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MYCOBACTERIUM - RESEARCH AND DEVELOPMENT

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Meet the editor

Wellman Ribón is a bacteriologist and clinical laboratory professional of the Industrial University of Santander and a specialist in Environmental Chemistry. He obtained his master's degree in Biochemistry from Universidad Pontificia Javeriana and is a PhD student of Public Health from the National Institute of Public Health of México and a senior researcher category COLCIENCIAS (Departamento Administrativo de Ciencia y Tecnología de Colombia). With more than 15 years of experience as public health adviser, he is a COLCIENCIAS researcher in scientific and technological development. He has worked at the National Health Institute of Colombia as coordinator of the Micobacterias Group, manager of research projects, and member of Centro Colombiano de Excelencia de Investigación en Tuberculosis (CCITB), the EurolabTB Consortium, and the SLAMTB. Mr. Ribón is currently a titular professor in Medicine School and works as a professor and researcher at the Industrial University of Santander. He is also the director of Mycobacterium Research Laboratory. Mr. Ribón has published articles about tuberculosis, leprosy, and mycobacteriosis diseases and has written four book chapters. He conducted a pulmonary tuberculosis research project in geriatric homes in the city of Bucaramanga, Colombia, and is currently carrying out the evaluation of the Tuberculosis Free Colombia Strategic Plan 2006–2015—"For the Expansion and Strengthening of the Stop TB Strategy." Mr. Ribón is the editor of *Hansen Diseases* book and *Tuberculosis: Expanding Knowledge*, and his major area of interest and research is *Mycobacterium* tuberculosis complex and other mycobacteria.

Progress obtained by mycobacteriology in recent years is undeniable. The constant knowledge generation that expands the frontiers of understanding is a key factor for finding solutions and successful activities for public health and industry.

Contents

Preface

In this book, you will find a diversity of information in which the teaching of mycobacteria continues to be supported by the conventional, molecular, and immunological advances that these microorganisms have had over time. The introductory part represents the significant knowledge of mycobacteria including epidemiological, clinical, molecular, and pathological information sufficient to address a topic of universal interest. The power of general analysis provides brief, concise, and updated information on those aspects of mycobacteria that are important in the fields of health, environment, and pharmacotherapy.

This book arose from the combination of diverse areas of knowledge, experience, research, and points of view that try to demonstrate that mycobacteria research is a complex science and very relevant to scientific studies that affect humans, because to search for sophisticated techniques for improving human health does not guarantee that the "battle" against mycobacteria has been won, since tuberculosis, mycobacteriosis, and leprosy are a daily world challenge.

Here you will find information about mycobacteria, dealing with major issues such as:

The physiological and biochemical characteristics that address the composition and cellular function of these microorganisms.

The conventional and molecular diagnosis of tuberculosis, mycobacteriosis, and leprosy that are of interest in public health and that through new and standardized methodologies allow to use in the diagnosis of all people, since these diseases do not discriminate the sex, age, and gender.

The virulence and pathogenicity factors of mycobacteria and their immune response by the host, for the ability to survive, require expressions of a series of determinants involved in the pathogen-host interaction.

The genetic study of mycobacteria has allowed researchers to infer techniques that allow the detection of genes or specific sequences for drug resistance.

The use of mycobacterial derivative agents that encode enzymes and proteins involved in metabolism and the cell membrane, associated in anticancer treatment and bioremediation of the environment.

How nontuberculous mycobacteria have been recognized over time as protagonists of lung diseases in immunocompromised and immunocompetent people throughout the world.

And finally we can't ignore diseases that have existed since biblical times, such as the leprosy, which presents a great challenge because of the difficulties for early detection and to cut the chain of transmission, and the lack of discernment on the part of the clinic has let this disease continue.

This book includes contributions made by prestigious experts and research groups in different areas of mycobacteria, and they have contributed new perspectives from their areas giving a comprehensive, important, and fascinating emphasis to this field that continues to offer challenges that lead various disciplines to understand their biology and pathogenicity. It is hoped that these chapters will be very useful for studying and discussion.

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Section 1

Introductory Chapter

Chapter 1

Introductory Chapter: Scientific Research on Mycobacteria and the Absence of Evaluation Processes

Wellman Ribón

Additional information is available at the end of the chapter

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1. Introduction

About 150 species belong to the *Mycobacterium* genus and are known as acid-fast bacillus. These bacteria are widely distributed in different ecosystems and it can be isolated from water, soil, or air. The *Mycobacterium* genus has a great metabolic diversity that has allowed them to adapt and survive in almost all environmental conditions. They are called slow-growing bacteria, because of their long generation times in comparison with other bacterial genus. Currently, there are new species which are being studied in order to be classified as new members of the *Mycobacterium* genus.

Some species of the *Mycobacterium* genus are known as nontuberculous mycobacteria (NTM). It can cause the mycobacteriosis disease in humans or animals. At present, it has not been established that mycobacteriosis is transmitted from person to person. This is one of the causes for not knowing precisely the number of cases of this disease and difficulties for an opportune diagnosis. The severity of the disease caused by NTM depends on multiple factors, the most relevant being the condition of the individual's immune system, the virulence characteristics of these bacteria, and the amount of NTM that is transmitted to the infected host. In the field of scientific research, there are studies on different aspects related to these microorganisms, nevertheless many characteristics of NTM and their implications in environmental processes, health or industrial use are not known in which given their great metabolic variety can help to solve environmental problems that threaten the human and animal's health and that compromise the quality of life of future generations.

Tuberculosis is an infectious disease caused by bacteria belonging to the *Mycobacterium tuberculosis* complex. Tuberculosis was declared a global emergency by the World Health Organization and it is considered a serious public health problem worldwide, given that it is

one of the infectious diseases that causes the most deaths worldwide along with the human immunodeficiency virus (HIV). Despite the strategies and efforts to achieve its control, the situation of tuberculosis is worrying and it is considered in several countries as an event of interest in public health. Therefore, the control implemented by each country is of vital importance to cut the chain of transmission. The foregoing evidences the priority need to conduct research on factors associated with tuberculosis control while continuing to produce new information in all areas of knowledge that may contribute to tuberculosis control.

Leprosy is a chronic disease, known as a peripheral neuropathy that can produce several and irreversible sequelae observed in the patient who has suffered from this disease, and as a consequence, it has generated a great stigma for all patients. Leprosy is an ancestral disease and has also been known as Hansen's disease, and there are currently studies documenting the great social and cultural component involved in the worldwide transmission and persistence of this infectious disease. The World Health Organization has declared leprosy as a disease in elimination due to the number of reported cases of patients, but there are countries that still report a recent transmission of the disease and in some of its provinces a larger number is diagnosed. A number of cases estimated by the World Health Organization consider that this country has reached the goal of elimination. *M. leprae*, the causative agent of leprosy, is not a cultivable microorganism in synthetic culture media, causing the diagnosis of the disease to be basically clinical even though there are currently molecular methodologies that can make an opportune diagnosis of the disease before the disability is observable in each individual affected by this alcohol-resistant acid bacillus.

The scientific research done on the species belonging to *Mycobacterium* genus can be classified into three groups. The following graph shows that a large number of publications in scientific research related to tuberculosis, leprosy, and some mycobacteriosis are produced. In the environmental field or application of mycobacteria to industrial processes, a smaller amount of new knowledge is produced that is useful for contribution to solving problems that threaten humanity but something very important is the little evidence generated by evaluation projects that must be carried out on the processes implemented in each country or region of the world so that the effect achieved can be attributed to the measure or knowledge implemented (**Figure 1**).

Given that tuberculosis, leprosy, and mycobacteria are diseases that affect a large population worldwide, control programs are performed in the regulatory framework of each country, and it is necessary and urgent that there is solid and incontrovertible evidence its impact and management in the control of these diseases that affect all individuals of different ages, genders, and social strata of a community, being of great importance the performance of periodic evaluations of the measures implemented by each government against the knowledge generated by the scientific investigation.

The evaluation of the projects, plans, policies, and strategies implemented must include the commitment of the authorities of each country and of the different territorial entities, in order to determine the effectiveness, cost-effectiveness, and attributions of the results obtained to the extent implemented in the community or country. These scientific evaluation projects should be based on official data collected through the information and monitoring system

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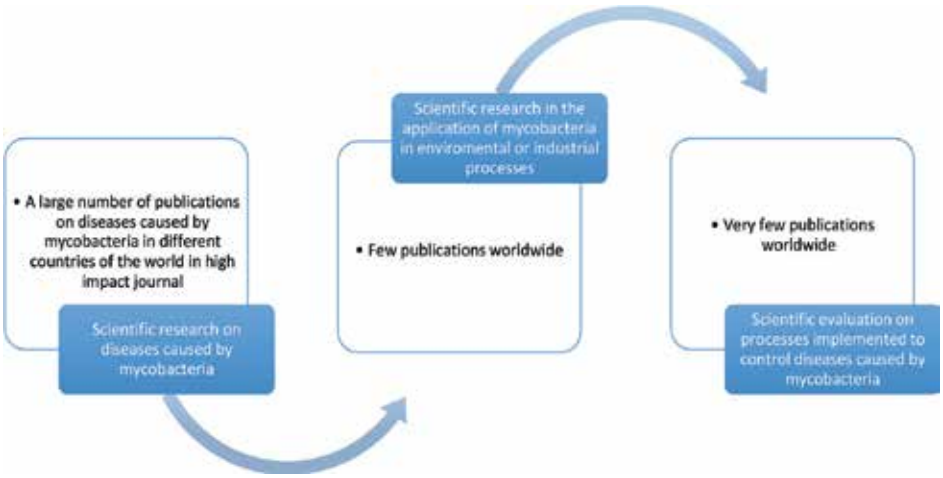

Figure 1. Research and scientific evaluation in mycobacteria.

of territorial entities for the measurement of performance and evaluation of the measures implemented. These evaluation projects make it possible to demonstrate the changes generated from the implementation of national or international policies, but especially should be evidence for decision-making and recommendations in health, public health, and international health.

One of the main limitations for the realization of scientific evaluation projects is to attribute the observed effect to the intervention implemented and evaluated. Because many of the processes in health, public health, or global health are assumed by the state governments through their national control programs, all control and surveillance activities must be authorized, assumed, and monitored by these control programs. Therefore, significant variations are only possible because of the implemented measures of an official nature or the sociocultural and political situation of each country that in any way affects the epidemiological situation of the disease independent of the adoption of measures or plans. In scientific research projects, many of the studies are conducted in environments and under highly controlled conditions and obtain reasonable cost-effectiveness levels, contrary to the result obtained in some situations on the costs that are required to reach the same level in the programmatic conditions of each country, requiring a large amount of human resources, infrastructure, and inputs that make the measure provided is not feasible to implement.

The great diversity of environments and social, cultural, and environmental determinants, scientific research and its evaluation in the species that make up the *Mycobacterium* genus, also presents the difficulty that its results and knowledge generated are representative for a community and applicable to other regions of the world. world that are similar in their conditions, it is almost imperative that each region or country performs its own evaluation processes to obtain evidence before implementing new control measures in their communities, complying with the provisions of the ethical considerations that shelter the population in the safekeeping of their human rights and a healthy environment.

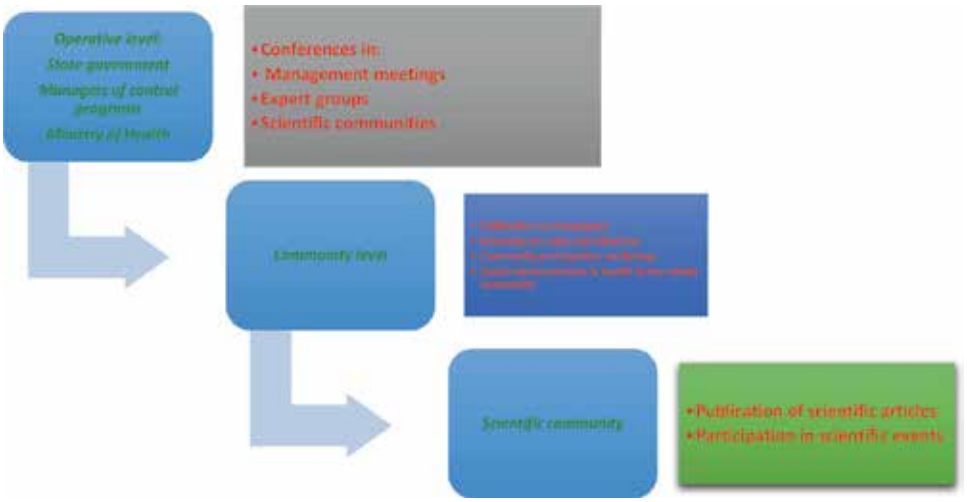

Figure 2. Flow of results of research and evaluation projects in mycobacteria.

The information obtained in the research and evaluation projects in the *Mycobacterium* genus should provide in the medium and long term that the results obtained contribute to the construction of new policies for the management and control of the disease, should serve as a critical input for the construction once again, knowledge must allow the evidence obtained to be able to reformulate or propose the operation of the indicators in time, form, and recourse. They should strengthen and contribute new knowledge in public health research and in the evaluation of broad coverage health programs.

The results of the research and evaluation projects must be translated into a language that is comprehensible to all through support provided by communicators with experience in health, politics, the environment, and the general community, who must objectively and appropriately carry out the adjustment of the vocabulary for dissemination in press, radio, television, and the general population.

The information must be widely disseminated and at all levels as illustrated in **Figure 2**.

The results obtained should be released to the community and the media in full report in large print and digital form, preferably with a slide presentation and a written guide to the presentation, to facilitate the management of information at management meetings and decision-making to finance new research or scientific evaluation projects, which leads to objective planning and decision-making based on the results of a rigorous scientific research or evaluation that will benefit the community and the different groups of policy-makers, decisions, and generators of knowledge.

2. Conclusion

This panorama and epidemiological record shows the difficult situation that is experienced worldwide against transmission, morbidity, and mortality caused by mycobacteria, and

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despite the strategies and efforts made by different countries through their national control programs, the situation has a high impact on the world economy. Therefore, the control carried out by each country is of vital importance to cut the chain of transmission, requiring that operational research be prioritized and evaluations be made of the measures, plans, and policies implemented by each country to successfully advance in its control. It is also necessary to carry out scientific research on the species that make up the *Mycobacterium* genus to know the metabolic characteristics that allow its application in the process of great benefit for industry, health, and the environment.

