effect through which tumor cell gets absorbed to nanosize bodies. In active mode of targeting, nanoparticles are attached with ligands such as proteins, peptides and antibodies which further interact with receptors present at the target site. Physical targeting includes use of external sources to interact with the receptors on target site and also controls the drug release from the nanoparticles (Ma *et al*., 2013; Egusquiaguirre *et al*., 2012).

The nanocarriers are synthesized by various techniques which produce sufficient consecutive antimicrobial actions. These can be organic, inorganic, hybrid NPs. The nanomaterials having highest antimicrobial potential are inorganic nanoparticles like gold, silver and alone/ mixed used with a variety of natural polymers. NPs are regularly developed and used as drug carriers and they can directly supply chemotherapeutics to the tumor tissues by reducing the toxicities associated with other organs (Beyth *et al*., 2015). Several metal oxides, metals, bimetals and metal halides in nanoparticulate forms have been reported for antimicrobial activity because the microbes are less prone to build up resistance for nanomaterials. These comprises of Gold (Au), Copper (Cu), Silver (Ag), Zinc (Zn), Magnesium (Mg), Titanium (Ti), Cerium (Ce), Nickel (Ni), Aluminium (Al), Selenium (Se), Yttrium (Y), Cadmium (Cd), Palladium (Pd) and super-paramagnetic Iron (Fe) (Ma *et al*.,2013). The nano carriers should be stable, non-immunogenic, biodegradable, easy to manufacture, cost efficient and should release their drugs solely at the target site. Regular nano-platform carriers are liposomes, polymeric nanoparticles, solid lipid nanoparticles, metal/metal oxide nanoparticles and dendrimers *etc.* (Chan *et al*., 2010). Nanocarriers may directly penetrate into biofilms preventing their formation and they also possess

some properties such as higher solubility, bioavailability, efficacy and maximum drug entrapment in comparison to traditional delivery systems (Ong *et al*., 2017).

Silver is widely used in nanocarriers due to its antimicrobial activity. Silver nanocarriers systems may hinder the development of microbial biofilms on medical devices which may be life- threatening. Silver nanoparticles against biofilms can be detected by acid-base indicator, scanning electron microscopy (SEM) and double fluorescent staining and Conofocal laser scanning microscopy (CLSM) (Ansari *et al*., 2014). Bacteriophages containing drug molecules in its protein coat and antibody on its tip tends to release drug molecules to its complementary receptors. The released drug molecule attack pathogenic bacteria and destroy them. OxiTitan has proved its antibacterial action against *Clostridium difficile* spores and other resistant bacterial strains. It is durable, cost effective and environment friendly (Sadekuzzaman *et al*., 2015).

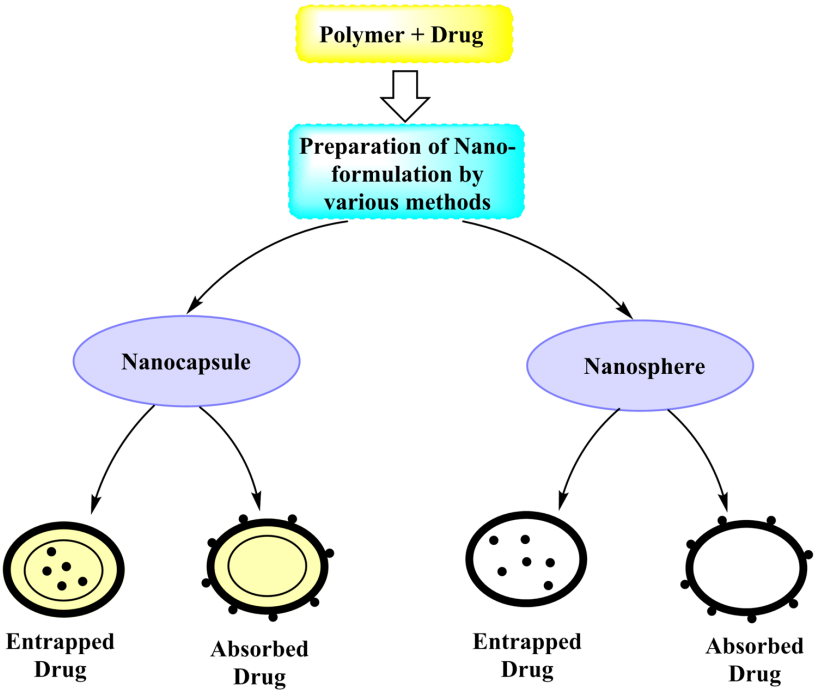
1.4 Nanocarriers Based Drug Delivery System

1.4.1 Polymer Based Nanoparticles

Polymer-based nanoparticulate systems are one of the potential carriers that are used in previous years. Two types of polymer based nanoparticles based on their method of preparation are nanocapsules and nanospheres (Figure 5). Nanocapsules are vesicular carriers in which the drug is entrapped into the cavity surrounded by the polymer membrane and nanospheres are matrix carrier systems where the drug is uniformly dispersed (Han *et al*., 2018). The utilization of polymeric nanoparticles results in optimized therapeutic outcomes by reducing toxic effects. The ideal polymers for nanoparticles should be easy to synthesize, biocompatible, inexpensive, non-immunogenic, biodegradable, non-toxic and water soluble. Polymers used in fabrication of nanoparticles may be classified as either biodegradable or non-biodegradable. Some of natural biocompatible and biodegradable polymers are gelatin, cellulose, chitosan, pullulan, gliadin and alginate. Various synthetic biodegradable polymers are poly-(lactide-co-glycolide) (PLGA), polylactic acid, poly-ε-caprolactone, polyanhydrides polyphosphazene and poly-alkylcyanoacrylates (Han *et al*., 2018). One of the study reported a formation of antimicrobial peptides (AMPs), which can act as the first line antibiotics to control resistance and better alternative to antibiotics. Sustained release (SR) of four potent ultra-short lipopeptides conjugated with an aliphatic acid chain (16-C) encapsulated into poly (lactic acid-co-castor oil) and ricinoleic acid-based poly (ester-anhydride) was studied. Better antibacterial efficacy along with anti-biofilm effect and membrane disruption was observed with these biodegradable polymer incorporated peptides (Eckhard *et al*., 2016). Various polymeric nanoparticles based antibacterial formulations have been

developed to overcome antibacterial resistance. For example **Qasim *et al.*, 2018** developed poly-*N*-isopropylacrylamide (pNIPAM)-based polymeric nanoparticles encapsulating silver nanoparticles (AgNPs). The prepared formulation (pNIPAM- and pNIPAM-NH_2-based) showed enhanced bacteriostatic action against Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. **Namasivayam *et al.*, 2017** formulated biocompatible ovalbumin nanoparticles encapsulating cefpirome using coacervation technique. The results showed good biocompatibility along with effective inhibition of pathogenic bacteria and biofilm formation. **Suchomel *et al.*, 2015** reported silver bromide (AgBr) nanoparticles using different polymers (PVP, PEG, PVA, and HEC) and studied the antimicrobial action of Ag and AgBr nanoparticles against both gram positive and negative bacteria along with several strains of *Candida*. PEG and HEC showed less interaction with silver ions. The AgBr NPs were more effective against gram-negative bacteria and tested yeast strains but Ag NPs were more effective against gram-positive bacteria. **Azhdarzadeh *et al.*, 2012** suggested enhanced efficacy of azithromycin (AZI) nanoparticles prepared by modified Quasi-Emulsion Solvent Diffusion to combat *Haemophilus influenzae (PTCC 1623), S. pneumoniae (PTCC 1240) Escherichia coli (PTCC 1330)* using agar well diffusion. The increased potency of AZI NPs

Figure 5. Different types of polymer based Nanoparticles



may be recognized to modify surface features as well as increased uptake of drug and adsorption. **Raval *et al*., 2014** suggested poly (e-caprolactone) microspheres loaded with doxycyclines formulated by double emulsion solvent evaporation for controlled-release drug therapy for longer periods (76 h).

The classification, applications, advantages and disadvantages of polymer-based nanomaterials are given in Table 4.

Table 4. Polymer based nanoparticles with advantages, disadvantages and applications

Classification	Materials	Advantages	Disadvantages	Application
Natural polymeric material	Chitosan	Biocompatibilty, easily degradable film formation, Antimicrobial Innocuous,	Poor spinnability, low water solubility, poor strength,	Hemostasis materials, medical dressings, drug delivery carrier, hydrogel, gene transfer
	Starch	Cost effective, non-degradable, non-toxic, anti-antigenic	Poor mechanical properties, water-resistant	Hemostasis material, bone repair material, tissue-engineered scaffold
	Alginate	Hypotoxicity, suppresses the tumor growth, immunity booster	Low biodegradability, poor cell attachment.	Pharmaceutical excipient, medical dressing.
	Cellulose	Extensive sources, cost effective	Rare Adverse reactions	Pharmaceutical adjuvant
Biosynthetic Material	Poly β-hydroxybutyrate (PHB)	Biodegradable, non toxic, safe, good physicochemical properties	Poor thermal stability, High Crystallinity	tissue engineering material, Drug delivery carrier
Chemosynthetic polymer(Copolymer)	Poly lactic acid	Biocompatible, safe, good mechanical properties, non toxic	Hydrophobicity, Poor toughness,	Anti-adhesion materials, drug delivery carrier, sutures, bone fixing device.
	Polyurethane	Low cost, good mechanical properties, rich resource,	Slow speed of degradation	Excipients, medical bandage
	Poly(lactic-glycolic acid) (PLGA)	Biocompatible, biodegradable	High cost	Tissue repair, sutures
	Polymethyl methacrylate resin	Easy to operate, biocompatible.	Cytotoxic, ease of oxidation.	Dental materials, artificial crystals.