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Tuberculosis

Chapter 2

Mycobacterium tuberculosis: Macrophage Takeover and Modulation of Innate Effector Responses

Khalid Hussain Bhat and Imtiyaz Yaseen

Additional information is available at the end of the chapter

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Abstract

Macrophages mediate the first line of defense in the host against various intracellular pathogens. They are armed with several immune-effector mechanisms to detect and combat pathogens. However, intracellular pathogens have developed strategies to overcome the macrophage protective immune responses and colonize inside the macrophages. Tuberculosis (TB), both pulmonary and extrapulmonary, is an infectious disease of global concern caused by *Mycobacterium tuberculosis*. *M. tuberculosis* is a highly successful pathogen and has acquired various strategies to downregulate critical innate-effector immune responses of macrophages such as phagosome-lysosome fusion, antigen presentation, autophagy, and inhibition of reactive oxygen (ROI) and reactive nitrogen (RNI) species to ensure its longer survival inside the macrophages. In addition to these, the bacilli also modulate T cell immune response which can help the bacilli to survive inside the host for a long time. In this chapter, we focus to describe important macrophage innate defense mechanisms and the signaling that can influence T cell adaptive response and the strategies adopted by the bacilli to exploit these signaling cascades to favor its replication and persistence inside the macrophages for establishing a productive infection.

Keywords: *Mycobacterium tuberculosis*, monocytes/macrophages, macrophage effector response and signaling cascades, host responses and *M. tuberculosis* pathogenesis

1. Introduction

Macrophages mediate the first line of defense in the host against various intracellular pathogens [1]. They are armed with several immune-effector mechanisms to detect and combat pathogens [2, 3]. However, intracellular pathogens have developed strategies to overcome the macrophage protective immune responses and colonize inside the macrophages. Tuberculosis (TB), is an

infectious disease caused by a extremely successful pathogen, *Mycobacterium tuberculosis*, as it has evolved numerous clever strategies over time to modulate important macrophage innate-effector immune responses such as phagosome maturation, antigen presentation, inhibition of reactive oxygen (ROI) and reactive nitrogen (RNI) species, and autophagy to ensure its survival inside the macrophages [4–6]. In addition to these, the bacilli also modulate T cell immune response which can help the bacilli to survive inside the host for a long time [7]. In this chapter, we focus to describe important macrophage innate defense mechanisms and the signaling that can influence T cell adaptive response and the strategies adopted by the bacilli to exploit these signaling cascades to favor its replication and persistence inside the macrophages for establishing a productive infection.

2. Monocytes/macrophages

2.1. History and development

Eli Metchnikoff's obsession, the "phagocyte" [phagos-to eat, cyte-cell], is a constituent of Ludwig Aschoff's reticuloendothelial system (RES) [8]; the macrophage plays a key role at almost all the stages of immune response including innate and adaptive immune responses. Macrophages provide the first line of defense against the invading pathogens. In addition to protecting the body against attacks by foreign organisms, macrophages regulate important physiological functions. Their role in homeostasis has been well established. Macrophages clear almost 2×10^{11} erythrocytes per day. This enormous metabolic turnover is crucial for iron homeostasis and to prevent formation of toxic intermediates [9]. Macrophages are equipped with scavenger receptors such as phosphatidylserine receptors, thrombospondin receptor, integrins, and complement receptors to clear the cell debris and rapidly remove the apoptotic cells to help in tissue-remodeling processes. Antigens from the engulfed cells are presented along with the MHC molecules to activate the adaptive immune responses [10]. Thus, macrophage serves as a professional scavenger of the dying cells that not only clears the corpus but also regulates the immune system.

The circulating monocytes that are considered to be the developmental intermediates between bone marrow precursors and tissue macrophages emigrate from the blood vessels and differentiate into tissue macrophages [11]. Macrophages and monocytes originate from hematopoietic stem cell-derived progenitors with myeloid-restricted differentiation potential [1]. The bone marrow progenitors, monocytes, and macrophages collectively were classified into mononuclear phagocytic system, a concept pioneered by van Furth [12]. Monocytes are initially identified by the expression of CD14 molecules and lack of CD16 expression on the surface. These monocytes are termed as "classical monocytes" with $CD14^{++}CD16^{-}$ phenotype and accounting for about 90% of human blood monocytes. However, later studies have proved the expression of CD16 on the surface of some cell populations that were termed as "non-classical monocytes" with $CD14^{+}CD16^{++}$ phenotype [13]. The replenishment of tissue macrophages with the circulating monocytes is well established, but in some instances like in microglial cells of brain, local proliferation of macrophages has been established. Owing to the adaptability and plasticity of macrophages and their responsiveness to different

microenvironments in different tissues such as lung, spleen, liver, gut, and brain, a considerable heterogeneity exists among them [14]. For example, lung alveolar macrophages being constantly exposed to a variety of antigens, express a high level of pattern recognition receptors and scavenger receptors on the surface. In contrast, the macrophages of the gut exhibit high levels of phagocytic and antibacterial activities compared to other macrophages [15].

2.2. Macrophage activation

Activation is defined as the acquisition of competence to execute a complex function [16]. The factor responsible for macrophage activation was found to be the interferon-gamma (IFN-γ) produced by $CD8^+$ cytotoxic T (Tc1) cells, $CD4^+$ T helper 1 (Th1) T cells, and natural killer (NK) cells. IFN-γ activation leads to conversion of macrophages to potent phagocytotic cells with increased production of reactive oxygen intermediates and reactive nitrogen intermediates, superoxides and proinflammatory cytokines helping the cells to efficiently kill the intracellular pathogens. These macrophages have increased antigen presentation activity, thus they mount an effective immune responses in the host. The IFN-γ-mediated activation is known as "classical activation" and the macrophages are classified as "type 1 or M1 macrophages" [3, 18] (**Figure 1**). IFN-γ stimulation is not enough for the classical activation of macrophages, and may require additional stimulation by TNF-α. As TNF-α is not

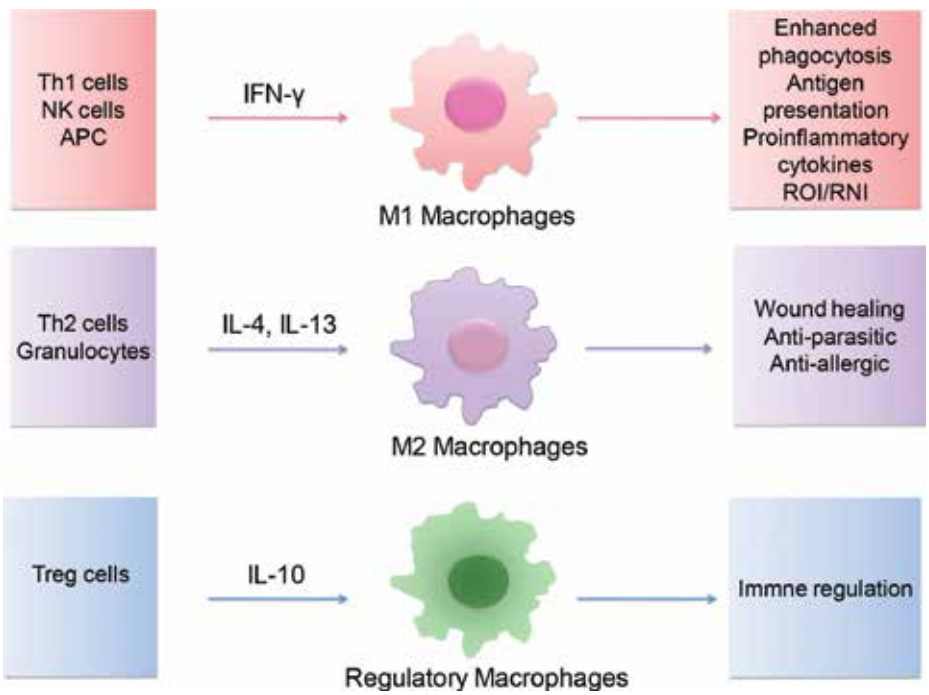

Figure 1. Schematic diagram showing different types of macrophages. Th1 cytokines primarily produced by T cells, natural killer (NK) cells and other antigen presenting cells (APC) result in the development of classically activated (M1) macrophages having microbicidal functions. While Th2 cytokines result in the development of macrophages that predominantly perform tissue repair and anti-inflammatory functions (M2), T reg cells result in the development of regulatory macrophages having immunosuppressive activities.

constitutively present in the environment, specific receptor ligands like lipopolysaccharides (LPS) and various microbial ligands may help in the induction of endogenous expression of TNF-α in macrophages [19, 20].

The T helper 2 (Th2) type of cytokines, IL-4 and IL-13, induce a response distinct from the one induced by IFN-γ with distinct set of genes being expressed and is known as "alternative activation" pathway of macrophages, and the cells are named as "alternative activated type 2 or M2 macrophages" [21]. In addition to T cells and B cells, IL-4 and IL-13 are also produced by various other cells such as mast cells, basophils, eosinophils, NK T cells, and macrophages that are involved in regulation of innate immune responses. Hence, alternative activation can be of both innate and acquired origin. Other than these two cytokines, immune complexes, IL-10, glucocorticoid, or secosteroid (vitamin D3) hormone can also contribute to the activation of M2 macrophages [22–25]. M2 macrophages are characterized by expression of scavenger, mannose [26], and galactose-type receptors, and markers such as dectin-1, arginase 1, Ym1, and FIZZ1 [27]. The M2 macrophages have anti-inflammatory properties and are associated with allergic and anti-parasite responses, and are thought to regulate humoral immunity [27, 28]. The alternatively activated macrophages are found to be recruited to wounds and other sites of tissue injury and are programmed to perform a wound healing function by expressing arginase. These macrophages are termed as "repair macrophages" or "wound healing macrophages" [19, 29, 30]. The M1 and M2 macrophages thus represent two populations of cells with different biological functions [31]. For example, the M1 macrophages, but not the M2 macrophages, produce high levels of reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF-α, IL-6), and have low arginase activity, express relatively high levels of CD86, and are efficient APCs. While the M1 cells have an IL-12^{high}, IL-23^{high}, and IL-10^{low} phenotype and play an important role in inducing a dominant Th1 response and provide resistance against intracellular pathogens and tumors [17, 23, 32–34], the various forms of M2 macrophages share an IL-12^{low} and IL-23^{low} phenotype, virtually devoid of the co-stimulatory molecules and fail to mount a strong T cell proliferation [35, 36]. The innate and adaptive immune responses can also lead to the production of the "regulatory macrophages" (M reg) (**Figure 1**). The M reg cells are shown to be very stable in their phenotype and have regulatory activity. These cells are a novel type of suppressor macrophage which induces tolerance during organ transplantation. They have potent T cell suppressive function [37] and inhibit production of the IL-12 cytokine [38].

The activated macrophages exhibit a profound change in their capacities and functions. In addition to other physiological changes, there is a rapid membrane turnover found in case of macrophages even in the resting stage. This membrane flow is enormously increased in the activated state as a result of enhanced phagocytic activity and lysosomal degradation of the ingested material [39, 40]. Phagosomes undergo a series of maturation steps resulting in gradual acidification and increase in the hydrolytic activity. In addition to hydrolases, lethal superoxide generating enzyme activities become prevalent toward the end of phagosome maturation [40]. The NADPH oxidase activity of the enzyme complex leads to formation of H_2O_2 in presence of superoxide dismutase enzyme [41].

3. *M. tuberculosis*: infection and disease

Mycobacteria are rod-shaped bacteria of phylum Actinobacteria mostly found in soil or water. The *M. tuberculosis, M. bovis, M. africanum, M. microti, M. canettii, M. caprae, M. pinnipedii*, and *M. mungi* all cause TB disease and are classified as *M. tuberculosis* complex [42–44]. The *M. tuberculosis*, a facultative intracellular pathogen, was discovered by Robert Koch in 1882 as a causative agent for TB disease in human, those days commonly known as "consumption" or "white plague" [45]. Different strains of *M. tuberculosis* differ in virulence and in distribution among different human populations. *M. tuberculosis* W-Beijing strain is one of the most pathogenic strains distributed throughout the world [46, 47]. The use of chemotherapeutics against *M. tuberculosis* has resulted in the appearance of drug-resistant strains [48]. Multiple drug-resistant (MDR), extensive drug-resistant (XDR), and total drug-resistant (TDR) strains are becoming increasingly prevalent [49–52]. The *M. tuberculosis* bacteria are highly aerobic, non-sporulating, and non-motile bacteria. They have a high guanine plus cytosine (G + C) content (61–71%) in their genomic DNA, and are characterized by the presence of large hydroxylated branched-chain fatty acids called mycolic acids in their cell envelope [53, 54]. Although they have been classified with other Gram-positive actinomycetes due to their lack of an outer cell membrane, mycobacteria stain weakly with crystal violet and are resistant to decolorization with acid-alcohol solutions after staining with alkaline arylmethane dyes such as carbol fuchsin, hence called acid-fast. *M. tuberculosis* primarily infects not only lungs (pulmonary) but can also colonize other body parts (extra-pulmonary). The symptoms of TB disease include chronic cough, blood with sputum, weight loss, fever and night sweats, cavitation, and fibrosis [55, 56].

TB is a major public health burden. Despite the availability of effective short-course chemotherapy (DOTS) and *M. bovis* bacillus Calmette-Guérin (BCG) vaccine, more than 9 million new cases of *M. tuberculosis* infections are reported every year that accounts to more than 2 billion (one third of world population) being positive for the infection, resulting in 2 million deaths every year and one fifth of all adult deaths in developing countries. Developing countries are the most affected by this pandemic with 30% of the cases being reported from Africa and 55% from Asia. India and China alone are harboring 35% of the cases. With no new drug in use for a while, TB has become increasingly resistant to drugs and multi-, extensive-, and total-drug resistant TB have emerged. Interaction with other infectious diseases like HIV is making it challenging to handle the disease [57]. Other pathological conditions and risk factors associated with TB such as diabetes mellitus, renal diseases, hematological disorders and use of anti-TNF-a drugs has complicated the problem [58]. Socioeconomic factors and variable efficacy of BCG vaccination are also responsible for further aggravating the already complex problem [55, 59].

After infection with *M. tuberculosis*, an individual may not necessarily develop active disease. In case the immune system is competent enough, an individual will either clear the infection or remain latently infected with no clinical signs of disease throughout the life or can have reactivation of TB during weakening of immune system or co-infection with other pathogens like HIV [60, 61]. The molecular factors or environmental conditions that influence the progression of latent phase to active disease are not well understood. During latency, the *M. tuberculosis* bacterium remains inside the infected macrophages in granulomas. These tiny granulomas show no clinical symptoms although they may be visible in chest X-rays and give

positive tuberculin skin test [62, 63]. In 5% of the cases where immune system is weakened, the microscopic primary lesion progresses to a larger primary caseous lesion. The caseation of the primary lesion may lead to hematogenous spread of bacteria causing miliary TB or extrapulmonary TB, where the infection spreads to liver, spleen, and kidneys [63]. In case the bacteria find their way into brain, they may cause tuberculous meningitis [63]. Patients with active pulmonary TB are diagnosed most commonly by sputum smear microscopy where bacteria are directly observed under microscope in the sputum samples of patients or culturing the samples to check for colony forming units and by chest X-ray. Diagnosis of extrapulmonary TB patients is carried out by tissue biopsy, urine culture, cerebrospinal fluid test, CT scan, or MRI. Latent TB has long been diagnosed by tuberculin skin test; however, its specificity has been questioned due to false positive results as a result of infection with other non-tuberculous bacteria or prior vaccination with BCG [64, 65]. Therefore, in recent years, interferon gamma release assays (IGRAs) have been used as an alternative for the diagnosis of both latent TB infection and active TB cases [66].