1.4.2 Metal and Metallic Oxides Nanoparticles

Metal nanoparticles (NPs) are promising candidate to fight against growing multi drug resistant strains. These nanoparticles help to create difficulty in building resistance to microbes *via* various mode of action (Ramos *et al*., 2018). Amongst metallic NPs, the silver NPs have been widely used in distinctive study as they are able to kill gram-positive and gram-negative bacteria. They are efficient against a wide range of antibiotic resistant bacteria such as methicillin resistant *S.epidermis* (MRSE), MRSA, Erythromycin-resistant *S.* pyogenes, Ampicillin-resistant *E. coli*,

and Multidrug-resistant *P. aeruginosa*. Multiple mechanisms can also be involved, which includes direct connections between Ag and DNA, Ag and bacterial cell membrane, Ag and enzymes/proteins, and indirect connections *via* development of reactive oxygen species (ROS) (Ramos *et al*., 2018). The nanohybrids of AgNPs were developed in combination with poly-methyl-methacrylate (PMMA) polymer to improve surface properties of AgNPs (found to adhere to 1nm-thick inorganic silicate clay platelets) and to decrease toxicity on mammalian cells (Zhu *et al*., 2014). Further, mixture of chitosan acetate polymer and AgNPs exhibited potential activity towards MRSA during its use in burn dressings (Rai *et al*., 2012, Huang *et al*., 2011). Gold nanoclusters have been developed with sufficient antimicrobial activity against resistant bacterial strains. Functional gold nanoclusters (AuNCs) have been formulated using lysozyme as sequestering and reducing agent. Titanium dioxide nanoparticles (TiO_2-NPs) were observed to improve the action of various antibiotics such as cephalosporins, beta-lactams, aminoglycosides and macrolides against MRSA (Waheed *et al*., 2015; Bonifacio *et al*., 2014).

Various studies on metal/metal oxide nanoparticles for antibacterial activity have been evidenced. For example, **Abdellatif *et al*., 2021** investigated antioxidant and antibacterial activity of formulated silver nanoparticles prepared by different cellulosic polymers. Ethyl cellulose and hydroxypropyl methylcellulose polymers were used due to its own antibacterial activity. The polymers showed its antibacterial activity against *E.coli*. **Gupta *et al*., 2020** synthesized metal oxide nanoparticles of copper, iron, lead and zinc. The synthesized nanoparticles were found in nano range and were found to have antibacterial activity against *E.coli* and *S. aureus*. **Holubnycha *et al*., 2018** synthesized chitosan-silver nanoparticles (AgNPs) solution against MRSA. Cetrimonium bromide (CTAB) was used for surface modification of silver nanoparticles. Chitosan-AgNPs solution showed enhanced antimicrobial activity over its pure form. **Eshghi *et al*., 2018** synthesized silver nanoparticles (Ag NPs) through microwave irradiation using extract of Juglans regia (J. regia) leaves, which act as reducing and stabilizing agent. The developed NPs showed enhanced antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus.* **El-Rashidy *et al*., 2018** developed zein scaffolds containing silver-doped bioactive glass and investigated antibacterial activity, biocompatibility, and compressive potency. Zein scaffolds with silver-doped sol-gel bioactive glass were effective against *E. coli* and *S. aureus*. **Holubnycha *et al*., 2017** developed copper nanoparticles (CuNPs) and Cu NPs/ chitosan solution against *MRSA* and multidrug resistant *E. coli* clinical isolates.

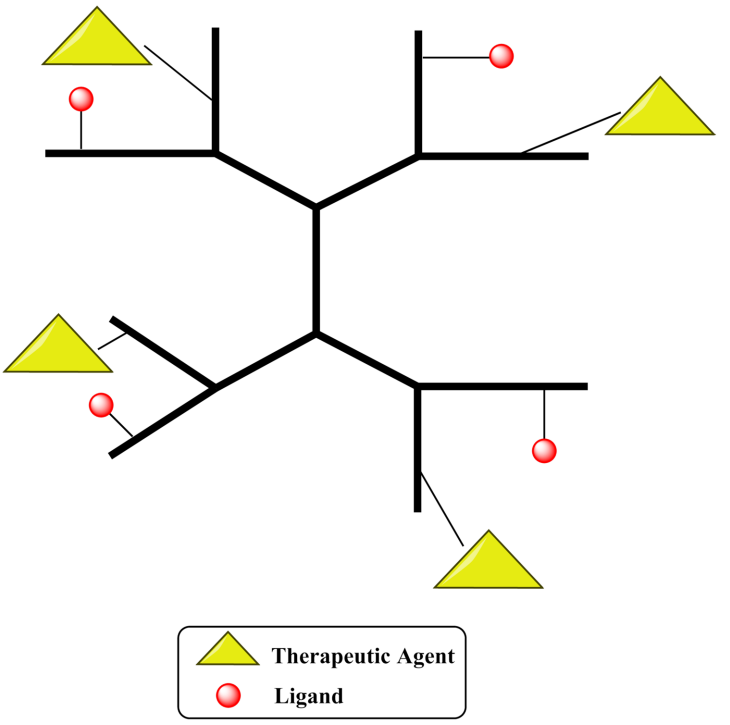
1.4.3 Liposomes

Liposomes are the main class of lipid-based nanocarriers for drug delivery (Waheed *et al*., 2015). The liposomes are synthesized by combining phospholipids (Emulsifiers) with oil phase. The core material (oil) can be used to encapsulate lipid miscible drugs such as hematoporphyrin, paclitaxel and lipid conjugated prodrug. The phospholipids used in fabrication of liposomes should be biodegradable and nontoxic (Kumar *et al*., 2012). Antigens and viral enveloped glycoproteins can be incorporated into the core to develop virosomes for treatment of influenza. Combination of 1,2-dioleoyl-3-trimethyl- ammonium propane (DOTAP) modified cationic liposome and a cationic polymer (usually protamine) condensed DNA are known as liposome polycation-DNA nanoparticles (LPD), an often used adjuvant delivery approach in DNA vaccine studies (Zhao *et al*.,2014). Liposome based antibacterial systems have been reported for enhanced antibacterial efficacy. For example, **Moya *et al.*, 2019** prepared and characterized liposomes encapsulating cefepime. Thus, it was concluded that cefepime liposomes were biocompatible nanocarriers for antibacterial activity. The formulation was effective against *E.coli* and showed better encapsulation efficiency than free drug. **Jung *et al*., 2015** formulated linolenic acid (Lipo LLA) liposomes and concluded an effective antibacterial activity for clinically isolated antibiotic-resistant strains of *H. pylori* and lead to destruction of bacteria within 5 minutes. **Solleti *et al*., 2014** reported liposomal carriers of azithromycin and examined its antimicrobial effects against *Pseudomonas aeruginosa*. The developed liposomes reduced the biofilm and suppressed the formation of various virulence factors along with a reduction of different patterns of bacterial motility. **Shafaa *et al*., 2008** developed cephalexin loaded neutral, negative and positive liposomes by phase transition measurements. Negative liposomes encapsulating cephalexin were superior in comparison to neutral and positive liposomes.

1.4.4 Dendrimers

Dendrimers are outlined as 3-D structures of nanorange about 1-100nm (Martins *et al*., 2013). Dendrimers are indicated as tremendous carriers for delivery of antibiotics because of its unique properties (Silva *et al*., 2012). The extremely branched structure of dendrimers (Figure 6) offers huge surface to size ratio which facilitate greater interaction with microorganism and it can incorporate hydrophobic and hydrophilic drug moieties into it (Medina *et al*., 2009).

Figure 6. Diagrammatic presentation of dendrimer



Dendrimer-based nanosystems hold three distinct units central core, polymeric branches and a terminal group at the dendrimers surface. The terminal group is PEGylated to improve biocompatibility and thereby, reducing toxicity. PAMAM [Poly (amidoamine)] was the primary synthesized and commercialized dendrimer and along with poly(propyleneimine) (PPI) that is extensively used as drug delivery agent (Martins *et al*., 2013; Kumar *et al.*, 2015). The marketed formulation based on this carrier is VivaGel® (http://www.starpharma.com) to prevent and cure bacterial vaginosis and sexually transmissible infections (Puri *et al*., 2009). VivaGel® is made from SPL7013 which is L-lysine derived dendrimer with methane series at the surface creating a polyanionic exterior. Dendrimer based antibacterial formulations with enhanced activity has been evidenced. For example, **Kannan *et al*., 2019** formulated poly(aryl ether) based amphiphilic dendrimers. The detailed mechanistic study reveals that optimal tuning of hydrophobicity of dendrimers play a vital role in membrane disruption of bacterial membrane. **Wronska *et al*., 2019** evaluated antibacterial activity of modified polycationic and polyanionic dendrimers in combination with levofloxacin against *E.coli, Proteus hauseri* and *Staphylococcus aureus.* The formulation showed satisfactory results due to a

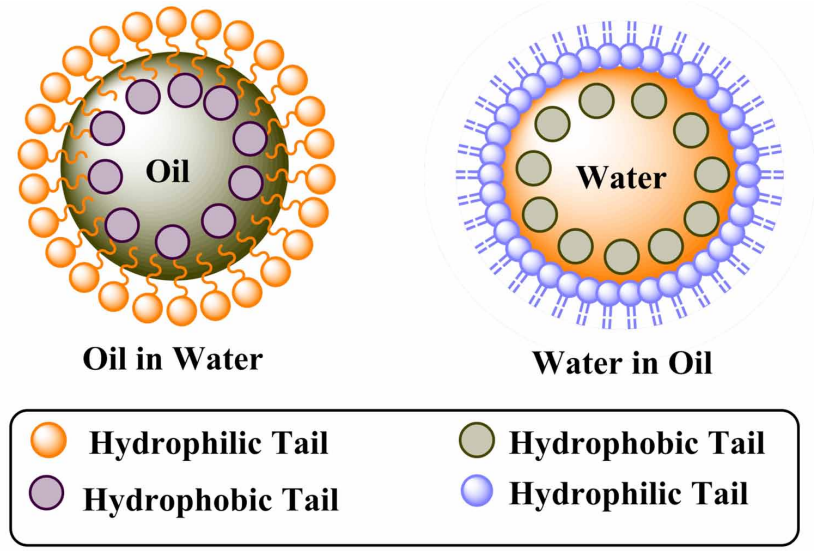
synergistic effect in antibacterial activity. **Gholami, 2017** reported antibacterial activity of poly(amidoamine) (PAMAM-G7) dendrimers was evaluated against gram-negative and gram-positive bacteria such as *P. Aeruginosa, E. coli, Acinetobacter baumanni, Shigella dysenteriae, Klebsiella pneumoniae, Proteus mirabilis, Bacillus subtilis* and *Staphylococcus aureus*. **Vembu *et al*., 2015** achieved sustained release of ciprofloxacin by formulating piperazine core (1,3,5-triazine) dendrimer. the developed formulation exhibited 5 times higher activity for tested microbes in comparison to pure ciprofloxacin. **Maleki *et al*., 2015** evaluated antibacterial activity of different generation (2 and 4) poly (amidoamine) dendrimer (PAMAM) using bacterial strains isolated from water resources like *E. coli, P. aeruginosa, k. oxytoca, B. subtilis,* and *S. aureus*. PAMAM dendrimer was found to be more effective against gram positive bacteria. Amino-terminated generation 2 poly(amidoamine) dendrimer showed positive effect on removal of main bacterial strains proving it as a safe and effective material for water disinfection in future. **Tang *et al*., 2013** synthesized dendrimers encapsulating silver nanocomposites. These were used as antibacterial agents (against *E. coli* and *S. aureus*) during manufacture of antibacterial cotton fabrics. Increased antimicrobial activity was observed with a decrease in the silver nanoparticle size. **Mazumder *et al*., 2013** fabricated antibacterial dendrimer-encapsulated zero-valent nickel nanoparticles (NPs) using fourth generation (G4)NH_2-terminated poly(amido) amine (PAMAM) dendrimer having antibacterial activity. **Winnicka *et al*., 2013** investigated the effect of PAMAM-NH_2 and PAMAM-OH dendrimers of second generation and third generation incorporating antibiotics such as erythromycin and tobramycin. Erythromycin loaded dendrimers exhibited 8 times increase in water solubility with a minor change in bactericidal action of erythromycin.

1.4.5 Microemulsion and Nanoemulsion

Microemulsions (MEs) act as potential drug carrier system in the pharmaceutical drug development (Shah *et al*., 2010). Drugs with distinct physicochemical properties can be delivered effectively to the tissues/organs with the help of microemulsion and nanoemulsion based nanocarriers. MEs are transparent emulsions or phase-transition systems (water microdroplets dispersed in oil or oil microdroplets dispersed in water) (Peek *et al*., 2008; O'Hagan, 2007). MEs or NEs are thermodynamically stable which consist of nanosize droplets in an inner phase and are enclosed within an amphiphilic compound (surfactant) or amphiphile along with an appropriate co-surfactant. MEs /NEs are able to carry antigens in the internal core for effective vaccine delivery. An oil-in-water emulsion of MF59TM was proved for influenza in more than 20 countries (Waheed *et al*., 2015). Microemulsions are of versatile nature having low surface tension, enhanced physicochemical properties such as stability, solubility

resulting in enhanced permeation and absorption (Mishra *et al*., 2014; Hung *et al*., 2007). Figure 8 showed a diagrammatic view of micro and nanoemulsion.

Figure 7. Diagrammatic representation of micro and nano-emulsions



Montanide ™ is an oil-in-water emulsion and water-in-oil emulsion (ISA 50V, 51, 201, 206 and 720). Montanide ISA 201 and 206 have been used in various infections associated with foot and mouth. Montanide ISA 51 and 720 was used in treatment of malaria (Aucouturier *et al*., 2002; Kumar *et al*., 2004). An oil-in-water nanoemulsion was developed using biosurfactant. Using this self assembling, the PEG moiety and a receptor specific antibody was encapsulated on the aqueous interface of nanoemulsion (Zhang *et al*., 2008). Researchers have synthesized various micro/nanoemulsion systems with enhanced bactericidal effect. For example, **Prakash *et al*., 2019** investigated coriander, cumin, lemongrass, pepper and fennel nanoemulsions for evaluation of antibacterial and anti-biofilm activities against *Salmonella enteric* Typhimurium. The formulation showed that particle size was in nanorange. The citral nanoemulsion can be further explored as natural antibacterial and anti-biofilm agent for food preservation applications. **Kumari *et al*., 2018** prepared an antibacterial nanoemulsion by using thymol, an essential oil component of plant and *Quillaja* saponin, a glycoside surfactant of *Quillaja* tree. The nano scale formulation could be a potential antimicrobial and plant growth promoting agent for agriculture. **Lu *et al*., 2018** reported Citral-in water nanoemulsion and

evaluated its antimicrobial activity. Citral can lose its bactericidal activity under normal storage. The antimicrobial activities of nanoemulsions were significantly large and therefore can be used as effective antimicrobials in cosmetics, food products and agrochemical industries. **Yildirim *et al*., 2017** prepared cinnamon oil based nanoemulsions /micro emulsions, which has good antimicrobial activity and also used for improving the quality, shelf life of foods. **Ma Q *et al.,* 2016** prepared microemulsions with a mixture of soybean oil with other oils such as cinnamon bark oil, eugenol/thyme oil. The antimicrobial activity of eugenol or thyme oil microemulsions was significantly greater than un-encapsulated drug. **Hu *et al*., 2016** developed a microemulsion to enhance the water solubility of the antimicrobials. This mixture consisted of N-methyl-2-pyrrolidone as solvent and dimethyl sulfoxide as co-solvent, polyoxyethylated castor oil, polyalkylene glycol, and polyoxyethylene tridecyl ether phosphate as surfactants. This formulation significantly enhanced the solubility of SecA inhibitors. The SecA inhibitors posses equal bactericidal activity as that of streptomycin. **Tian *et al*., 2016** reported stable self emulsifying nanoemulsion using cinnamaldehyde. The formulation provided a long-term inhibition towards *E. coli* as compared with pure cinnamaldehyde. **Shaaban and Edris, 2015** prepared carvacrol microemulsions to be used as preservatives and disinfectants. The carvacrol microemulsion exhibited significantly higher antibacterial activity. It was concluded that cationic carvacrol microemulsions can also be used as disinfectants and preservatives. **Jerobin *et al*., 2015** formulated neem oil based nanoemulsions (O/W) using high energy ultrasonication method. The developed systems proved antibacterial against *Vibrio vulnificus* by disrupting their cell membrane. It was nontoxic at lower concentration to human lymphocytes. **Hwang *et al*., 2013** reported cetylpyridinium chloride (CPC) nanoemulsion for antimicrobial action against *A. baumannii.* The formulation contains 10% Triton X-100, 1% and 25% soybean oil which indicated the best efficacy against *A. baumannii.* A specific amount of CPC is responsible for death of planktonic forms of *A. baumannii* while the destruction of biofilm was obtained by emulsified oil and its detergent fractions. **Anjali *et al*., 2010** developed refined sunflower oil and tween 20 based o/w microemulsion. A decline in bacterial growth was observed with all the formulations prepared.

1.4.6 Solid Lipid Nanoparticles

Solid lipid nanoparticles are colloidal system alternative to emulsions, polymeric nanoparticles, liposomes *etc*. The lipid-lipid (oil) has been substituted by a solid-lipid in these submicron sized lipid emulsions. Solid lipid nanoparticles have high surface area, small in size, high drug loading capacity and potential approach to target the drugs. They exhibited several advantages such as low toxicity, compatibility and high stability (Ekambaram *et al*., 2012). Solid Lipid Nanoparticles of various

antibiotics have been formulated and studied. For example, **Anjum *et al*., 2020** developed anacardic acid loaded solid lipid nanoparticles by homogenization method. The results suggested the enhanced efficacy of developed formulation to overcome the biofilm-mediated antimicrobial resistance. **Bolla *et al*., 2019** formulated furosemide-silver complex for antibacterial activity. The particles were spherical and in nanorange. The formulation could be considered as promising topical antibacterial agent against bacterial infections. **Pignatello *et al*., 2018** synthesized ciprofloxacin loaded solid lipid nanoparticles using quasi-emulsion solvent diffusion and solvent injection method. Positively charged and stable formulation was formed by incorporation of cationic lipid (didecyl dimethylammonium bromide; DDAB). The formulation showed effective antibacterial effect and could be promising nanocarrier for delivery of antibiotics by reducing toxicities associated with pure drug. **Gaspar *et al*., 2017** reported the anti-mycobacterial activity of solid lipid nanoparticle containing rifabutin (RFB) *via* pulmonary administration. SLN's were loaded into uniform sized microspheres to facilitate their pulmonary administration. The particle diameter indicated a uniform distribution of drug in the alveolar region. The *in vivo* bio-distribution studies of microencapsulated RFB-SLNs suggested that it could be used for delivery of antibiotic. **Shazly *et al*., 2017** formulated SLNs loaded with ciprofloxacin *via* ultrasonic melt-emulsification method for controlled release and improved bactericidal effect.