4. Infection of macrophages with *M. tuberculosis* and host immune responses

4.1. The host-bacilli interplay

M. tuberculosis infection is transmitted via aerosol route. Prolonged and close contacts with infected patients result in the transmission of the pathogen in healthy persons as it is known that survival of the bacterium ranges from one to few hours in the aerosol droplets, which are about 1–2 μm or less size [67, 68]. Once the pathogen enters the respiratory track, it is finally engulfed by the alveolar macrophages of lung through surface receptors. A number of studies reveal that complement receptors and complement-mediated opsonization are majorly involved in the entry of *M. tuberculosis* inside the macrophages. One of the most important receptors for mycobacteria is complement receptor 3 (CR3), while other receptors such as CR1 and CR4, mannose receptor, surfactant protein A receptor, CD14, Fcγ receptor, scavenger receptors, etc., have also been implicated in phagocytosis and internalization of the bacteria inside the macrophages [69–71]. For alveolar dendritic cells (DC), DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is the main receptor for *M. tuberculosis* [72]. Though complement receptors are found to be important for phagocytosis of both the avirulent and virulent strains of *M. tuberculosis,* the decline of mannose receptors was found to be associated with reduced binding of only the virulent strains [73]. The mycobacterial surface glycoprotein, mannose-capped lipoarabinomannan (Man-LAM) is recognized by the C-type lectins and the macrophage mannose receptor (MMR) [74, 75]. An important role of toll-receptors, mainly the TLR2, has been demonstrated for the attachment of mycobacteria to macrophages [76]. Interestingly, a large number of surface proteins of *M. tuberculosis* interact with the TLR2 receptors [76]. After binding, the bacteria are internalized and engulfed into phagosomes, where they can be killed by several defense mechanisms. Thoma-Uszynski et al., (2001) have shown a role of the TLR2-triggered signaling to induce cytotoxicity against *M. tuberculosis* in alveolar macrophages [77]. Soon after

the first contact of *M. tuberculosis* with alveolar macrophages, generally a robust proinflammatory immune response is induced that confers protection against the bacilli. It is observed that dampening of the proinflammatory signaling can increase *M. tuberculosis* infection burden in mice [78]. Following intracellular infection, adaptive immunity is generated against the invading pathogen via activation of $CD4^+$ T cells and $CD8^+$ T cells. Many times this adaptive immunity fails to provide a sterilizing immunity resulting in longer persistence of the infection and reactivation of *M. tuberculosis* bacteria. The bacilli are found to inhibit the class I, class II, and cross presentation of mycobacterial antigen to T cells, thus avoiding immune recognition by T cells. It has been observed that integrity of bacterial cell wall is important for *M. tuberculosis* in evading adaptive responses. At the site of infection, proinflammatory IFNs and cytokines are secreted, which help in the recruitment of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells, and neutrophils [79]. Many a time, the induction of acquired immune response against *M. tuberculosis* is slow and the establishment of infection wins against the induction of full-fledged response [4, 80]. The cell-mediated immune response initiated at the sites of infection is found to be modulated by the bacilli. After establishing the infection, the *M. tuberculosis* antigens move with the help of alveolar dendritic cells to the draining lymph node, which leads to the stimulation of naive $CD4^+$ T cells. The active role of $CD4^+$ T cells in fighting against *M. tuberculosis* infection was proposed in 1974 in mice. In addition, the HIV mediated depletion of $CD4^+$ T cells and a defective macrophage activation in some genetic disorders has been associated with worst TB prognosis [4, 81]. Furthermore, $CD4^{-/-}$ knockout and MHC-$II^{-/-}$ knockout mice have been found to be prone to infection by *M. tuberculosis* [82]. The inhibitory effect of stimulated Th1-type $CD4^+$ T cells is by the production proinflammatory cytokines such as IFN-γ and TNF-α, which inhibit bacillary growth [81]. In comparison, when stimulated in the context of MHC class II, Th2-type $CD4^+$ T cells proliferate and produce anti-inflammatory cytokines such as IL-4, IL-5, and IL-10, which are favorable for the bacilli to establish a productive infection [79, 81]. Many studies indicate that *M. tuberculosis* bacilli suppress the pro-inflammatory cytokines such as IL-12 and IFN-γ and activate production of anti-inflammatory cytokines like IL-10 to skew the anti-mycobacterial immune response from a protective Th1 to a non-protective Th2-type. At the early stages of infection, activation of $CD8^+$ T cells by APC leads to bacterial killing. However, the role of $CD8^+$ T cells at later stages of infection has not been established [82]. The $CD8^+$ T cells can also be activated by the cross presentation of *M. tuberculosis* antigen along with MHC class I molecules. Post infection, $CD8^+$ T cells migrate to the infected tissue and produce IFN-γ. This migration of $CD8^+$ T cells is a characteristic of the granuloma establishment [83]. Higher bacterial load was found in mice deficient in some of the components of class I presentation and $CD8^+$ T cell activation pathways like β_2 microglobulin, transporter associated with antigen processing protein (TAP), and T cell co-receptor CD8α [83].

4.2. Formation of granuloma

M. tuberculosis infection of alveolar macrophages leads to the activation of alveolar dendritic cells which migrate to lymph nodes. In the lymph nodes, $CD4^+$ T cells, $CD8^+$ T cells, and $\gamma\delta$ T cells proliferate in response to activation by the alveolar dendritic cells. At the site of infection, the resulting immune activation leads to a microenvironment of cytokines and chemokines which induces the expression of integrins, selectins, and addressins on the surfaces of

lymphocytes and endothelial cells. This facilitates a mass migration of immune cells resulting in the formation of a focus of immune cells called "tubercle" or granuloma around the primary site of infection. Macrophages and giant nucleated epithelioid cells form layers around the granuloma. While the inner core of granuloma becomes necrotic, the outer surface is covered by fibrous tissue and vasculature is developed. The granuloma is formed by the immune system to contain *M. tuberculosis* to the site of infection and not allowing its spread to other normal tissues. The latent infection may persist and remain dormant for life time without proceeding to diseased condition [79, 84]. At the nascent stage of granuloma, the recruitment of uninfected and susceptible macrophages to the site of infection may lead to their infection due to apoptosis of the existing infected macrophages. This may lead to initial proliferation of the bacteria till equilibrium is attained by containment of bacteria in the granuloma and control of infection due to adaptive immune response. Some of the newly infected macrophages egress from granuloma and nucleate the formation of new granulomas at other uninfected parts of the lungs (**Figure 2**) [85]. It has been noticed that bacterial dissemination and rapid disease progression are related with larger necrotic granulomas [57], however, containment of disease is found to be associated with smaller solid granulomas [86]. Furthermore, *M. tuberculosis* contributes to the formation of granuloma by secreting pathogenic factors

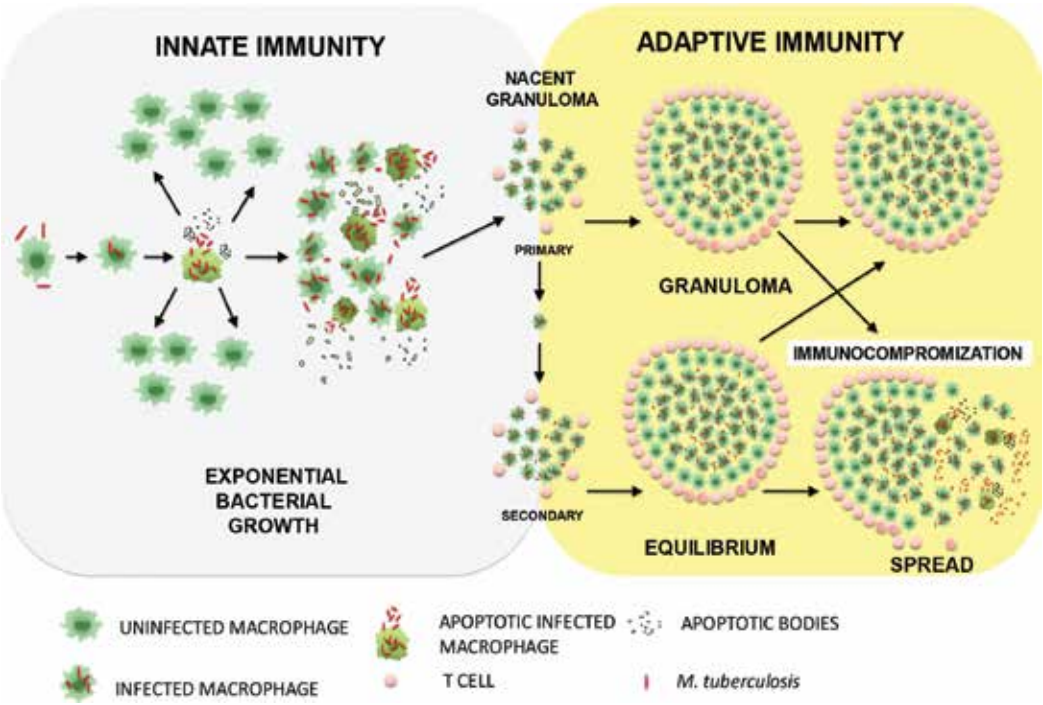

Figure 2. Development of granuloma. Immediately post infection, innate immune response is induced. The immune activation and apoptosis of infected cells lead to a microenvironment of cytokines and chemokines which attracts more uninfected macrophages. At this stage, some newly infected macrophages can migrate to uninfected areas and initiate the formation of new granulomas. This results in initial proliferation of the bacilli. Adaptive immunity although inherently slow in development, checks further spread of infection resulting in an equilibrium state. Immunocompromization of an individual at later stages can lead to caseation of the granuloma and the bacteria are rapidly disseminated.

as RD1 deficient bacteria show attenuated granuloma formation and macrophage migration to the primary site of infection in zebra fish model [85]. Studies in non-human primate, rabbit, and guinea pig models indicate hypoxic inside the environment of granuloma. In combination with nutrient deficiency, hypoxia induces a dormancy program in the bacilli which is characterized by changes in gene expression and alterations in the bacterial metabolism. Hence probably a latent infection is established which may be activated latter during immunocompromization. Thus, after entry in the human lungs, *M. tuberculosis* faces a series of host defense attacks. However, the overall outcome of infection with *M. tuberculosis* depends on the balance between (i) outgrowth and killing of *M. tuberculosis* and (ii) the extent of tissue necrosis, fibrosis, and regeneration.

4.3. Phagosome maturation response

After phagocytosis, *M. tuberculosis* bacteria reside inside the endosomes of the macrophages. Normally, endosome fuses with lysosome to degrade the pathogens, but *M. tuberculosis* bacteria are capable of inhibiting the process of phagosome maturation, as a result of which acidification of phagosome is compromised. The intracellular survival and persistence of the tubercle bacilli rests upon its ability to prevent phagosome-lysosome fusion, thus avoiding degradation, antigen processing, and cidal properties of the phagolysosome. Lipoarabinomannan capped with mannose (Man-LAM), a cell wall component of *M. tuberculosis* and SapM, a phosphatidylinositol 3-phosphate (PI3P) phosphatase secreted by the bacilli, are found to interfere with phosphoinositide metabolism of macrophages by depleting PI3P in phagosome [87, 88]. The latter is used as a docking molecule by peripheral proteins of lysosomes [7]. *M. tuberculosis* also possess protein phosphatases (Ptp A and B) that may interfere with host trafficking process possibly by modulating vacuolar sorting proteins [89].

The transport of *M. tuberculosis* containing vacuoles to lysosomes is mediated by a class of GTPases called Rab GTPases. A phagosome, when it normally matures into the phagolysosome, undergoes a transition between the stages marked by early endocytic Rabs (e.g., Rab5) and late endocytic GTPases (e.g., Rab7). Under normal circumstances, Rab proteins are actively recruited to the vesicles and assembled resulting in fusion of different vesicular compartments. In case of *M. tuberculosis* infection, it was observed that recruitment of Rab7 to the vacuole containing *M. tuberculosis* was inhibited while Rab5 was recruited normally indicating a maturation block between a Rab5 and Rab7 stage in infected macrophages that causes inhibition of phagosome-lysosome fusion. The exchange of Rab5 protein with Rab7 was later named as Rab conversion [90, 91]. Rab5 interacts with early endosomal autoantigen 1 (EEA1), which in turn interacts with phosphatidylinositol 3-phosphate (PI3P). Thus, inhibition of PI3P production by *M. tuberculosis* appears to be also critical for the inhibition of maturation of endolysosomes [91]. A series of Rab proteins are bind to phagosomes to ensure its acidification and recruitment of cathepsin D, while the process is severely inhibited in case of *M. tuberculosis* infection. A soluble eukaryotic-like protein kinase PknG of pathogenic *M. tuberculosis* is shown to be crucial for the prevention of phagosome-lysosome fusion [92]. A recent study has also shown that the *M. tuberculosis* secretory proteins, ESAT-6 and CFP-10 encoded by RD1 region play crucial roles in preventing phagolysosomal fusion [93]. Genetic screens using comprehensive mutant libraries of *M.*

tuberculosis and BCG suggest that additional mycobacterial products directly or indirectly can influence trafficking processes which probably are important for intracellular survival [94, 95]. Coronin1, exclusively recruited to *M. tuberculosis* containing phagosome, is an important host factor that specifically prevents the lysosomal delivery and death of mycobacteria inside macrophage [96]. Coronin1 prevents phagosome-lysosome fusion by regulating Ca^{2+}-dependent signaling processes when macrophages are infected with *M. tuberculosis* [97]. The *Nramp1* (natural-resistance-associated macrophage protein 1) gene involved in macrophage activation and mycobacterial killing [98] becomes part of the phagosome following phagocytosis and displays reduced phagosomal maturation and acidification [99].

A family of IFN-γ-inducible GTPases, also called immunity-related GTPases (IRGs), was found to play a critical role in host innate immunity against intracellular pathogens [100–102]. A member of IRG family, 47 kDa Irgm1 (also called LRG-47) protein (which is strongly inducible by IFN-γ and *M. tuberculosis* infection in mice) is an important anti-*M. tuberculosis* protein [100]. The anti-mycobacterial role of Irgm1 is due to its interaction with phosphatidylinositol-3,4-bisphosphate (PtdIns (3,4)P2) and PtdIns(3,4,5)P3 present on the phagosomal surface harboring *M. tuberculosis*. Irgm1 also increases phosphorylation of lipids by augmenting the PI3K activity. Normally, Irgm1 interacts with a membrane trafficking protein Snapin, which interacts with SNARE to ensure the fusion of phagosomes with lysosomes resulting in the elimination of bacilli. *M. tuberculosis* has developed a way to counter Irgm1 effect by exploiting a natural pathway of Irgm1 inhibition by Rab14 protein that is critical for phagosome-lysosome fusion. It has been shown that the mycobacterial phagosomes recruit and retain Rab14 [103]. Rab 14 is inhibited by unphosphorylated form of AS160 protein. Manipulation of Rab14-pathway by *M. tuberculosis* is mediated by the activation of Akt1 that phosphorylates AS160. Phosphorylated AS160 is unable to inhibit Rab14 hence leaving it free to inhibit Irgm1 recruitment to phagolysosomal compartment resulting in the failure of phagolysosome maturation (**Figure 3**) [103–105]. The fusion of phagosomes with lysosomes has been shown to be dependent on Ca^{2+} ions. Ca^{2+} ions and Ca-binding protein calmodulin are critical for the delivery of lysosomal components to phagosomes using PI3P-dependent pathways. Blockade of Ca^{2+}/calmodulin pathway by *M. tuberculosis* is also found to be one of the ways to block phagosomal maturation [70].

4.4. Autophagy

Autophagy (also called xenophagy) is an evolutionary conserved basic homeostatic mechanism of a cell to digest intracellular organelles and large protein aggregates that are difficult to digest by normal proteasomal pathway. The engagement of TLRs with mycobacterial ligands induces autophagy using both MyD88-dependent and TRIF-dependent pathways [106, 107]. This suggests that autophagy is an effector of innate immune response. The IFN-γ induces IRG proteins that are mainly involved in induction of autophagy and elimination of *M. tuberculosis* bacilli [108] and polymorphism at IRG locus is shown to be associated with resistance to *M. tuberculosis* [109]. Many autophagy-associated proteins are shown to be involved in phagosome-lysosome fusion process [110]. Expression of a number of host genes involved in autophagy is shown to be modulated at the onset of *M. tuberculosis* infection. The pathogen has therefore acquired

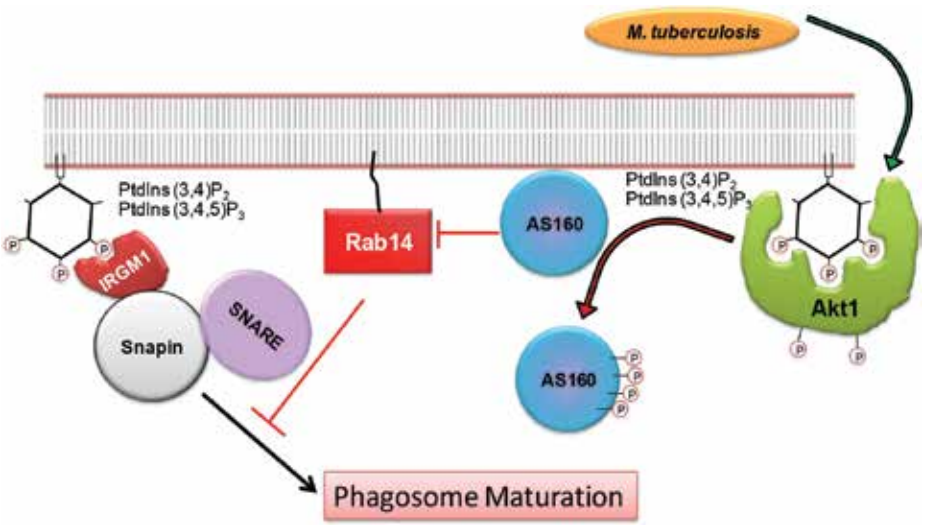

Figure 3. Critical balance between Rab14 and Irgm1 is important to counter *M. tuberculosis* infection. Pathogen results in the production of phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2) and PtdIns(3,4,5)P_3 which act as a docking site for Akt1. The phosphorylated Akt1 phosphorylates AS160 which is inactive and is unable to deactivate Rab14. The active Rab14 blocks the phagosome maturation. PdlIns also recruit Irgm1 which along with Snapin protein may help in maturation of phagosomes and elimination of intracellular bacteria.

mechanisms to subvert the autophagy to induce a favorable condition for its persistence inside the host [111]. *M. tuberculosis* can also inhibit autophagy by skewing the immune response toward the Th2-type. While proinflammatory cytokines such as TNF-α and IFN-γ promote autophagy, Th2 cytokines such as IL-4 and IL-13 inhibit autophagy in human and murine macrophages, and this is dependent on the Akt-STAT6-signaling pathway [112]. Autophagy is known to help in antigen presentation via both MHC class I and class II pathways and, by inhibiting autophagy, *M. tuberculosis* also achieves the goal of suppression of MHC class I- and class II-mediated antigen presentation [112]. Vaccines that are designed to elicit a strong autophagic response can prove to be effective against latent TB infection and also drugs designed to modulate autophagy can be effective against the drug-resistant strain of *M. tuberculosis* [113].

4.5. Apoptosis

Macrophages use apoptosis as an effector mechanism to eliminate *M. tuberculosis* and to constrain the spreading of infection [114]. The apoptotic vesicles are readily engulfed by the neighboring dendritic cells. The dendritic cells in turn activate $CD8^+$ T cells by cross-presenting the processed antigens in the context of MHC class I. This results in an effective immune response against the bacilli. Necrosis, however, is favorable for the dissemination of bacteria and spread of infection. Hence, *M. tuberculosis* has developed mechanisms to suppress apoptosis and favor necrosis during active infection state [6, 63, 115, 116]. Inhibition of host cell apoptosis by *M. tuberculosis* has been implicated as a potential virulence mechanism. In fact, an inverse correlation between virulence of mycobacterial strains and their capacity to induce apoptosis has been reported [114]. Infection with virulent *M. tuberculosis* strain H37Rv is found to be associated with

reduced expression of several pro-apoptotic genes and increased expression of the anti-apoptotic gene as compared to uninfected macrophages [117]. Studies using H37Rv and H37Ra strains of *M. tuberculosis* to infect alveolar macrophages have indicated that although both could induce apoptosis, the virulent H37Rv induce less apoptosis than the avirulent H37Ra by upregulating expression of the anti-apoptotic gene Bcl-2 in macrophages [118]. TNF-α is shown to play important role in host cell apoptosis infected with avirulent H37Ra strain [114, 119]. Also, the role of Bfl-1/A1 is realized in inhibition of apoptosis by *M. tuberculosis*, as decreased intracellular H37Rv growth was observed in Bfl-1/A1 siRNA-treated macrophages [120]. These cells show enhancement of phagosome-lysosome fusion and Caspase-3 activity indicating that expression of Bfl-1/A1 in H37Rv-infected macrophages provides the bacteria a survival strategy to overcome host defense. Nuclear factor-kappaB (NF-κB) activation is shown to play an essential role in the inhibition of host cell apoptosis by *M. tuberculosis* H37Rv [119]. Infection of macrophages with virulent *M. tuberculosis* confers resistance to apoptotic stimuli like Fas ligand (FasL) or TNF-α by reducing the cell surface expression of Fas receptors or secreting soluble TNF-α-receptor, respectively [121, 122]. Interestingly, Divangahi et al. have shown that virulent *M. tuberculosis* strains inhibit the production of prostaglandin E_2 by interfering with lipoxigenase pathway leading to inhibition of apoptosis and promotion of necrosis [115]. The other mechanism by which *M. tuberculosis* could possibly inhibit apoptotic process is via the *nuoG* gene, which can neutralize the NOX-2-mediated increase in ROS and TNF-α production in phagosomes containing *M. tuberculosis* thereby inhibiting apoptosis [123, 124]. Also, secA2 and pknE play roles in resistance against apoptosis as mutants of these genes induce more apoptosis in macrophages as compared to the wild-type *M. tuberculosis* strains [124–126].

4.6. Reactive oxygen and nitrogen intermediates (ROIs and RNIs)

ROIs and RNIs are produced in cells like macrophages in response to proinflammatory cytokines [127, 128]. ROIs and RNIs being small molecules defuse easily through the membranes and have a detrimental effect on the pathogens been engulfed in phagocytic vacuoles [129]. Studies have indicated that these molecules are important in providing innate host defense against *M. tuberculosis* [4, 129]. ROIs are produced by phagocytes, particularly the polymorphonuclear leukocytes and the activated macrophages, while RNIs are produced mainly by the activated macrophages. ROIs ($O^{2}\bullet^{-}$, H_2O_2, $OH^{\bullet}$,) are generated through the action of phagocyte oxidase (also called NADPH-oxidase). They further react with halides and amines to generate more reactive species (**Figure 4**). The important role of NADPH-oxidase in defense against *M. tuberculosis* and other pathogens is proven by the susceptibility of mice deficient in NADPH oxidase [129–131]. Children carrying mutations in gp91phox subunit of phagocyte oxidase enzyme are found to be more susceptible to TB infection than normal population [132]. In macrophages, IFN-γ in synergy with the TNF-α induces production of nitric oxide (•NO) with the help of inducible nitric oxide synthase (iNOS) (**Figure 4**). Not only does NO exert its effects on its own but also reacts with ROIs and the reaction products like peroxynitrite $^{(OONO-)}$ and other reaction intermediates can be even more toxic [127, 133]. After production, NO is not restricted to the area but readily defuses to other areas by using S-nitrosothiols, S-nitrosylated proteins, and nitrosyl-metal complexes as transport vehicles. NO produces heterogenous and diverse effects owing to its non-specific reactivity with a number of regulatory proteins [129,

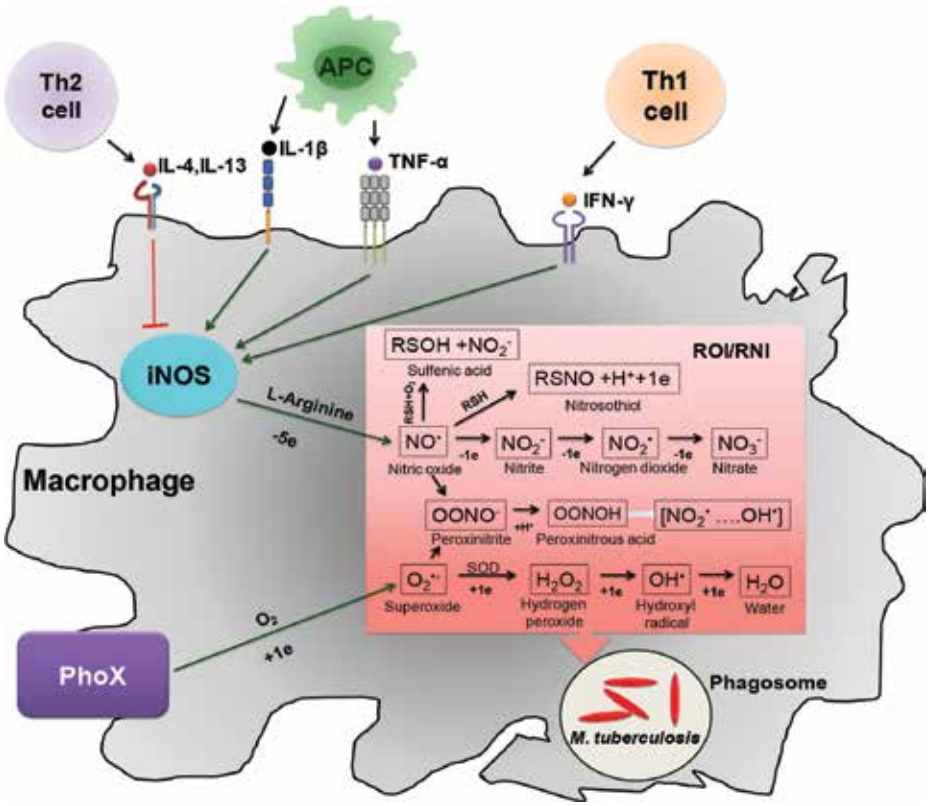

Figure 4. Regulation and function of iNOS during *M. tuberculosis* infection: Th1 cytokines like IFN-γ, TNF-α and IL-1β produced by Th1 T cells or antigen-presenting cells (APC) induce nitric oxide synthase (iNOS) enzyme. IL-4 and IL-13 secreted by Th2 cells negatively regulate iNOS expression. The products of phagocyte oxidase (Phox) and iNOS react and produce even more toxic intermediates like peroxynitrite against *M. tuberculosis*. NO also gives rise to sulfenic acid and nitrosothiols on reaction with sulfhydryl groups.

134]. In mouse macrophages, activation of TLR2 by various bacterial ligands induces iNOS promoter activity, production of NO, and killing of intracellular *M. tuberculosis*. However, protective role of NO in human TB is controversial. An essential role of NO/iNOS in anti-mycobacterial immunity was established by infection studies using iNOS knock-out mice [135, 136]. Mouse homozygous for knock-out allele for iNOS gene $^{(iNOS-/-)}$ when challenged with *M. tuberculosis* showed a very high susceptibility to infection [135]. Although most of the studies were conducted in mouse model, recent studies reveal that NO/iNOS is also important in killing the bacteria in human TB [134, 137, 138]. The iNOS activation through TLR2 pathway was found to enhance the killing of intracellular *M. tuberculosis* [77, 139]. All these studies indicate that probably iNOS also plays crucial role in anti-mycobacterial immunity in human. A comparative study of mice deficient either in phagocyte oxidase or in iNOS showed higher anti-mycobacterial activities by RNIs as compared to ROIs [131]. ROIs and RNIs damage the DNA and react with variety of other chemical moieties such as Fe-S clusters, tyrosyl radicals, hemes, sulfhydryls, thioethers, and alkenes to inactivate important components of the invading pathogens to compromise its survival inside the host.

Like other effector mechanisms, *M. tuberculosis* has evolved effective strategies to counter RNIs- and ROIs-mediated toxicity. The inhibition of the recruitment of iNOS to the infected phagosome would be one of the ideal strategies used by the bacilli [140]. EBP50, a scaffold protein in activated macrophages, targets iNOS to phagosomes [141]. *M. tuberculosis* downregulate EBP50 in the infected macrophages thereby reducing the transport of iNOS to the infected phagosome [141]. The bacteria also induce various genes to protect them from intracellular oxidative stress [109]. For example, mycobacterial catalase peroxidase (*katG*) and alkyl hydroperoxide reductase (*ahpC*) have role in antioxidant defenses, defend bacteria from intracellular oxidative stress [142, 143], and play roles in the virulence of *M. tuberculosis* [144, 145]. It has been shown that virulence of different clinical as well as recombinant strains of *M. tuberculosis* is correlated with varying expression levels of the peroxidase enzymes [143, 144]. NO induces a low oxygen state (hypoxia) in mycobacteria resulting in the overexpression of *katG* and *ahpC* [129, 146]. These genes were also reported to be overexpressed *in vivo* during infection with mycobacteria and studies indicate that these responses are abrogated in the phagosomal environment of $NOS2^{-/-}$ mice [147–149]. Oxidative stress has been established to induce a two-component system called DosR/DosS, consisting of a sensor histidine kinase DosS/DosT and a response regulator DosR [150–152]. The system helps the bacilli to cope oxidative and other stress (S-nitrosoglutathione, ethanol, etc.) conditions by initiating a complex response.