1.4.7 Immunostimulating Complex (ISCOM)

ISCOMs are nanorange spherical, hollow, cage like assembled particles consisting of saponin Quil A adjuvant, phospholipids, cholesterol and antigen protein. The particle without antigen is known as an ISCOM matrix (Zhao *et al*., 2014). Quil A possesses affinity for cholesterol which induces stability in the ISCOM matrix. Hydrophobic molecules can be only loaded within these complexes. The assembly of ISCOM is facilitated by hydrophobic interaction between these components and the antigen. **Kostetsky *et al*., 2011** developed tubular immunostimulating complex which was able to increase the immunogenicity (three to four folds) for protein antigens from *Yersinia pseudotuberculosis.*

Various nanocarriers incorporating antibacterial drugs have been summarized in Table 5.

Table 5. Nanocarriers incorporating antibacterial drugs

S.No.	Drug Carrier	Drug	Micro-organism	References
1	Chitosan-AgNPs	Cetrimonium bromide	Methicillin-resistant *Staphylococcus aureus* (MRSA)	Holubnycha *et al*., 2018
2	AgNPs	Juglans regia leaf extract	*E.coli and S.aureus*	Eshghi *et al.,* 2018
3	Silver doped bioactive glass	Zein scaffolds	*E.coli and S.aureus*	El-Rashidy *et al*., 2018
5	CuNPs/Chitosan solution	Ginger and ascorbic acid	MRSA and *E.coli*	Holubnycha *et al*., 2017
6	PAMAM-G7 dendrimers	Trimethoprim-sulfamethaxozole, Ampicillin, Tetracycline, Chloramphenicol	*Pseudomonas aeruginosa, E.coli, Acinetoacter baumanni, Shigella dysenteria, Klebsiella pneumonia, proteus mirabilis, S.aureus, Bacillus subtilis*	Gholami, 2017
8	PAMAM(G2-G4) dendrimer	Quaterinary ammonium salts using halogen groups(Cl, Br, I)	*E.coli, P.aeruginosa, klebsiella oxytoca, Bacillus subtilis* and *staphylococcus aureus*	Maleki *et al*., 2015
9	PAMAM dendrimer(low generation)	Silver nanocomposites	*E.coli* and *S.aureus*	Tang *et al*., 2013
10	PAMAM dendrimer(G4)	Zero-valent Nickel "Ni(O)" NPs	*Gram positive* and *Gram negative*	Mazumder *et al*., 2013
11	PAMAM dendrimer NH2 and OH (G2 and G3)	Erythromycin and Tobramycin	*S.aureus, Bacillus Subtilis, E.coli, Pseudomonas aeruginosa* and *Proteus mirabilis*	Winnicka *et al*., 2013
12	1,3,5-Triazine dendrimer	Ciprofloxacin	*Gram positive* and *Gram negative bacteria like (S.aureus, Bacillus Subtilis, E.coli, Pseudomonas aeruginosa, Proteus mirabilis)*	Vembu *et al*., 2015
13	Liposomal formulation	Azithromycin	*P.aeruginosa*	Solleti *et al*., 2014
14	Liposomal formulation	Linolenic acid	*H.pylori*	Jung *et al*., 2015
15	Liposomal formulation	Cephalexin	*S.aureus*	Shafaa *et al*., 2008
16	Ovalbumin NPs	Cefpirome	*Vero cells* and *blood cells*	Namasivayam *et al*., 2017

continues on following page

Table 5. Continued

S.No.	Drug Carrier	Drug	Micro-organism	References
17	PLGA polymer	Azithromycin	*E.coli, Haemophilus influenza, streptococcus pneumonia*	Azhdarzadeh *et al*., 2012
18	Poly(lactic acid co Castor oil) {PLACO} and Ricinoleic acid based poly(ester anhydride){PSA-RA}	Ultra short lipopeptides conjugated to an aliphatic acid chain	*E.coli, S.aureus*	Eckhard *et al*., 2016
19	Poly(e-caprolactone)	Doxycycline	*E.coli, Haemophilus influenza, streptococcus pneumonia*	Raval *et al*., 2014
20	Poly-N-isopropylacryl amide (pNIPAM)	AgNPs	*E.coli, S.aureus*	Qasim *et al*., 2018
21	PEG, PVP, PVA and HEC	AgBr NPs	Gram positive and Gram negative bacteria	Suchomel *et al*., 2015
22	ISCOM	Curcumarinoside A2-2-cholestrol-MGalDG	*Yersinia pseudotuberculosis*	Kostetsky *et al*., 2011
24	Micro emulsion	Soyabean oil with Cinnamon bark oil, eugenol or thyme oil, tween 80	*Listeria monocytogenes, Salmonella entrica, E.coli*	Ma Q *et al*., 2016
25	Micro emulsion	Refined sunflower oil, tween 20	*E. coli*	Anjali *et al*., 2010
26	Micro emulsion	SecA of Candidatus Liberibacter asiaticus	*Agrobacterium tumefaceins, liberibacter crescens, Rhizobium etli, Buadyrhizobium japonium, mesorhizobium loti, sinorhizobium melikoti*	Hu *et al*., 2016
27	Nanoemulsion and microemulsion	Cinnamon oil	*Campylobacter jejuni, Salmonella enteridis, E.coli, S. aureus* and *Listeria monocytogens*	Yildirim *et al*., 2017
28	Microemulsion	Triton, Soyabean oil, Cetylpyridinium chloride	*A.baumanii*	Hwang *et al*., 2013
29	Microemulsion	Carvacrol	*E.coli, S. aureus* and *Listeria monocytogens*	Shaaban and Edris, 2015
31	Nanoemulsion	Citral	*Bacillus cereus, B. subtilis, Haemophilis influenza, Neisseria gonorrheae, S. pneumonia, Vibrio cholera*	Lu *et al*.,2018
33	Nanoemulsion	Thymol and Quillaja	*Xanthomonas axonopodis*	Kumari *et al*., 2018

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Table 5. Continued

S.No.	Drug Carrier	Drug	Micro-organism	References
34	Nanoemulsion	Cinnamaldehyde	*E. coli*	Tian *et al*., 2016
35	Nanoemulsion	Neem	*Vibrio vulnificus*	Jerobin, *et al*., 2015
36	SLNs	Rifabutin	*Mycobacterium tuberculosis*	Gaspar, *et al*., 2017
38	SLNs	Ciprofloxacin	*E.coli*	Pignatello, *et al*., 2018
40	SLNs	Ciprofloxacin	*Gram positive, Gram negative bacteria*	Shazly, 2017
42	SLNs	Minocycline and ciprofloxacin	*P.aerugiosa, S. aureus*	Valdes *et al*., 2018

1.4.8 Bacteriocins

Bacteriocins are a kind of ribosomal synthesized antimicrobial peptides produced by bacteria, which can kill or inhibit bacterial strains (Yang *et al*., 2014). In other words, antimicrobial peptides or proteins produced by bacteria are categorized as Bacteriocins. Bacteriocins can be classified on the basis of origin. Bacteriocins from gram-negative bacteria include colicins and microcins and Bacteriocins from gram-positive bacteria are generally divided into class I (modified peptides, lantibiotics), class II (unmodified peptides, non-lanthionine) and class III (large proteins, heat unstable) (Cotter *et al*., 2013). The bacteriocins come under GRAS status (generally recognized as safe) and have no side effects. The use of various nanocarriers such as liposomes, nanofibers, chitosan, and metallic nanoparticles can be used to shield bacteriocins from degradation (Dimov *et al*., 2005). Bacteriocins are safe for human use as they are acted upon by gastrointestinal enzymes (proteases). Bacteriocins exhibit antibacterial activity against various Gram positive bacteria including *B. cereus, S. aureus*, *C. botulinum,* and *L. monocytogenes* (Saavedra *et al*., 2004). Bacteriocins are associated with several disadvantages such as restricted antimicrobial spectrum, high doses required for inhibiting multi-drug resistant strains, high sensitivity to gastrointestinal proteases, high production cost and low production yield due to incomplete recovery during purification. The commonly used bacteriocins include pediocin and nisin. Nisin is a polypeptide, synthesized as pre-pro-peptide and carries 57 amino acid residues and it undergoes post-translational modifications (Alishahi, 2014). It is found to have activity against various pathogenic food-spoiling bacteria such as *S. aureus* and *L. monocytogenes* (Zohri *et al*., 2013). However, it has been observed that it is less effective against gram negative bacteria, yeasts and moulds (Zacharof and Lovitt, 2012). Nisin is not poisonous for human consumption as per toxicological studies and it is restrained in acidic food materials due to its less

stability at neutral pH (Jeevaratnam *et al*., 2005). Pediocin polypeptide is composed of 44 amino acids. Unlike nisin, it does not show any post-translational changes (Ovchinnikov *et al*., 2016; Cotter *et al*., 2005). Nanoformulations of bacteriocins include conjugation of bacteriocins with nanoparticles (Fahim *et al*., 2016); encapsulation of bacteriocins in nanoliposomes, chitosan nanoparticles and metallic nanoparticles (Sidhu and Nehra, 2017) etc. Bacteriocin nisin is widely used and it does not develop resistance due to its narrow antimicrobial spectrum (Zendo, 2013; Rea *et al*., 2013; Sidhu and Nehra, 2017). Table 6 depicts various nanocarriers developed by bacteriocins along with targeting bacteria.