The DosR is characterized as a transcription factor responsible for transcription of the genes in response to oxidative stress. Genes expressed during the stress response, like α-crystallin was shown to carry sequences in the regulatory regions for DosR binding. In response to upstream activation signals, DosR is phosphorylated at Asp54 that results in its binding to DNA via its C-terminal domain and subsequent activation of DosR responsive genes. The sensor kinases, DosS and DosT, respond to redox environment and hypoxia, respectively. Both the proteins contain two GAF domains at N-terminal harboring a heme prosthetic group which interacts with O_2, NO or CO to induce autophosphorylation of the kinases and induce transcription of genes by activating DosR (**Figure 5**). CO is produced by heme oxygenase (HO) enzyme of macrophages. The enzyme is significantly upregulated during *M. tuberculosis* infection, oxidative stress, hypoxia, and stimulation with various cytokines. The enzyme catalyzes degradation of heme to biliverdin, free iron, and produces sufficient physiological amount of CO to induce dormancy program via binding primarily to DosS while DosT plays a little role in CO sensing. Although CO is sufficient to induce dormancy regulon, it was established that iNOS is required for optimal induction of response. Under aerobic condition, both the sensors are blocked and maintain a basal level of DosR responsive genes, while in anaerobic condition, they respond to the ligands and upregulate the DosR responsive genes to help the pathogen to survive in the stress caused by ROIs and RNIs (**Figure 5**) [153–155].

4.7. Vitamin D3

Vitamin D has long been known to be one of the nutritional therapeutic agents with a capacity to modulate the immune system in the case of an *M. tuberculosis* infection [156]. There has been a greater incidence of TB during the spring/summer months in temperate countries like UK [157], which can be correlated to a decrease in Vitamin D production on account of lower sun

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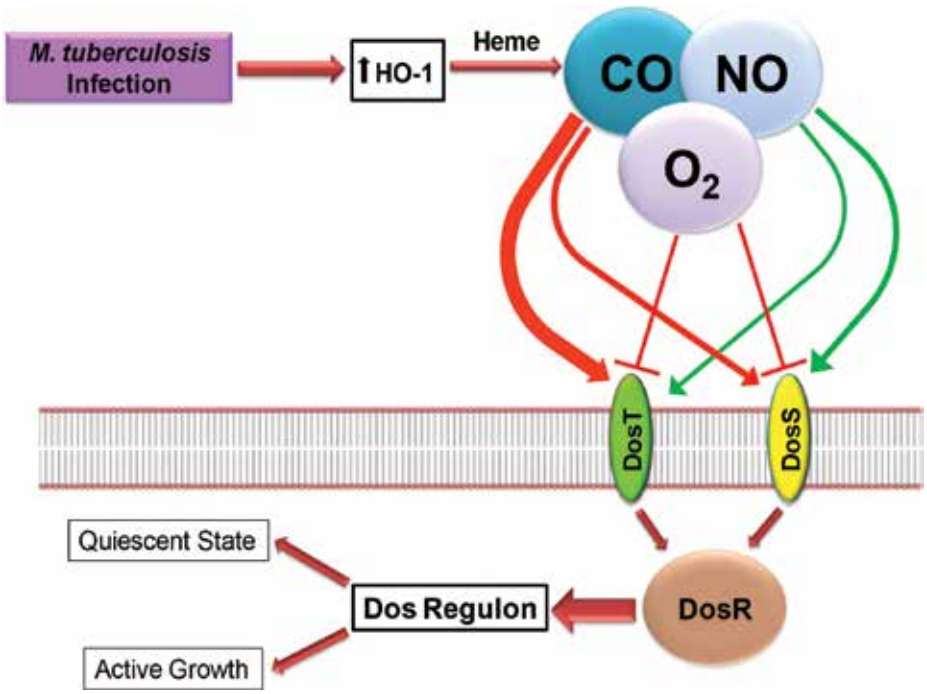

Figure 5. Role of NO, O_2 and CO in regulation of DosR/S/T regulon: NO, O_2, and CO are recognized in conjunction by DosS and DosT in a concentration gradient-dependent manner. CO mostly signals via DosS than DosT, while O_2 inhibits both DosS and DosT. *M. tuberculosis* infection upregulates the production of heme oxygenase-1(HO-1) which metabolizes heme to produce CO.

exposure in the winter months. Dietary composition also seemed to have a bearing with a predisposition toward TB, since a study conducted on an immigrant Gujarati Indian population in UK showed a marked increase in TB cases. It must be mentioned here that this population is a vegetarian one and hence dietary intake of Vitamin D is markedly less [158]. The genetic reason for this preponderance has been worked out to be an association with the VDR polymorphisms associated with the Gujarati Asian population concerned and Vitamin D deficiency [159]. Various studies indicate that Vitamin D can help the body fight against *M. tuberculosis* infections [160]. In a recent study, it has been found that patients administered with Vitamin D in combination with antibiotics recovered from TB more quickly than the patients administered with only antibiotics [161].

The mechanisms for a correlation of Vitamin D and TB are unknown, but it could be the antimicrobial peptides in association of Vitamin D generated by the pattern receptor stimulation in lieu of an infection with *M. tuberculosis* [162]. This stimulation then induces expression of cathelicidin [162] and β-defensin 2 (DEFB4) [163]. Cathelicidin induces phagolysosomal fusion which is necessary for killing of *M. tuberculosis*. In addition, 1,25(OH)2D, which is another downstream biochemical produced in Vitamin D biosynthetic pathway, induces autophagy [160, 164] and downregulates metalloproteinases (MMPs) [165]. All these processes help in the formation of phagolysosomes and the subsequent killing of *M. tuberculosis*. Vitamin D also seems to

affect the adaptive immune system albeit in a regulatory manner. This is ensued as the downstream product 1,25(OH)2D upregulates regulatory responses with a skew toward the Th2-phenotype pattern. This can be ascertained by the anti-proliferative effects of 1,25(OH)2D on $CD4^+$ T cells [166]. It also seems to inhibit Th1 cytokine production [167, 168], while promoting T regulatory function at the same time [169]. It also seemingly upregulates Th2 cytokine production [170]. Studies using 1α,25-dihydroxyvitamin D3 indicate that vitamin D3 increases generation of oxygen intermediates via NADPH-dependent phagocyte oxidase involving the phosphatidylinositol 3-kinase [171]. In addition to this, Vitamin D3 can downregulate transcription of tryptophan-aspartate containing coat protein [172], which is important for the entry and survival of *M. tuberculosis* in human macrophages [173]. Thus, the role of proper nutrition in the control of *M. tuberculosis* is evident. Vitamin D which has otherwise been associated as the "anti-cold" Vitamin seems to have critical roles in the control of *M. tuberculosis* infection at the innate and the adaptive levels of the immune system of the host.

5. Conclusion

TB remains a global pandemic and despite thorough and constructive measures to eradicate TB, it has flourished and continues killing people. It has evolved into various MDR, XDR, and TDR strains, notwithstanding the best healthcare available, which are resistant to the obsolete group of drugs. This necessitates the need to find new drug targets as well as drugs to counter the menace of TB. Therefore, it becomes imperative to understand the biology of *M. tuberculosis* and the host response modulation mechanisms it has evolved. The same can be achieved by dissecting the biochemical processes throughout the life cycle of the pathogen and by understanding the host-pathogen interaction mechanisms in TB, both of which are prerequisites for the development of effective anti-TB vaccines/drugs. More importantly, the processes associated with the so-called "dormant stage" needs to be identified since this stays the biggest challenge in identifying asymptomatic TB patients, and understanding this Trojan can therefore escalate our steps to eradicate this menace by eons. In this chapter, we have attempted to address various host processes that are subverted by *M. tuberculosis* to survive inside its host as well as launch an assault when the host immune defenses are weakened. Right from when *M. tuberculosis* enters inside the body, it counters the host innate defenses by downregulating the oxidative burst inside the macrophages. It also subverts other macrophage effector functions like inhibition of phagolysosomal fusion which is critical for the action of lytic enzymes and therefore forms an important block where it escapes the host defenses. Most importantly, it modulates the host signaling targeting the PAMP receptors, more importantly the TLR2 or to downregulate the proinflammatory signaling cascade known to be detrimental for its intracellular survival. The adaptive responses are similarly affected as one of the major mechanisms, viz. antigen presentation seems to be downregulated. Both MHC class I and class II and even cross presentation are affected. This results in a very less or delayed outcome of protective adaptive immune response which again helps the pathogen to survive very efficiently inside the macrophages. It also affects critical host processes like apoptosis which could clear the pathogen in a controlled manner

without allowing it to spread. Downregulating the proinflammatory signaling cascade also involves skewing the macrophage responses from a protective Th1- to a non-protective Th2-type. This involves most importantly a shift in the cytokine secretion pattern which subverts the host favorable macrophage signaling and effector functions in favor of the host. We have described important pathophysiological events during *M. tuberculosis* infection and the virulence processes by which the bacilli can escape the macrophage surveillance mechanisms, and use it as their safe refuge which may help in designing suitable interventions against *M. tuberculosis* infection.

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Chapter 3

The Existence of *Mycobacterium tuberculosis* in Microenvironment of Bone

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Additional information is available at the end of the chapter

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Abstract

Mycobacterium tuberculosis is an obligate aerobe bacteria requiring oxygen in its metabolism. In normal condition, bones have pH of 6.9–7.4 and temperature of 37°C. With the composition mentioned, bones fall in the group of tissue with less rich oxygen (<35%) which theoretically means, *M. tuberculosis* is hard to grow in the bone environment. Bone microliving environment is formed by the cells constructing the bone itself and the active cells which periodically interact with the bone cells. Activation of these cells gives impact to the temperature, pH, gas concentration, and liquid concentration, and at the same time triggers calcium, phosphor, and other minerals to be deposited in the bone. In the process of new bone formation, the osteoblast cells produce matrix and release them to the microenvironment that needs a high concentration of calcium and phosphor. The survival of *M. tuberculosis* in the microenvironment of bone is reflected in interaction of the bacteria and the non-immune cells, the bacteria and the organic environment, and the bacteria and the inorganic environment. In addition, the immune system also threatens the survival of *M. tuberculosis*. The results of these interactions will affect the lives of bacteria and has an impact on the bone microenvironment.

Keywords: bone microenvironment, survival of *M. tuberculosis*, cell host interaction, organic bacteria interaction, inorganic bacteria interaction

1. Introduction

Mycobacterium tuberculosis is an obligate aerobe bacteria requiring oxygen in its metabolism processes. Because of this oxygen requirement, *M. tuberculo*sis manifests in the lung of mammals

that have very high volume of oxygen. The optimum growth condition for this bacterium is at 37°C, pH 6.4–7.0, and oxygen level of >95% [1, 2]. Bones are composed of matrix comprising 60–70% inorganic components, 5–8% water, and the rest is organic components. In normal condition, bones have pH of 6.9–7.4 and temperature of 37°C. With the composition mentioned, bones fall in the group of tissue with less rich oxygen (<35%) which theoretically means, *M. tuberculosis* will be hard to grow in the bone environment [3].

In reality, however, *M. tuberculosis* can live in and infect the bone. This is proven in the cases of tuberculosis spondylitis and osteomyelitis. How this happens, what are the mechanisms that exist, and what compounds and conditions in control to enable *M. tuberculosis* growth in the bone environment will be discussed in this chapter.

2. *M. tuberculosis* complex

2.1. Cellular structure of *M. tuberculosis*

M. tuberculosis is a type of Actinomycetales bacteria of the Mycobacteria family and *Mycobacterium* genus (**Figure 1**). Shaped as tiny thin rod-shaped tubercle bacilli, *M. tuberculosis* is straight or slightly curved with 2–4 μm long and 0.2–0.5 μm wide, depending on the environment condition. When observed under the light microscope, this bacterium is usually conjoined forming a chain, filament, or branched forming into X, Y, or V shape [4].

M. tuberculosis does not have any capsules, and the cell walls (**Figure 2**) comprising peptidoglycan and DAP (diaminopimelic acid), with lipid content of +60%, have metachromatic granules known as *Much* granules. The fat in the cell wall associated with

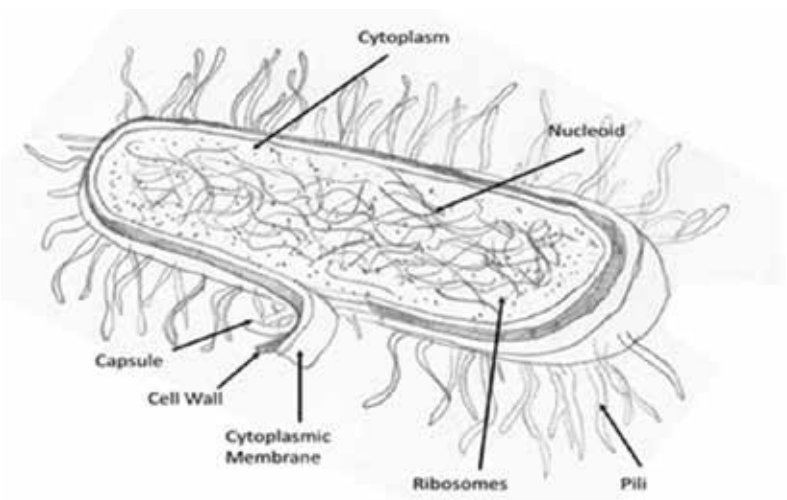

Figure 1. The structure of *M. tuberculosis* [4].

arabinogalactan and the peptidoglycan below forms a structure causing decrease in cell wall permeability that results in the reduction of antibiotic effectivity. Another molecule in the cell wall, lipoarabinomannan, is involved in the interaction between the host and the pathogen, making this bacterium survive in the macrophage [5].

Although *M. tuberculosis* does not produce spores, this bacterium is relatively heat resistant. The capability of adaptation in various microenvironment of *M. tuberculosis* is presented in **Table 1**.

Dormant is the effort of the bacteria to transform into the most stable form with a very low metabolism process and stop growing. This transformation is a response to the unsupportive environmental condition to grow normally. If one day the environmental condition becomes normal again and enable growing, this bacterium can revive and become active again [6].

2.2. Pathogenicity

2.2.1. Types

The known *M. tuberculosis* species which can infect human is classified into seven spoligotypes: The East African-Indian (EAI) strain and the Manu (India) strain, Beijing strain, Central Asian (CAS) strain, Ghana dan Harleem (H/T) strains, Latin America-Mediterranean (LAM) and X strains, *Mycobacterium africanum,* and Horn of Africa strains. This classification is according to the evolutionary demography of the bacteria [7, 8].

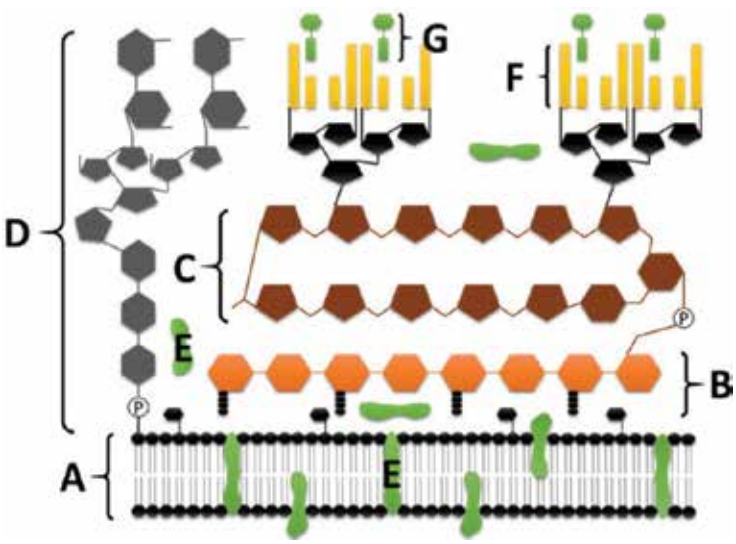

Figure 2. Structural and functional analysis and spatial organization of the "cell envelope" constituent of *M. tuberculosis* consisting of: plasma membrane (A), peptidoglycan (B), arabinogalactan (C), mannose covered with lipoarabinomannan (D), plasma membrane and the related cell-envelope-protein (E), mycolic acid (F), and glycolipid surface molecule associated with micolic acid (G) [5].

Environment	Description	Survival	Survival mechanism
Sunlight	Environment exposed to sunlight will result in low humidity and oxygen level. The dominant factor is when there is direct exposure with temperature reaching more than40°C and the period of the exposure	Survive in 2 hours	The cell wall thickened and produce liquid to protect from light exposure and high environment temperature
Dark and humid	Humid and dark condition will make the environment cool and rich in water. The oxygen level tends to be normal than the pH tends to be neutral	Survive according to the incubation period	Like in normal condition
Sputum	Sputum's temperature follows the temperature of the body, which is 36–37°C, pH of the sputum also follows the condition of other body fluid. The oxygen level inside the sputum is relatively high because the sputum lies inside the cavity of the lungs and the respiratory tract where oxygen flows	Survive in 20–30 hours	Like in normal condition
Storage cabinet	Storage cabinets have an advantage where the desired environmental temperature can be set according to the optimum condition favorable for the growth and proliferation of *M. tuberculosis*	Survive up to 2 years	Depends on the condition and the temperature set, normal or transformed into dormant
Mucosa of upper respiratory tract	The mucose of the respiratory tract has pH between 3.5 and 5.5, high oxygen level, and temperature close to body temperature	Ideal condition, reproduced and proliferated according to the period of incubation	Like in normal condition
Cavity of lung alveoli	The pH of the lung alveoli microenvironment is relatively higher compared to the pH of the upper respiratory tract, but still close to neutral pH for alveoli has the function to maintain the pH of the body. The oxygen level is relatively high and the temperature is warm because the activity of gas exchange and very active cell metabolism	Ideal condition, reproduced and proliferated according to the incubation period	Ideal condition
Macrophage's intracell	Ideal condition for the growth and development of *M. tuberculosis*. The intracell environment has a neutral pH with temperature of 36–37°C and oxygen level of >60%	Ideal condition, reproduced and proliferated according to the incubation period	Ideal condition
In tubercle	The environment inside the tubercle is also an ideal environment just like in the intracell, only this environment formed by the immune response trying to isolate the *M. tuberculosis*	Ideal condition, reproduced and proliferated according to the incubation period	Ideal condition

Environment	Description	Survival	Survival mechanism
Interstitial space of cancellous bone	Depends on the microenvironment, generally the oxygen level is low, the pH tends to be acid, and the temperature follows the body temperature	The condition is according to the normal incubation period	Normal or transformed into dormant
Interstitial space of cortical bone	Depends on the microenvironment, generally the oxygen level is lower compared to the interstitial space of the cancellous bone, the pH tends to be acidic, and the temperature follows the body temperature	The condition is according to the normal incubation period	Normal or transformed into dormant

Table 1. The survival of *M. tuberculosis* in various microenvironment.

2.2.2. Virulence factors and survival of M. tuberculosis

Full genome sequence of *M. tuberculosis* strain H37Rv was done successfully in 1998, but not all functions of the genes in the genome were known [1, 9]. From the discovery, it is known that *M. tuberculosis* do not have virulence factors like those discovered in other bacteria, such as toxin, capsule, or fimbriae, but some of the structures and physiological systems of *M. tuberculosis* itself contribute to the virulency. The virulence factors of *M. tuberculosis* among others are:

(a) *M. tuberculosis* can interfere with the toxic effect of reactive oxygen intermediate produced in the process of phagocytosis.

(b) *M. tuberculosis* has an antigen complex function to protect the bacteria from immune system and facilitate the formation of tubercle.

(c) Slow regeneration time of *M. tuberculosis* causes the immune system not recognizing this bacterium and eliminating it.

M. tuberculosis also have self-defense mechanism related to the virulence factors, one of them may be seen surviving extracellularly and intracellularly. Extracellularly, *M. tuberculosis* tries to survive by adapting to the environment through various mechanisms, such as decreasing metabolism, thickening the cell walls, reducing the surface area, and increasing the effectiveness of cell communication with the external environment. Intracellularly, when phagocyted by macrophage and finding a new environment different from the extracellular environment, the bacteria will feel threatened and tries to adapt by proliferating actively and inhibiting the fusion process of phagosome-lysosome so that it could not be digested [9].

In the effort to adapt to the environment and as a self-defense mechanism, bacteria could turn themselves into an inactive state, a condition where the bacteria do not give any respond to the environment they are in. The factors causing inactive bacteria to be infectious are: immune system and bacterial virulence. These two factors are highly related to one another in causing infection. For example, a weak immune system and a strong bacterial virulence will result in infection; this is also true in the condition where the immune system is weak and the bacterial virulence is also weak, the infection will still occur. However, it is not the case when the bacterial

virulence is strong and the immune system is also strong, because the infection will not occur in this condition. In MHC polymorphism, the host, genetically, has a condition most favorable to the bacteria to grow and develop.

Besides the immune system and bacterial virulence, the condition of the bone microenvironment, such as temperature, pH, oxygen level, and liquid, may also influence the existence of the bacteria as well as the interaction between the host and the bacteria. This phenomenon occurs because the living cells, indirectly, will influence the microenvironment from the metabolism products of the living cells. As an example, the debris of *M. tuberculosis* could influence the growth of the bone's active cells to form a favorable environment for bacterial growth. The bacteria will be active and dominant that the growth expanded and causing the bone's cells death. The death of bone cells will result in the formation of sequesters, which will then be deserted by the bacteria in order to find a new environment more favorable to maintain the bacterial existence and life (creeping phenomenon).

2.2.3. Immunology and defense mechanism

Response mechanism of the host to the intracellular pathogen bacterial infection depends highly on the location of infection. In immunology reaction or inflammatory reaction, a substance in the form of hormone and other cells functioned as intracell signal will be released by T lymphocytes, known as lymphokines. There are several lymphokines important to the process of *M. tuberculosis* infection, i.e., macrophage chemotactic factor, lymphocyte activation factor, and gamma interferon. In tuberculosis lesion, lymphokines from the T cells will cause macrophage accumulation and activation, and increasing number of TNF-alpha and TGF-beta lymphocytes will result in tissue damage [8].

In the first phase of *M. tuberculosis* infection, phagocytosis by the macrophages will occur as the result of bacterial activity in phagosomes. For 2–6 weeks, granuloma formation facilitated by CMI will happen, and the bacteria will then live and sit forever in the middle of the granuloma. The macrophage-bacteria interaction is initiated by the linkage between the bacterial cell wall and the macrophage at the time of phagosome-lysosome fusion. The inhibition of bacterial growth, even death, will further occur, inflammatory reaction and T cells antigen presentation will subsequently appear [8].

In the first stage of infection, right after the host exposed to the bacteria, detectable symptoms or immune response have not yet appeared. If the process of infection develops into the next stage, the signs of infection will then appear, for example, the skin tuberculin test and roentgen examination will give positive results. However, this process is not linear with the results of testing in the cell level and in the host organism level, where there will be shift between latent infection and the newly developed infection with the reactivation of previous infection [8, 10].

M. tuberculosis is an intracell microorganism that is needed in cellular immune response, which is the function of T-lymphocytes. In the thymus, T cells express surface antigens, CD4, CD5, and CD8, which in further development reside and mark the subset of T cells. The lymphocyte cells that act in the CMI reaction in tuberculosis infection are helper T lymphocyte cells (CD4), and suppressor T lymphocyte cells (CD8) are cells with specificities and functions tightly controlled by MHC (**Figure 3**). Based on the distribution in the tissues and molecule structure, MHC

antigens in human are divided into two main classes: class I antigen comprises HLA-A, HLA-B, and HLA-C, and class II antigen comprises HLA-D, HLA-DR, HLA-DQ, and HLA-DP [9].

M. tuberculosis is phagocyted by macrophage functioning as APC (**Figure 4**). This antigen is secreted by the bacteria together with MHC Class II and will react with CD4 on the T receptor and release IL-1, which further will replace CD4. This signal will give sign to lymphocytes to produce lymphokines, including gamma interferon, IL-2, BCGF, and chemotactic factor. Gamma interferon will activate macrophage to destroy the intracell *M. tuberculosis*. In this condition, the reaction between the somatic part of the bacterial antigen, which reacted with CD4 through the expression of MHC Class II with macrophage as APC, will occur. This active macrophage will cause some changes, such as increasing activity of hydrolase and increasing glucose metabolism [8].

Helper T cells composed of two subpopulations with different functions in producing cytokines (**Figure 5**). Th1 cells produce gamma interferon, IL-2, and lymphotoxin which are functioned to alter the macrophage's microbicide activity and strengthen DTH reaction. Th2 cells produce IL-4, IL-5, IL-6, and IL-10, which are functioned to assist the growth and differentiation of B cells and strengthen the humoral immune response. Th1 and Th2 cells will also produce IL-3, GMCSF, and TNF [11].

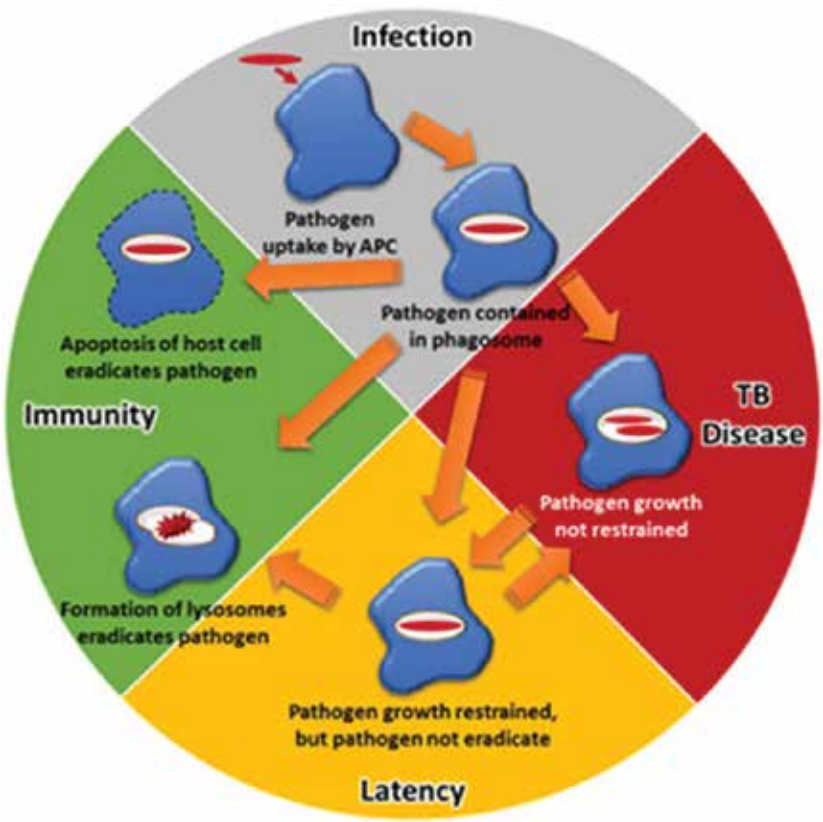

Figure 3. The relation between *M. tuberculosis* infection (new and latent) and immunity in infected host in the cell level (macrophage) [10].

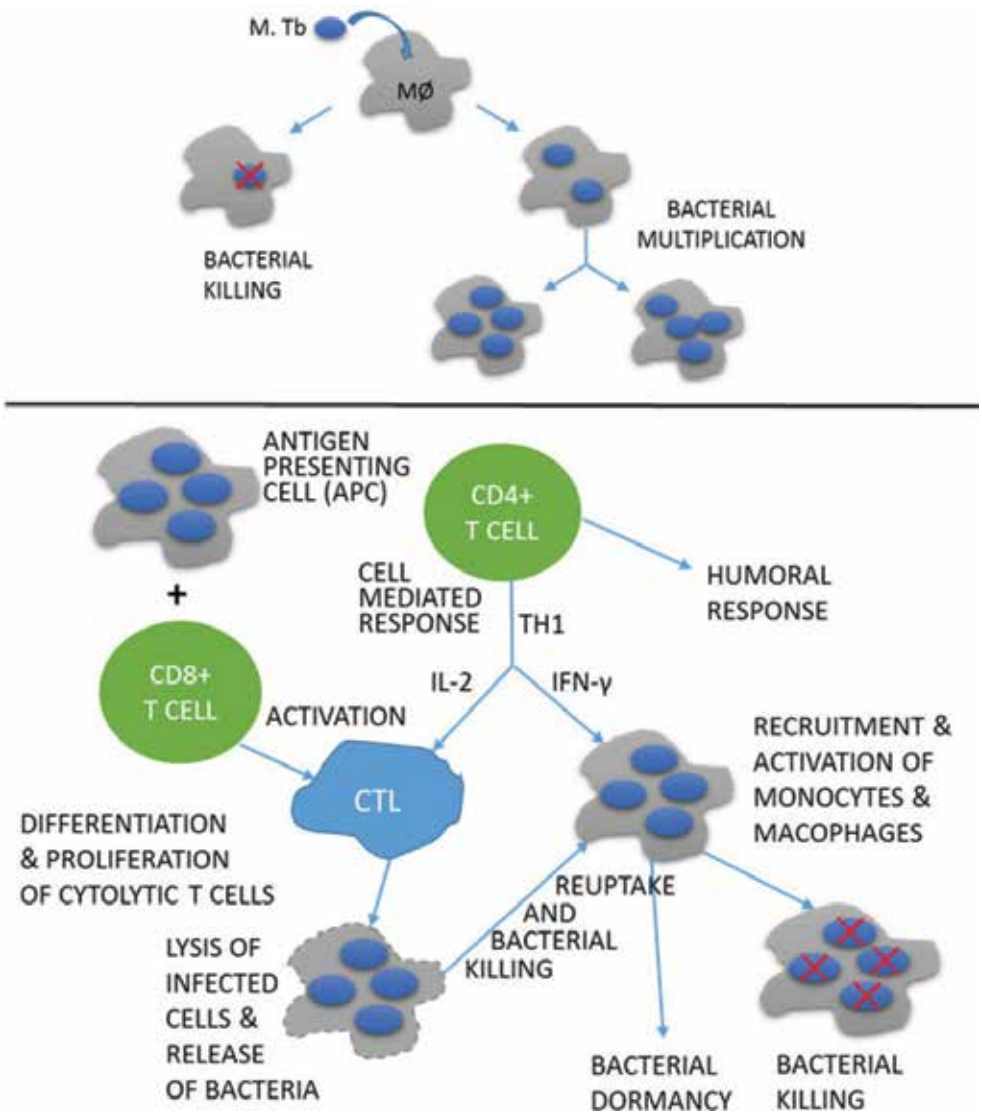

Figure 4. CMI reaction of *M. tuberculosis* [8].