Table 6. Various nanocarriers systems incorporating bacteriocins

Approaches	Bacteriocins	Drug delivery	Results	Test organisms	References
Encapsulation of bacteriocins in nanoliposomes	Nisin Z produced from bacillus licheniformis P40	Nanovesicles of phosphatidylcholine	Inhibitory action	*Listeria monocytogenes*	Teixeira *et al*., 2008
Conjugation with chitosan NPs	Nisin	Dipalmitoyl phosphatidylcholine/ Dicetylphosphate/ cholesterol chitosan NPs	Antimicrobial activity	*Bacillus subtilis*	Colas *et al*., 2007
	Nisin	Nanoparticles of Chitosan	Antimicrobial activity two folds	*E.coli*	Alishahi, 2014
	Nisin	Chitosan-alginate nanoparticles	Greater antimicrobial activity	*S. aureus*	Zohri *et al*., 2010
	Bacteriocin	Chitosan nanoconjugates	More antimicrobial potential	*S. aureus*	Namasivayam *et al*., 2015
Bacteriocin Conjugation with metallic nanoparticles	Enterocin	Silver NPs	Broad antimicrobial activity against food spoiling bacteria	*Listeria monocytogenes, S. aureus*	Sharma *et al*., 2012
	Nisin	Silver NPs	High antimicrobial spectrum	*Listeria monocytogenes*	Kumari, 2012

1.4.9 Carbon Nanotubes (CNTs)

Carbon nanotubes are cylindrical molecules made of hexagonal arrangements of sp^2 hybridized carbon atoms which are classified as the members of fullerene and belongs to family of carbon allotropes. The size varies from 0.5 to 1 nm of diameter to several hundred in length. When the CNTs walls are made of single graphene sheets then these are referred as single-walled carbon nanotubes (SWCNTs) and when these are

made of several graphene sheets then these are called multi-walled carbon nanotubes (MWCNTs). These possess unique mechanical, electronic and structural properties which make them potential nanocarriers for delivery of antibacterial drugs. The organic functionalization of CNTs can improve its biocompatibility profile with low cytotoxicity. CNTs can be used as carriers for anticancer molecules, immunoactive compounds, proteins and genetic materials (Tostes *et al*., 2013). Table 7 comprises patents on CNTs (Martins *et al*., 2013).

Table 7. Various patents on carbon nanotubes

Patent no.	Title	Nanocarriers	Therapeutic indications	References
US20100324315 A1	Poly (citric acid) functionalized CNTs DDS.	CNTs-g-PCA	Therapeutic agents and cancer	Atyabi *et al*., 2010
WO2012031164A2	Drug delivery via CNT arrays	CNTs	Therapeutic agents and cancer	Aria *et al*., 2012
US20120220921 A1	Immunologically modified CNT for cancer treatment	SWCNTs	Photodynamic therapy and cancer	Chen *et al*., 2014
WO 2010123989 A1	Chitosan/CNT composite scaffolds for drug delivery	Chitosan coated SWCNTs/ MWCNTs	Therapeutic agents and antibiotic delivery	Jennings *et al*., 2010
WO 2009070380 A3	Water-soluble CNT compositions for drug delivery and medical applications	SWCNTs	Therapeutic agents and medical applications	Tour *et al*., 2014
WO 2013101983 A3	Targeted self assembly of functionalized CNT on tumors	Oligo-morpholino coated SWCNTs	Therapeutic agents and cancer	Scheinberg *et al*., 2013
US 20130034610 A1	Hydrophobic nanotubes and NPs as transporters for delivery of drugs into cells	SWCNTs/ MWCNTs	Therapeutic agents	Dai *et al*., 2012
US 20130158377 A1	Drug delivery and substance transfer facilitated by nano enchanced device having aligned CNTs protruding from device surface	Coated CNTs	Therapeutic agents and medical applications	Gharib *et al*., 2012

1.5. Synergistic Effect of Antibodies with Nanomaterials to Combat Superbugs

Antibodies are used since 19th century; the first antibody (immune-boosting serums) was developed to treat tetanus and diphtheria patients. The first noble prize for medicinal drug was awarded to Emil Von Behring in 1901 for his efforts in field of antibiotics (Cal *et al*., 2017). Researchers (New York City) developed an antibody serum that captured the interest of the state health commissioner for treatment of pneumonia in 1910. Thomas Parran Jr conducted a pneumonia control program in which the serum was dispensed to human beings all over the country. Monoclonal antibodies (MAbs) are the targeting molecules with high specificity, affinity and versatility. Antibody-conjugated nanoparticles were prepared to reach directly at a target site by overcoming physiological barriers (Hu *et al*., 2010). The delivery of targeted nanomaterials to the infection site can be achieved by surface modification with ligands such as polyethyleneimines, chitosan and glucosamine which may also improve the therapeutic efficacy and reduce the side effects of antimicrobial drugs (M Cardoso *et al*., 2012). A study reported an antibody-antibiotic conjugate consisting of an anti-*Staphylococcus aureus* antibody conjugated to an exceptionally efficacious antibiotic that was activated solely after its release in the proteolytic environment of the phagolysosome. The antibody-antibiotic conjugate proved to be effective to vancomycin for treatment of bacteraemia and provided direct evidence that intracellular *Staphylococcus aureus* represents the necessary component of invasive infections (Azevedo *et al*., 2014; Peng *et al*., 2017).

Table 8. Various antibodies with their target organism

Target organism	Antibody	References
Bacillus anthracis	Raxibacumab	Subramanian *et al*., 2005
Bacillus anthracis	Anthim(ETI-204)	www.elusys.com
Clostridium difficile	CDA1/CDB1	Lowy *et al*., 2010
Bacillus anthracts	Valortim (MDX- 1303)	www.pharmathene.com
Shiga toxin- Producing E.coli	Urtoxazumab	Lopez *et al*., 2010
Shiga toxin- Producing E.coli	ShigamAbs	Bitzan *et al.,* 2009
Pseudomonas aeruginosa	KB001	www.Kalobios.com
Staphylococcus aureus	Pagibaximab	Weisman *et al*., 2009
Pseudomonas aeruginosa	Panobacumab(KBPA101)	Lazar *et al*., 2009
Pseudomonas aeruginosa	Anti- Pseudomonas IgY	Nilsson *et al*., 2008

1.5.1 Emergence of Natural Antibodies in Form of Nanomaterials

Anti-bacterial antibodies attach directly with pathogen or intend to deactivate toxins or other virulence factors (Subramanian *et al*., 2005). The bacteria bind to antibodies by opsonizing process. Some antibody-antibacterial drugs are presently being evaluated in clinical trials and are shown in Table 8.

1.6 CONCLUSION AND FUTURE PERSPECTIVE

Nanotechnology has become a revolutionary approach to improve and investigate novel pharmaceutical drug products with antibacterial activity. Nanoparticles present promising choices in the plan of next generation therapeutics towards fungal, bacterial and viral threats. The development of new nano antimicrobial drugs with multiple functions will revolutionize medical medicines and it will also play a role in pacifying infectious diseases (Sharma *et al*., 2012; Stern *et al*., 2005). Thus, it has become important to fight multi drug resistant (MDR) microorganisms using conventional antibiotics in clinically proved nano formulations (MacRae *et al*., 2003). The nanoparticles improve the shelf life of drugs by nano-based aspects like decreased lipophilicity, increased solubility, cost-effectiveness, patient compliance, reduced toxicity, high bioavailability and sustained-release (SR) of a drug over a long period of time. Scientists have engineered quantum dot nanoparticles made up of cadmium that makes bacteria more vulnerable to antibiotics telluride (Aruguete *et al*., 2013). This will hopefully be a step forward in fighting against drug-resistant pathogens, like superbugs and the infections caused by bacteria's. Currently, some nano based antibiotics had been approved clinically for the use of human beings in several infectious diseases. Lipoquin™ and Pulmaquin™ (S.A., Grifols, Spain, Barcelona, and Aradigm Corporation, Hayward, CA, USA) are the liposomal formulations (inhalable) of ciprofloxacin, for curing the diseases such as cystic fibrosis (CF) or in non-CF bronchiectasis. Thus, future research will present a great potential to fight against multidrug-resistant superbugs.

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Chapter 11
Harnessing the Capability of CADD Methods in the Prediction of Anti-COVID Drug Likeliness

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ABSTRACT

The COVID-19 pandemic has claimed many lives and added to the social, economic, and psychological distress. The contagious disease has quickly spread to almost 200 countries following the regional outbreak in China. As the number of infected populations increases exponentially, there is a pressing demand for anti-COVID drugs and vaccines. Virtual screening provides possible leads while extensively cutting down the time and resources required for ab-initio drug design. The chapter aims to highlight the various computer-aided drug design methods to predict an anti-COVID drug molecule.