2.3. Immunologic response to *M. tuberculosis*

As intracellular pathogen bacteria, *M. tuberculosis* develops various strategies to be able to survive in the macrophage and form granuloma in the organ of the host. Under the same way, the infected phagocyte cells and the surrounding tissues will respond to the presence of this interfering pathogen. Today, DNA array and proteomic examinations have been used to study the gene expression and the composition of bacterial protein from various strains of *M. tuberculosis* living in different microenvironments. The objective is to study the mechanism of interaction between *M. tuberculosis* and the host.

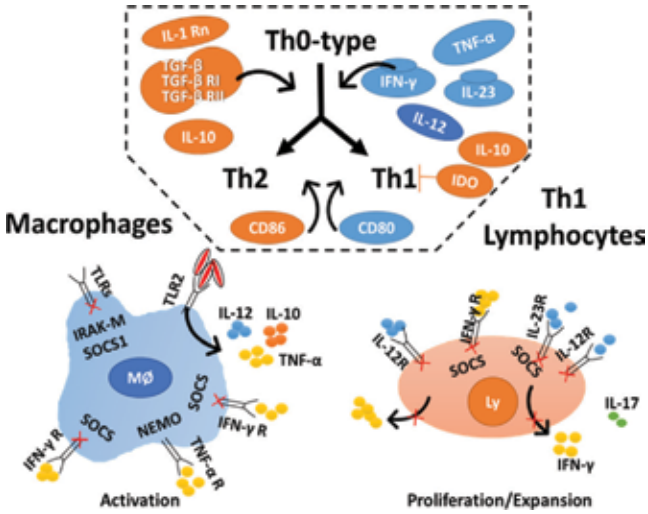

Figure 5. Helper T cells mechanism in *M. tuberculosis* infection [11].

When breathe in droplets containing *M. tuberculosis*, the infectious droplets will be throughout the respiratory tract. The majority of the bacilli in the droplets will be caught in the upper respiratory tract, where the goblet cells secrete mucus. The mucus will catch foreign substrate and the cilia in the cell surface will keep moving the mucus and catch the particles released by the upper respiratory tract. This mechanism provides the body a physical defense system to prevent further infection. The bacteria in the droplets that are managed to go through the mucociliary system and reached the alveolus rapidly will be surrounded and phagocyted by a large number of alveolar macrophages in the alveolus chamber. Macrophage is the next body defense system against *M. tuberculosis* which is able to interfere with the invasion process and prevent infection. After being ingested by the macrophages, *M. tuberculosis* continues to propagate slowly by dividing every 25–32 hours. Regardless, the infection will be controlled or continue to progress, and the initial development of bacterial cells will involve proteolytic enzymes and cytokines produced by the macrophages in order to degrade them. Cytokines produced will attract T lymphocytes and macrophages will then present microbacterial antigen on the surface of T cells. This process of initial defense will continue for 2–12 weeks. *M. tuberculosis* will continue to grow until the number is sufficient to avoid cellular immune response detectable by tuberculin test.

In people with good cellular immune system, the next stage of the body defense mechanism is granuloma formation around the *M. tuberculosis.* The mechanism will generate nodular-type lesion originated from the accumulation of T cells and activated macrophages. The accumulation of the cells creates a microenvironment limiting the replication and the spread of the microbacteria. This environment impaired the macrophages and produce necrosis liquid in the center of the lesion. However, in this condition the *M. tuberculosis* bacilli will still be able to adapt to survive. *M. tuberculosis* can alter the expression of its phenotype, such as the regulation protein to increase the survival. In 2 or 3 weeks, the necrosis

environment will resemble cheese tissue, often called as cheesy necrosis, with characteristics of low oxygen level, low pH, and limited nutrition to limit bacterial growth. Lesion in people with sufficient immune system is usually through fibrosis and calcification that success to control infection that the bacilli will be in the dormant state, and the lesion will then improve. Lesion in the people with insufficient immune system will develop to primary progressive tuberculosis.

3. Laboratory detection to diagnose the infection of *M. tuberculosis*

In this subchapter, the method of laboratory diagnosis of *M. tuberculosis* existence consisting of smear microscopy, culture, genotyping, immunology, and other examination modalities like radiology and histopathology will be discussed.

3.1. Smear microscopy

There are several methods to make accurate diagnosis by using a common smear microscopy: Ziehl Nelseen staining dan auramine staining. Cell morphology observation may be conducted by using a standard light microscope and a fluorescence microscope. Today, light-emitting diodes (LED) microscope has been developed, which is more efficient, consumes low power, and does not require a dark room. However, diagnosis of tuberculosis infection by using smear microscopy has some weaknesses, such as the number of cells per milliliter sample required is quite large (10,000 CFU/mL sample) and it quite often gives negative results, especially in patients with immune system disorder and paucibacillary [12].

3.2. Culture

Culture is a gold standard for tuberculosis diagnosis. This method only needs a relatively small number of germ cells (10–150 CFU/mL sample). Culture method is divided into two, liquid culture and solid culture (egg and agar-based). BACTEC 460TB, MGIT 960, MB/BacT system, MB Redox, and ESP Culture System II are the examples for liquid culture. Lowenstein-Jensen is an egg-based solid medium while Middlebrook 7H10/7H11 is agar-based solid medium. The growth in liquid culture medium is relatively fast compared to the solid culture medium, even though the liquid culture generally cannot be used to determine directly the nontuberculosis species based on the morphology of the colony [13, 14].

3.3. Genotyping

This method is based on the amplification of a specific target gene based on the principles of polymerase chain reaction. Today, it has been applied as a detection method for *M. tuberculosis* directly from the patient's sample, which detects the presence of the suspected bacteria altogether with the resistance to the antituberculosis, rifampicin. This device is called GeneXpert MTB/Rif test and has been endorsed by WHO. Some identification methods of *M. tuberculosis* which are based on the attachment of the target DNA amplification product to the probe of

HAIN Lifescience have also been used, such as Line Probe Assay, GenoType®Mycobacteria Direct assay, and INNO-LiPA MYCOBACTERIA of Innogenetics N.V. Genotyping method has some advantages from the time of examination that is relatively short and accurate. However, this method has some weaknesses such as requiring well-trained operators, presence of inhibitor in the sample, and easily contaminated [15].

3.4. Immunology

3.4.1. Tuberculin skin test (TST)

This test is more common to detect miliary tuberculosis than lung tuberculosis. Tuberculin anergy ranges from 35 to 74% in pediatric and 20–70% in adults. However, positive results of TST do not always indicate active tuberculosis [16].

3.4.2. Interferon-gamma release assay (IGRAs)

This *in vitro* examination is based on the production of gamma interferon by the T cells that may be detected with enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISPOT). This test will give a good result if applied to pediatric tuberculosis patients, patients that received BCG, and patients with HIV-AIDS. Like TST, IGRAs is also not able to determine the presence of active tuberculosis infection, besides the cost of examination is relatively expensive [17].

Serological examination methods, up until now, are not recommended by WHO to detect tuberculosis infections, both lung tuberculosis and miliary tuberculosis.

3.5. Other diagnostic methods

Some other diagnostic methods have also been used to determine tuberculosis infection, especially in miliary tuberculosis patients, such as ultrasonography, in which method can help detecting ascites, focal hepatic and splenic lesion, intra-abdominal lymphadenopathy, etc. CT and MRI have also been successfully used to detect lesion in the liver, spleen, intestine, and several other inner organs. Whenever possible, tissue biopsy could be done and histopathology examination may then be done by using hematoxylin-eosin staining to see the presence of granuloma and giant cells indicating infection by *M. tuberculosis* [17].

Mycobacterium tuberculosis detection using smear microscopy, genotyping, immunology, and other examination methods cannot confirm the existence of living bacteria in the sample preparation. Culture method is the only one that can assure to find living bacteria in the preparation, but if the culture gives negative result, other examinations do not necessarily give negative results.

4. Bone anatomy and histology

Bone tissue is different from other tissues in the body. Bone is a hard tissue, the main support for the body structure, which is composed of connective tissue and strengthens with continuous calcification process, in which function is controlled by the joint. In this subchapter,

the general anatomy of the bone, bone as organ, and bone as tissue together with the functions will be discussed [18].

4.1. General anatomy of bone

4.1.1. Long bone

Long bone has two parts, diaphysis and epiphysis (**Figure 6**). Diaphysis is the part between proximal and distal ends. The empty part in the diaphysis is called medullar cavity and filled with yellow bone marrow.

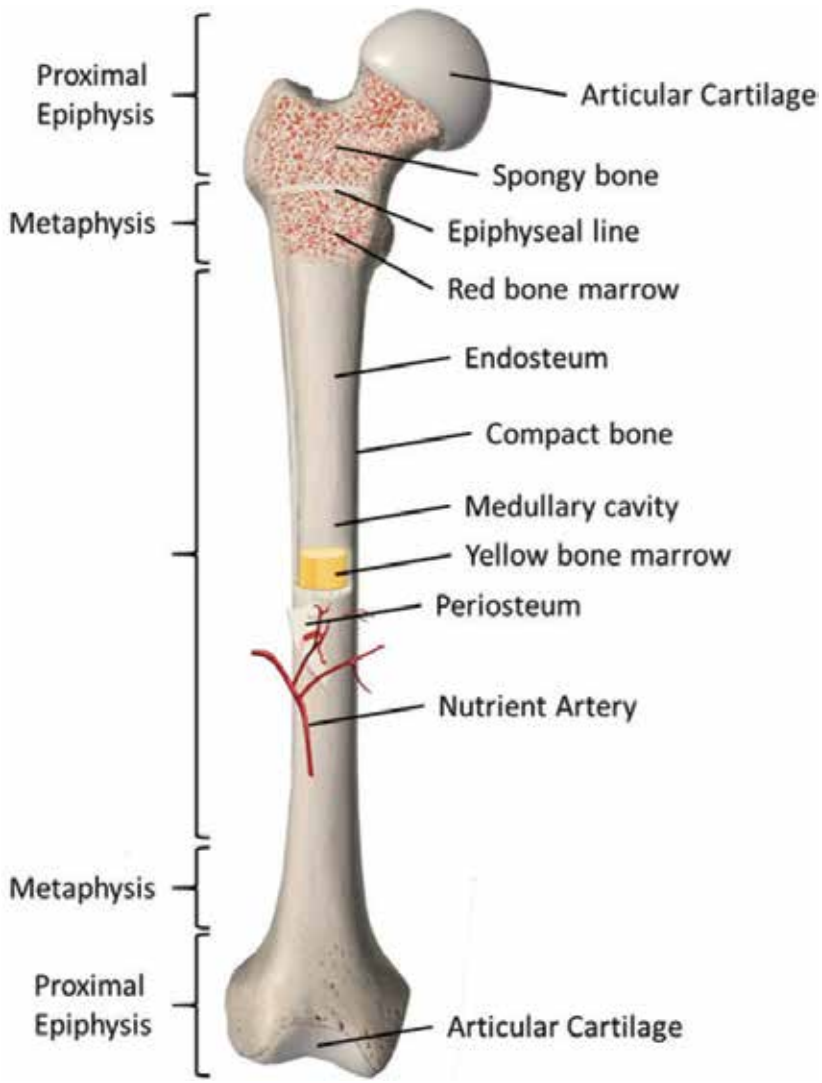

Figure 6. Bone anatomy [19].

The wider part, located near the proximal and distal ends, is called epiphysis, and this part is composed of spongy bone. Red bone marrow filled this spongy bone. Epiphysis and diaphysis meet in metaphysis, a narrow part containing epiphyseal plate (growth plate), which is a hyalin layer (transparent). When the bone stops growing, this cartilage will be replaced with osseous tissue and epiphysis plate will alter to epiphysis line [19].

Medullary cavity has a membrane layer called endosteum, where the bone growth, improvement, and remodeling occur. The outer part of the bone is covered with fibrous membrane called periosteum. Periosteum contains blood vessels, nerves, and lymphatic vessels, providing nutrition to the compact bones. Tendon and ligament also attach to the periosteum covering all bone's outer surface, except the part where epiphysis meet the other bone's end and form a joint. In this part, epiphysis is covered with articular cartilage, a thin layer of cartilage functioned to reduce friction and act as a shock absorber [19].

4.1.2. Cortical bone

Cortical bone is a part of compact bone found below the periosteum and the diaphysis of the long bone, the function of which is to support and protect. Microscopic structure of the cortical bone is called osteon or haversian system. Each osteon consists of concentric ring comprising of calcified matrix called lamellae (or called lamella if single). Down to every osteon is centralis canalis or haversian canal, where blood vessels, nervus, and lymphatic vessels found. These vessels and nervus will be branched in the canal cavity, called Volkmann's canal, and then extends toward the periosteum and endosteum [19].

4.1.3. Spongiosa bone

Like the cortical bone, spongiosa bone (also known as cancellous bone) contains osteocytes inside the lacuna but not arranged in concentric circle (**Figure 7**). Lacuna and osteocytes are arranged in grid-like form called trabeculae (or called trabecula if single). Trabeculae look like a random connection but every trabecula is formed to provide strength to the bone. The spaces in the nets formed by the trabeculae give balance to the compact bone by making the bone total mass lighter that the muscles could move the bone easily. In addition, the cavity

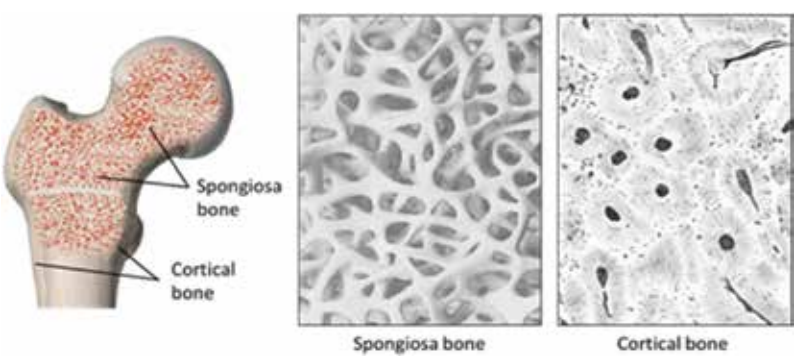

Figure 7. Spongiosa and cortical bone structure [19].

inside the spongiosa bone contains red bone marrow protected in the trabeculae, here the process of hematopoiesis also occur [19].