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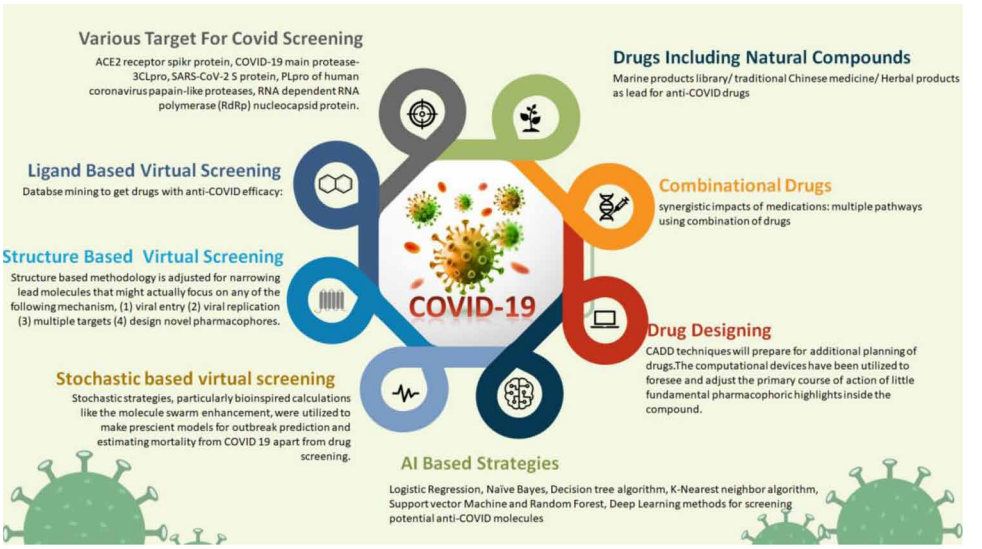
1. INTRODUCTION

Nearly the entire year 2020 was marred by the deleterious effect of the pandemic, COVID 19 infection. The infection claimed many lives and added to the social, economic, and psychological distress. The contagious disease that had an initial regional outbreak in Wuhan, China, quickly spread to almost all countries. The World Health Organization declared the disease as a pandemic in March 2020. As the number of infected populations increased exponentially, the medical research fraternity faced pressing demand for anti-COVID therapeutic solutions like drugs and vaccines. Despite some recent success claims in vaccine development, the search for an ideal drug candidate continues. Predominantly, three drugs that have ambiguous success include hydroxyquinoline- an antimalarial drug with quinoline as the pharmacophore, remedesivir- a nucleoside analog with an interfering role in RNA replication, and dexamethasone- a steroid that modulates the inflammatory response. However, as none of the above candidates are "the drug" against COVID-19, the scientists continue searching for drugs. With limited workforce resources due to the infected personnel or preventive lockdown and time-pressures for early therapeutic solutions, computer-aided drug design emerged as a preferred tool with many scientific reports that focussed on drug repurposing using screening methods.

Researchers applied computational screening methods to the FDA approved drugs to cut down on cost and time as the already tested and approved drugs were assumed to be safe for immediate clinical application. Media reports included mega-projects of Scripps Research, US announcing compound library screening of over 14000 drugs, the screening of drugs for augmenting remedisivir by Calibr scientist, and many more.

The quantum of the reported work in a short span is unparallel. However, in the absence of uniform protocols, though the protocol may be complete and standardized in itself, there have been contradictory and ambiguous findings. The reliability of the screening data hence needs to be ascertained. Further, as the predicted drugs, especially the established antivirals, did not elicit desired results in clinical settings, the scientists were forced to include varied categories of the molecules while screening. While mining extensive databases with varied scaffolds, newer screening protocols based extensively on machine learning methods were also developed. The chapter aims to highlight the various computer-aided drug design methods to predict an anti-COVID drug molecule. (Figure 1)

Figure 1. Highlights of the chapter



2. TARGETS USED FOR COVID SCREENING

The targets for anti-COVID drug development play vital role in virus pathogenesis and are classified as targets during (1) viral entry, (2) viral replication, (3) viral repackaging and release, and (4) immune response modulators of the host (human). The initial and widely reported targets for the anti-COVID drug screening have been the proteins, particularly proteases involved in the various COVID life cycle stages (Hu et al., 2020)

A detailed literature covering the biochemical role of these targets already exists. For computer aided drug design (CADD) applications, the targets and associated relevant information is summarized in Table 1. Among the various targets, the spike protein has been of the particular interest, inhibition of which can potentially impede virus entry into the host cell. The 3D structure of the variants of coronavirus spike protein are available in RCSB PDB repository, for example PDB 6ACD as a complex with the ACE2 receptor (Song et al., 2018), PDBs 6CRV, 6CRW, 6CRX, 6CRZ, 6CS0, 6CS1 and 6CS2 as cryo-EM analyses of ACE-2 bound trimeric SARS-CoV spike protein, and various trypsin-cleaved, stabilized SARS-CoV spike protein variants (Kirchdoerfer et al., 2018). Homology predictive model of the spike protein using the SARS-CoV-2 sequence (GenBank accession number: MT159721.1, length: 29882 bp) was used as a target when the structure was not known (Bharath et al., 2020). In March 2020, SARS-CoV-2 RBD–ACE2 complex cryo-structure was deposited as PDB 6M0J, that has been used extensively for CADD applications (Lan et al., 2020).

As listed in the Table 1, structured and non-structured proteins and accessory proteins have been indicated as potential targets at different stages of the viral cycle. Apart from existing knowledge of virus invasion, the targets have also been identified through computational assessment using the pathogen-host interactome.

The detailed analysis of the interactome built using the spike protein's domain interaction with the ACE2 receptors, the topological and functional analysis, and the network distribution reflect the role of at least fourteen proteins with varied functions.

3. METHODOLOGY AND APPROACHES APPLIED

A vast number of published data following the COVID pandemic focussed on virtual screening using structure-based methods and explored docking energy and interactions.

The docking protocols used systematic and stochastic searching for conformers library. The screening protocols included antivirals like antimalarials, anti-HCV (hepatitis-Cvirus, *etc.*, antibacterials, antifungals, diuretics, immune-stimulants, and naturally occurring compounds. However, due to the absence of well-defined protocols, the results have varied key molecules. Few compounds also showed the ability for multi-targeting. Such compounds have been predicted to have better pharmacological utility. The results of published data have been summarized in the review article by Mohamed et al., 2020. Reports used structure-based (SBVS) and ligand-based virtual screening (LBVS) methods alone or in combination, and frequently, the results were also supplemented using molecular dynamics. In screening, multiple databases and softwares have been used.

3.1 Databases for Anti-Covid Screening

Databases are the backbone for *in silico* drug design. The databases list not only the compounds, but the pharmacological data, activities, potential targets, mechanism of action and various other important parameters. Conventionally and for COVID -19 repurposing of drugs screening has been carried using FDA approved drug databases, DrugCentral, ZINC, ChEMBL, Chemical Abstracts Service (CAS) that offers $\leq$50,000 compounds with tested or predicted antiviral activities, Natural compounds databases listing compounds in marine and traditional medicines, PubChem - a convenient access to antiviral chemicals and related information, SuperDRUG2 that is a repository of more than 4000 drugs, etc. In addition dedicated databases came into effect to save the time and resources, for example COVID-19 Drug-Repurposing Database (COVIDDRD) that provides data for COVID-19 drug-repurposing, DrugBank designed "COVID-19 Information Dashboard" that enables the direct access to pharmacological data of ~40 repurposed drugs, IUPHAR/BPS

Guide to PHARMACOLOGY (GtoPdb) having expert-curated SAR relationships on investigative COVID-19 drugs, and Therapeutic Target Database (TTD) provides data of clinical/preclinical COVID-19 drugs. (Wang et al., 2020)

Table 1. Various COVID Targets reported in the studies referred in the chapter and their representative available PDBs

Targeted pathway in viral life cycle	Protein Target	Role	PDBs
Viral Entry and structural protein	ACE2 receptor-spike protein interface	restrict the binding of SARS-CoV2 spike protein to the ACE2 receptor	Templates reported for predictive homology modelling: 6ACD, PDBs 6CRV, 6CRW, 6CRX, 6CRZ, 6CS0, 6CS1, 6CS2
			Crystallographic PDB:6M0J, 6VXX (Walls et al., 2020)
Cleaving RNA genome	EndoRNAse (nsp15)	Cleave both single-stranded and double-stranded RNA.	6W01 (RNA uridylate-specific endoribonuclease (NendoU) Kim et al., 2020)
viral replication and transcription	COVID-19 main protease-M^{pro}/C30 endopeptidase/ 3CLpro	Cysteine proteases	6LU7 (Jin et al., 2020)
Targeting viral replication	PLpro of human coronavirus papain-like proteases/ ORF3a protein	1. responsible for the N-terminus cleavage of the replicate poly-protein to nsp1-3 2. Essential for correcting virus replication. 3. significant in modulation of the innate immunity of the host	6W9C (Osipiuk,et al., to be published)
Replicating Viral Genome	RNA dependent RNA polymerase (RdRp)/ nsp 12	Viral replication	7BTF, 6M71 (Gao et al., 2020)
	helicase (nsp 13)	Blocks the ATPase activity to inhibit the replication of viral genome	6JYT (Jia et al., 2020)
	3′-to-5′ exonuclease (nsp 14)	Critically involved in SARS-COV-2 RNA synthesis	Models reported using predictive modelling
transcription and assembly/ structural proteins	nucleocapsid protein	forms complexes with genomic RNA of the virus	6M3M (Kang et al., 2020)
RNA synthesis and processing	2'-o- methyltransferase/ nsp 16	involved in the capping of coronaviral mRNAs	6W75 (Rosas-Lemus et al., 2020)
structural coronaviral protein	small envelope (E) glycoprotein (ORF 4) / membrane (M) glycoprotein (ORF 5)	Involved in several aspects of virus life cycle such as assembly, envelop formation and pathogenesis	Homology modelling: Apo form (PDB-ID: 6M03) and Holo form (PDB-ID: 6LU7, bound with N3 inhibitor) forms
Non structural proteins (nsp)	Various classes: nsp1, nsp 2, nsp4	Multiple functions in replication, packaging and release	6YWL for nsp3 (Schroeder et al., to be published, RCSB)
Host target	transmembrane protease serine 2 (TMPRSS2)	Modulates Viral entry	Predictive modelling using 2OQ5 as reported Elmezayen et al., 2020

3.2 Ligand based Virtual Screening (LBVS)

Ligand based virtual screening has been reported using representative softwares like FLAP (Fingerprints for Ligands and Proteins: Baroni et al., 2007), JChem fingerprint along with in-house developed softwares like D3Similarity (Yang et al, 2021).Purely based on ligand-based approach, Bocci et al. narrowed down on nine hits while screening a library of 4000 approved drugs. The database molecules were compared and ranked based on Glob-Prod (GP) score evaluated using different molecular interaction fields along with other structure-activity parameters. Hydroxyquinoline was regarded as the cut-off molecule and the potential molecules were then assessed *in vitro* on a cell line for cytotoxicity on SARS-CoV-2.

3.3 Structure Based Virtual Screening (SBVS)

Schrödinger's Glide, Autodock Vina, rDOCK, UCSF Chimera platform are among the different softwares that were used to carry SBVS for COVID 19 potential drug molecules. Structure based methodology was adapted for narrowing drugs that could potentially target, though not limited to, any of the following mechanisms: (1) inhibit entry of the virus by binding with the viral S-protein (Choudhary et al., 2020), (2) inhibit replicase machinery of the virus like disrupting nsp 9 (Chandra et al., 2021), (3) screening inhibitors for multiple implicated COVID 19 targets (Hosseini, et al., 2020), (4) to study interactions with COVID 19 targets like interaction studies of Mpro with therapeutically indicated drugs for COVID (Marinho, et al., 2020).

Dedicated servers to carry docking studies were also reported like the DINC-COVID that account for the flexibility in protein through ensemble docking algorithm. The server was validated through inhibitor prediction for Mpro (Hall-Swan et al., 2021).

The poses obtained from the docking studies were also subjected to dynamic studies that indicate the stability of the protein-ligand complex. For instance, 50 to 100 ns simulations using different platforms like AMBER/ GROMACS (GROningenMAchine for Chemical Simulations) / YASARA have been performed on docked poses of synthetic or naturally derived chemical compounds with COVID target proteins to understand the dynamic behavior of ligands (Quimque, et al., 2020: Sen Gupta et al., 2020).

Various parameters like the surface areas (solvent–accessible surface area (SASA) and molecular surface area (MolSA)), bond parameters, radius of gyration (Rg), binding energy per-frame, root-mean-square deviation (RMSD), hydrogen-bonding (HB), root-mean square fluctuation (RMSF), center-of-mass (CoM) distance and principal component analysis (PCA) can be studied to arrive at stable conformation and interactions of the ligand with protein. In addition, for a more accurate analysis

binding energies (ΔGbinding) can also be calculated using the Molecular mechanics (MM), MM-GBSA (generalized Born surface area) and MM/PBSA (Poisson–Boltzmann surface area) approaches.

3.4 Stochastic Methods Based Virtual Screening

Stochastic methods, especially bioinspired algorithms like the particle swarm optimization, were used to make predictive models for outbreak prediction and estimating mortality from COVID- 19. Probabilistic models were also reported by Czuppon et al., 2020, to predict the prophylactic activity of antivirals when used alone or in combination. The model included within-host viral dynamics based on viral kinetics in epithelial cell along with parameters for humoral immune response and virion release. The prophylactic prediction of the antiviral therapy accounted for (i) viral infectivity reduction β, (ii) enhanced viral clearance c, (iii) decrease in viral production p, and (iv) enhanced infected cell death δ. The critical efficacy and time scales for viral load and extinction were also reported using the model. Using mathematical modeling, viral dynamics has been studied after developing suitable within- host viral models using single and dual (upper and lower respiratory tract) physiological compartments. The models were used to predict viral load, infectiousness of an individual, infection to symptom onset, probability of transmission etc. (Ke et al., 2020).

Genetic algorithms using the GOLD software was used for docking and predicting the inhibitors for Mpro protein. The results indicated that Apixabin, an anticoagulant can be a potential candidate molecule (da Silva Hage-Melim et al., 2020).

3.5 AI Based Methods

Apart from the conventional docking programs, artificial intelligence and machine learning methods found wide application, not only in the utilization to analyze the clinical data of chest X-rays and CT data but also for virtual screening. As the AI methods are designed to analyze, categorize and predict with some degree of in-built decision making, AI methods distinguish from the conventional algorithms for screening. Few algorithms used for AI methods are listed in Table 2.

The AI methods were applied to predict the antibody variants, prediction of targets apart from the conventional proteases, and screening of massive drug/ compound datasets for the novel specific scaffold. The methodology typically followed the following pattern: (1) Compound database with approved or drug-like molecules including natural products, (2) in silico representation of the compounds through descriptors, (3) conformational searching through extensive sampling, or activity screening through docking or other parameters or both and (4) parallel application of

AI based methods. For a rigorous validation of results, combined scores of different algorithm programs were also used. The concept can be appreciated through the research published by Onawole et al., wherein scoring obtained from machine learning method based virtual screening and molecular docking were considered in parallel to evaluate surface glycoprotein inhibitors. The approach is termed as vote rank method.

When different methods for example docking and molecular vector- / structure based screening using deep learning methods gave different lead molecules while screening TargetMol-Approved-Drug-Library, molecular dynamics studies were carried to ascertain the results. Thus, hybrid screening methods can help to identify potential lead molecules. (Zhang et al., 2020(b))

Because of several AI algorithms, Xu et al., 2020 evaluated the algorithms for their capability in virtual screening. A combination of LB and SBVS dataset screening of 2030 Chinese natural medicine was carried using six commonly used ML algorithms. The six algorithms used were Logistic Regression (LR), Naïve Bayes, Decision tree algorithm (DT), K-Nearest neighbor algorithm (kNN), Support vector Machine (SVM) and Random Forest (RF). When compared on the basis of the statistical parameters like area under the curve (AUC), the LR outperformed the other five ML algorithms. Similarly, Zhang et al., 2020(a), evaluated eight AI algorithms (DT, kNN, SVM, RF, ERT, AdaBoost, GBT and XGBoost) using the various statistical parameters. Random forest (RF) regression models combined with Vina docking scores were used to screen library of 19000 molecules that can have potential to disrupt virus entry by binding with the S protein. Important molecular fragments in the compounds were oxolanes, benzene sulfonate groups and imidazoles that aid in binding. The results also indicated the importance of certain descriptors like the topological surface area and ring count. (Batra et al., 2020)

The protein-protein interactions are vital for the entry of the viral particles into the host. Learning vector Quantization in conjugation with multiple supervised learning algorithms was used for predicting as many 1326 potential human proteins as a target for anti-COVID strategies due to viral protein and human protein interactions (Dey et al., 2020). Deep docking protocols are machine learning methods that utilize QSAR deep models trained on docking parameters for screening billions of compounds with a certain level of accuracy. In the study (Ton et al., 2020), Deep docking protocols were developed for mining as many as 1.3 billion compounds from the ZINC dataset. The QSAR model utilized Morgan fingerprinting for screening, along with a neural network that works on the feed-forward back-propagation principle.

Table 2. Examples of few algorithms for AI based methods used in references and cited in the chapter

AI methods	support vector machine	Random Forest	Naive Bayes	K-nearest neighbors' algorithm	Decision Tree	Logistic Regression
	(SVM)	(RF)	(NB)	(k-NN)	(DT)	(LR)
Type of learning method	Supervised machine learning technique (instance based method)	Supervised machine learning technique (Ensemble learning methods)	Supervised learning methods (Probabilistic classifier, non-linear model)	Supervised learning methods (non-parametric instance-based learning, /lazy learning/ memory-based method)	Supervised learning method (Regression analysis methods, non-linear model).	Supervised learning algorithm (classification algorithm, statistical model, linear model)
Primary tasks	Classification, and regression	Classification, and regression	Used for constructing classifiers	Classification, and regression and making predictions	Classify, or predict values for input data	classify, or predict values for input data
classification and regression includes pattern recognition and outlier's detection						
Ideal for	Variable separation	Large datasets with multiple features.	Large datasets with random noise.	Calculating properties with strong locality, e.g. protein function prediction	Dimensionality reduction	solving classification related problems.
Based on	vectors that help in creating the hyperplane.	Multitude of uncorrelated decision trees as an ensemble	Applies bayes' theorem with the "naive" assumption of conditional independence between features and the class variable.	Local approximation for classification	Flowchart-like /Tree-shaped structure having representations of chains of decisions.	Utilizes a sigmoid function and works best on binary classification problems.
Aides in	Attributing missing data, working with outliers, and estimating characteristics for classification.	Attributing missing data, working with outliers, and estimating characteristics for classification	Predicting biological properties. E.g.. Toxicity, bioactivity classification for drug-like molecules	Predict the class, property, or rank of a molecule based on nearest training examples.	Multiclass problems, and aides l in handling non linear data sets	Hypothesis testing, model coefficient and classification data interpretation.
Error frequency/ Limitation	Least number of errors	Least number of errors	Least number of errors	Irrelevant attributes in high-dimensional space may mislead and may not handle large volume of data	Poor handling of noisy or incomplete data	Target variables classification important
Applications (*though not restricted to)	Categorization, classification of images, classification of data like SAR	Regression and classification task	Medical data classification and real-time predictions	Detecting outliers.	Classification	Prediction.
	Drug-target interactions: distinguishing between active and inactive compounds: ranking compounds Regression model		target interactions, novel targets classification tools for non-related information biomedical data, and Predicting ligand-target interactions	Chemical similarity search, Predict other bioactivities of drug-like molecules.	For predicting QSAR relationships, Fast conformational search Promising new compounds	

(Wang et al. 2005; Segal et al. 2004; Madhukar et al. 2019: Lavecchia, et al 2015: Zhang et al. 2017: The features listed are in general though exceptions may exist.)

4. APPLICATIONS OF CADD METHODS FOR DRUG SCREENING

4.1 Combinational Drugs

Researchers also explored not only multi-targeting but synergistic effects of drugs. Zhou et al. used network proximity analysis and complementary exposure patterns to identify drugs that target the various aspects of the viral cycle and other potential host cellular pathways. Three combinations were proposed; (1) Sirolimus plus Dactinomycin can be used to target mTOR signaling, and RNA synthesis pathway, (2) structural interference in viral glycoproteins and disruption of interaction of spike protein with ACE2 could potentially be effected using Toremifene plus Emodin combination, and (3) Inhibition of PLP and modulation of the inflammatory response through a combination of Mercaptopurine plus Melatonin (Zhou et al., 2020). Synergistic effects of drugs (A) lopinavir, (B) oseltamivir and (C) ritonavir was studied through sequential docking (Muralidharan et al., 2020).

4.2 Designing of Drugs

CADD methods will pave the way for further designing of drugs. Zhang et al. proposed alpha-keto amides as peptidomimetic compounds that can potentially serve as broad-spectrum antiviral agents for alphacoronaviruses, beta coronaviruses, and enteroviruses. The genesis is based on docking results with crystal structures of the main protease of different viruses. The *in silico* results were validated by wet-lab synthesis and *in vitro* validation (Zhang et al., 2020). The computational tools have also been used to predict and fine-tune the structural arrangement of small essential pharmacophoric features within the compounds. For instance, in the report by Kumar et al., the authors using computational structure-activity relationship predicted the role and structural arrangement of hydroxyethyl amine (HEA) within antiviral compounds. Similarly, an effective inhibitor for $3CL^{Pro}$ was speculated to have a preference for an aromatic bicyclic scaffold bearing N and O atoms and flexible linkers with NH groups to facilitate interactions with the substrate site. The presence of carboxyl or trifluoromethyl groups in the rings was also noted among the scorers in the virtual screening of compounds (Olubiyi et al., 2020). Heterocyclic bearing scaffolds like the oxadiazole and isatin moieties were screened as potential inhibitors for the main protease M^{pro} (Badavath et al., 2020)

For example, *in silico* and machine learning methods, an unsupervised Markov Cluster algorithm was also applied to identify aptamers as recognition molecules against COVID. The 51- and 67- base containing hairpin structured aptamers were screened as potential candidates using the SMART-Aptamer algorithm. Unique

scoring methods viz., motif enrichment score (K), aptamer abundance (F), stability score (S), and penalty score for families to counter opposing changes (P) were used. After optimizing the aptamers, MD simulations offered an insight into the binding of the aptamers with the spike protein (Song et al., 2020).

1.3 Novel Classes of Drugs Including Natural Compounds

Other classes of drugs that have been explored include antipsychotics, aptamers, and drugs from other natural sources. Antipsychotic drug molecules were screened *in silico* and *in vitro* for their inhibitory effect via binding with the ACE2 receptor. Nineteen drugs were docked using the ACE2 PDB (6MOJ), of which five antipsychotics, especially phenothiazines, were realized to block SARS-CoV-2 interaction with ACE2 (Lu et al., 2020). Histamine-2 receptor antagonist, Famotidine was also investigated computationally for exact mechanism of action as an anti-viral, after reports of its usage in clinically on COVID-19 patients. Though an indirect effect of Famotidine can be traced in modulation of immune response Sen Gupta et al., 2020 investigated the molecule as potential inhibitor against COVID protein targets using SBVS and molecular dynamics of 100 ns.

Bacterial compounds have for long exhibited antibacterial and antiviral compounds. Gentile et al. narrowed down on phlorotannins (brown alga), flavonoids such as Apigenin-7-*O*-neohesperidoside, Luteolin-7-rutinoside, and Resinoside and Peptidomimetic derivatives like pseudotheonamides based on docking and molecular dynamics results with SARS-CoV-2 M^{pro} after screening the marine natural product library with nearly 14000 compounds. The group initially screened the library using a binding-site-derived pharmacophore model to realize the importance of dimensional pharmacophore that accounts for stearic and electronic arrangement. The objective was to retain compounds that can serve as Michael acceptors covalent inhibitor and, if not non-peptidic molecules, then should have peptidomimetic regions. A combination of the supervised machine learning algorithm, Random forest, and docking protocol was utilized to classify and screen marine natural compounds library with 494 entries that ultimately lead to the identification of five potential inhibitors for M^{pro}. (Gaudêncio et al., 2020).

Lianhuaqingwen (LH) is a traditional Chinese medicine (TCM) preparation with broad-spectrum antiviral effects on different influenza virus strains. LH also showed anti-2019nCoV activity. Having honeysuckle and forsythia as active compounds in LH with antiviral potency, the LH components were examined for anti-Covid activity. The reported application of LH includes its usage in the treatment of SARS in 2003. In this study, the binding of chloroquine, remdesivir, ribavirin, and luteolin (the active component of honeysuckle) with the main proteins of 2019-nCoV (3CLpro, PLpro, RdRp, and S) were carried out by computational methods. (Yu et al., 2020)

Xu et al., while screening Chinese medicinal compounds not only evaluated different AI methods (referred above) but also narrowed on active compounds like Rutin as effective inhibitor for 3CLpro.

Natural products belonging to Nigerian and Indian plants were also screened for efficacy against COVID-19. Over 3000 natural products of Nigerian plants origin, majority being flavonoids, along with the ZINC database compounds were screened for inhibitory activities against 3CLpro.Amentoflavone, Glabrolide and zeylanone were some of the lead molecules. The work was based on structure based screening using AutoDock. (Olubiyi, et al., 2020)

Rather than targeting viral proteins, host (human) proteins were also proposed to serve as therapeutic targets against COVID 19. For example, Transmembrane Protease Serine 2 or TMPRSS2, a host protease or cathepsins that plays important processesing step for viral entry. Indian herbs phytochemicals were virtually screened in the study by Vivek-Ananth et al., 2020 through docking interactions using AutoDock Vina and molecular dynamics simulations.

More than 35,000 compounds in the Natural Product Activity and Species Source (NPASS) database were screened through SBVS for anti-COVID activity by targeting multiple targets (endoribonuclease, exoribonuclease, RNA-dependent RNA polymerase, methyltransferase and 3C-like proteinase) (Naik et al., 2020).

With modeling tools, ninety seven compounds that are antiviral secondary metabolites from fungi were screened for potency. The five SARS-CoV2 targets PLpro, 3CLpro, RdRp, nsp15, and the spike binding domain were docked with the compounds followed by molecular dynamics simulation. Three fumiquinazoline alkaloids, the polyketide isochaetochromin D1, and terpenoid based metabolite exhibited high binding affinities. (Quimque et al., 2020)

5. CONCLUSION

CADD tools can edge scientists in preliminary screening by cutting short the time and resources investment. However, the lead molecules obtained as results of CADD need extensive testing before any clinical applications. Past evidences exist wherein even when designed for a dedicated application drugs failed during pre-clinical to clinical translation. Repurposed drugs will certainly require testing on actual targets before any conclusive efficacy.

The other aspect is the standardization of CADD tools. With different algorithms and scoring functions, arriving on a single molecule during screening may not be feasible. Validating the CADD workflows will help to address the ambiguity in results.

However, CADD tools have certainly proved their utility, especially in the urgency during the COVID-19 infection.

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Related Readings

To continue IGI Global's long-standing tradition of advancing innovation through emerging research, please find below a compiled list of recommended IGI Global book chapters and journal articles in the areas of microbial infections, drug resistance, and solutions to avoid future epidedemics. These related readings will provide additional information and guidance to further enrich your knowledge and assist you with your own research.

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