4.2. Bone as tissue

Bone contains cells that are resided in the collagen matrix that prepare the surface for salt crystals adherence (**Table 2**). These salt crystals are formed when calcium phosphate and calcium carbonate are joined to form hydroxyapatite that will deploy other inorganic salts like magnesium chloride, fluoride, and sulfate toward the collagen fibers. The crystals of hydroxyapatite give strength and rigidity to the bones while the collagen fibers give flexibility. There are four types of cells found in the bone tissue: osteoblast, osteocyte, osteogenic cells, and osteoclast [19, 20].

4.3. Blood circulation in the bone

The circulation system in the bone begins with two entries: from outside to inside (from periosteum to bone medulla) and from inside to outside (from the medulla to periosteum). These systems intersect or meet in the bone cancellous area filled with cavities and in the cortical area filled with haversian canal [21].

The important thing in blood circulation in the bone is that this system, in one part, has already filled with capillary blood vessels, and the other part is flowed by the Arterial system. Generally, capillary blood vessels are the end of the arterial blood vessels, where the cells will be released to the tissue and then charge exchange happens. Oxygen-containing blood will be released to the tissue, and the blood containing CO_2 will enter the vein capillary and so forth, then return to the heart. Other things to consider is that a part of the blood vessel will then end in the medulla wall, which subsequently become the place of exchange of hematopoietic cells where the cells form, develop, and mature. In the bone medulla, regeneration and degeneration occur. Regeneration is a process of bone maturation and degeneration is a process of dead bone destruction [21].

Cell	Function	Location
Osteoblast	Bone formation	Periosteum, endosteum, and at the part of bones growing
Pluripotential stem cell	Differentiated into osteoblast	Inner layer of periosteum and bone marrow
Osteocyte	Maintain the mineral concentration in the matrix	In the matrix
Osteoclast	Bone resorption	Bone surface and in the area that have been wounded

Table 2. Types of bone cells with the function and location.

5. Microenvironment of bone

Microenvironment of the bone is determined by the histologic structure of the bone that it may be divided based on the types of bones, i.e., cancellous bone and cortical bone.

(a) Cancellous bone:

Histologically, cancellous bone has the characteristics to contain many blood vessels and loose intracell chamber to form a hollow structure. This hollow structure enables the blood vessels and red bone marrow to reside there and creating an oxygen-rich environment to increase the metabolism processes and become slightly acidic. This condition is a favorable environment for the growth of *M. tuberculosis.*

(b) Cortical bone:

Histologically, the cortical bone has a more compact structure with more bone cells. With this more bone cells, there will be more bone matrix produced. This liquid bone matrix will attract bone minerals, such as calcium, magnesium, phosphor, etc., to be deposited and make the cells trapped in there. Because of the compact and solid structure of this bone, there are very little blood vessels found creating an oxygen-poor environment, there is only little metabolism and the condition is slightly basic. This becomes a nonfavorable condition for the *M. tuberculosis* growth.

5.1. Living environment

Bone microliving environment is formed by the cells constructing the bone itself and the active cells which periodically interact with the bone cells. Activation of these cells gives impact to the temperature, pH, gas concentration, and liquid concentration, and at the same time trigger calcium, phosphor, and other minerals to be deposited in the bone. This activity of these cells maintain the bone growth, bone strength, endurance against attack, and trigger the bone metabolism process [21–23].

In a condition with low temperature bone macroenvironment, the cells are trying to increase the microtemperature by doing metabolism activities. When the environment is basic, the bone cells will produce CO_2 that will make the microenvironment back to its normal pH. In a condition where the oxygen in the microenvironment of the bone drop as a result of blood vessels obstruction, the bone cells will try to reduce their oxygen consumption in order to be able to maintain the oxygen level. In another condition, like hypoxia, the bone cells will decompose CO_2 to O_2 and CO [23, 24].

In the process of new bone formation, the osteoblast cells produce matrix and release them to the microenvironment that it is needed in a high concentration of calcium and phosphor. This enables the formation of new bone and bone regeneration, like in cases of fracture. For bone strengthening, other inorganic minerals are required to strengthen the bone structure [21, 23, 24].

5.2. Acidity-alkalinity (pH)

Basically, the acidity or alkalinity of the bone microenvironment is determined by comparing the pattern of the acidity-alkalinity of cancellous bone and cortical bone.

There are many blood vessels in the cancellous bone which allow improvement in the metabolism process and oxygen exchange therein resulting in a relatively more acid environment in the cancellous bone than in the cortical bone. This is possible, because logically,

in a condition with a very active metabolism process the exchange in O_2 and CO_2 is very high and so is in other substances, i.e., the products of metabolism that will give acid environment (pH decrease).

There should be a mechanism of the body and the existing system to return the pH to be ideal again when there is a pH decrease. The function to restore and maintain the ideal condition is suspected to be provided by the immune system, such as leucocytes and macrophages.

When compared to the metabolism process in the cancellous bone and in the cortical bone, the metabolism process in the cortical bone is considered as less active. This could be caused by:

(a) The cortical bone environment has a solid character with narrow intercell spaces making the development of the bone cells smaller, which in turn will make the pH in the cortical bone relatively higher or the microenvironment more alkaline.

(b) This situation will be controlled by the immune system that will make the pH of the microenvironment of the cortical bone approximately the same as the cancellous bone.

5.3. Temperature

When the temperature in the microenvironment of the bone is discussed, it means how to create a physiological optimum temperature. Naturally, the condition of normal temperature will be maintained by the body through the thermostat mechanism controlled by the brain.

Temperature is determined from the result of metabolism and chemical mechanism and the interaction among the living cells, for example, between the osteoblast and the immune system, the formation of the calcium, phosphor deposits, etc. In determining the temperature of the microenvironment of the bone, it should differentiate between the temperature outside the cells and the temperature in the intercell spaces. Temperature will be created by the heat rises as a result of chemical reaction, such as:

(a) Biochemical reaction outside the cells producing heat triggered by, for example, H_2O, CO, CO_2, O_2, and other carbon chains.

(b) Reaction inside the cells, both aerobe and anaerobe, producing ATP and releasing heat from the cells.

(c) Metabolism inside the cytoplasm and in the nucleus.

5.4. Gas level in the interstitial chamber

The gases influencing the microenvironment of the bone are oxygen (O_2), carbon dioxide (CO_2), carbon monoxide (CO), nitrogen (N_2), and other gases in small number, where O_2 and CO_2 become the most dominant. The existence of these gases will form particular composition of the interfacial environment of the bone.

In the bone, both gases will exchange in the interstitial chamber, meaning that if O_2 is brought by the red blood cells from the lung and then delivered through the blood vessels to the

tissues, the red blood cells will get into the interstitial chamber and then release the O_2 to the environment. Further, O_2 will be taken up by the bone cells to perform metabolism processes and subsequently, the cells will get energy, produce energy, and metabolite concurrently while releasing CO_2 and O_2 again. In a particular situation, for example, in the condition of poisoning, O_2 will also bring another gas, such as N_2, which physiologically cannot be caught by the red blood cells to be released in the tissues.

5.5. Liquid

Among the bone cells, the osteoblast will produce bone matrix in the form of liquid comprising of protein and mineral salts that will attract calcium, phosphor, and other materials from the environment or metabolite products to be deposited into the matrix.

This deposit will cause solidification that makes the bone structure hard. In the cancellous bone, there are still cavities enabling the interaction among the bone cells (osteoblast, osteocyte, and osteoclast). These interbone cavities are formed when osteocytes trapped by the solidified bone matrix and leaving chambers which still contains liquid that is able to carry nutrition, gas, and important substances like hormones, enzymes, cells, etc., so that the osteocytes are still able to be active [24].

This environment certainly has an ideal concentration, where the composition of the liquid flows in the bone cavities or intermatrix cavities and contribute to the metabolism processes. Based on the above, this microenvironment is largely determined by the protein, blood cells, gas, and mineral transportation.

It is needed to specify the level of protein that could be delivered and form liquid so that it does not disturb the metabolism process, so that the possible ideal concentration of various structures in the bone remains capable of performing activities.

6. Interaction of *M. tuberculosis* and bone microenvironment

The interaction between *M. tuberculosis* and the microenvironment in the bone may be differentiated by the interaction of the bacteria and the nonimmune cells, the interaction of the bacteria and the organic environment, and the interaction of the bacteria and the inorganic environment.

6.1. Interaction of bacteria and nonimmune cells

The interaction between *M. tuberculosis* and nonimmune cells, like the bone cells (osteoblast, osteocyte, and osteoclast), is not mutually destroying or weakening, but this interaction will cause indirect disturbance in the form of metabolism disturbance and bone cell growth disturbance. As an example, the presence of *M. tuberculosis* debris will cause disturbance in the communication in both intercells and cells and the environment in performing metabolism, although it is not yet clear at what level the disturbance occur.

The communication in both interbone cells and the cells and the environment may occur through the following mechanisms:

(a) Direct exchange, the extracellular materials and intracellular materials directly exchange as a result of high difference in the cell wall permeability, for example, in the Na-K pumping.

(b) Indirect exchange, occur through intermediary mechanism that will change the outer and the inner part of the cell wall charges resulting in charge gradient causing the extracellular materials only adhere to the receptor of the outer cell wall.

6.2. Interaction of bacteria and organic environment

The interaction between *M. tuberculosis* and the organic environment is marked with the response of the bacteria to the organic substances in the bone. The organic substances composing the bone are, among others, collagen (bond of protein fibers arranged lengthwise and elastic), polysaccharide protein, and glycosaminoglycan (mucopolysaccharide).

M. tuberculosis will isolate and utilize the proteins from the cell's metabolism products as a medium to grow. The utilization will start with protein denaturation and protein compounds breakdown into simpler compounds, the availability of oxygen supply will create a condition and a new microenvironment that will be used by the bacteria as the media to grow.

6.3. Interaction of bacteria and inorganic environment

Like in the organic environment, the interaction between *M. tuberculosis* and inorganic environment is marked with the response of the bacteria to inorganic substances in the bone.

Inorganic substances making up the bone are, among others, calcium phosphate $Ca_3(PO_4)_2$, i.e., an ionic compound composed of Ca^{2+} ion and PO_4^{2-} ion, and also bicarbonate ion (HCO_3^-). The inorganic substances form a mineral compound called hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) function as a hardening material, provide rigidity, and bone strengthening.

The interaction between *M. tuberculosis* and the bone inorganic environment may be seen as the impact of the structure of the inorganic substance to the bacteria and the way the bacteria utilize the inorganic environment.

Basically, the bone inorganic environment is the respond to the system in the bone. Deposition of calcium and phosphate occur because of the infiltration capacity of the bone cells generating bone matrix. This matrix then attracts calcium and phosphate into the bone structure. The presence of *M. tuberculosis* will affect the condition of the microenvironment by inhibiting the infiltration of calcium and phosphate to the bone and inactivate the bone cells in order to not produce matrix so that the deposition of calcium and phosphate will be disturbed.

Other mechanisms that occur is *M. tuberculosis* colony tries to utilize the inorganic materials in their metabolism by isolating the inorganic matrix into the colony so that brittle bones will generate as a result of calcium and phosphor deposition without matrix.

7. Sex hormones' role in *M. tuberculosis* infection

The presence of *M. tuberculosis* in the body happens incidentally. When the bacteria stranded in the droplets enter the respiratory tract and then go into the alveoli and finally spread in the body, *M. tuberculosis* undertake efforts to survive and adapt to the new, continuously changing, environment. This is different with the immune system that always considers the presence of foreign body (including bacteria) as a threat, and macrophage will then come to the location of threat and try to eliminate, isolate, and destroy the foreign body. If the survival of *M. tuberculosis* is not sufficiently high to withstand the immune system attack, an extensive damage in the tissue will occur, and that is the time when the hormonal system will respond by releasing steroid, cortisol, and anabolic hormones aimed to recover the immune system in order that the damage is not getting wider.

Hormonal system is known to be able to strengthen or weaken the immunity system. During the immunity reaction, TNF alpha, interleukin-1, interleukin-6, and interleukin-12 will affect the endocrine system activation through the vascular system. It is also known that hormones can inhibit the proliferation of lymphocytes, cytotoxicity, and strengthen the secretion of IL-2, IL-10, IL-6, and IFN Y.

Sexual hormones have some contribution in the body resistance to tuberculosis infection. At the time of immunity reaction due to *M. tuberculosis* invasion into the body, tissue damage will give feedback to the endocrine gland, this process is known as immuno-endocrine mechanism. This mechanism is a process in which the adaptive and innate immune systems meet the hormone produced by the cytoplasmic endosomes and ended when the cytokines synthesis and T and B cells activities are strengthened. Cytokines, which is an immune response, will mediate and control the process of inflammation, and then influence the endocrine system in order to allow hormonal change [25].

7.1. The role of sexual hormones in tuberculosis infection

7.1.1. Androgen

Androgen has some effects to the cellular and humoral immunities that sometimes this hormone is categorized as an anti-inflammatory hormone, while estrogen will strengthen the humoral immunity and influence the balance of B and T cells. The host's control to *M. tuberculosis* is facilitated through Th1 cells and macrophage cells will be activated.

7.1.2. Testosterone

Testosterone is the main androgen hormone in men with immunosuppressive effect. This hormone disturbs the activation of macrophages and has an important role in lowering the production proinflammatory cytokines, including TNF alpha, and reduce the expression of toll-like monocytes functioning as a pathogen bacteria identifier. Testosterone will reduce the NK cells and induce the production of anti-inflammatory cytokines, such as IL-10, and reduce the production of proinflammatory cytokines, such as TNF, through NF B inhibition.

7.1.3. Esterogen

Esterogen is a proinflammatory hormone stimulating the production of TNF alpha and other proinflammatory cytokines. This hormone also has humoral immunity capability influencing the balance of T and B cells, strengthen the natural killer cells, and prevent immune cells from apoptosis.

7.1.4. Progesterone

Progesterone produced during maternity is suspected to inhibit the development of tuberculosis infection. Progesterone acts as an immunomodulator, which will suppress NK cells and induce IL-4, IL-5, and IL-10, increase the expression of SOCS1, and induce the release of IFN and TNF that will prevent bacteria multiplication.

7.1.5. Estradiol

Estradiol, which is one of maternity hormones, also functions as an immune system activator. Estradiol will strengthen the activity of NK cells through NF B activation; this hormone will induce the production of TNF, IL-1, IL-6, IL-17, and IL-23, and on the other hand, will inhibit the production of IL-4, IL-10, and IL-12.

7.2. The role of endocrine hormones in tuberculosis infection

Immunoendocrine disturbance is related to tuberculosis spread because hormones and cytokines affect the energy release and metabolism. This is especially applied in chronic tuberculosis infection where the pathogen and the immune system are fighting each other for a long time. There are increasing evidences supporting that the stress due to the hormonal alteration could directly stimulate the proinflammatory cytokines production that will then affect the condition related to the disease [26]. Therefore, in imbalance immunoendocrine condition, there will be increase in morbidity and mortality. The role and relation of immunoendocrine in tuberculosis infection can be seen in **Table 3**.

Hormone	Profile and immune response	Hormone concentration during disease	Description
Glucocorticoids	Facilitating Th2 and inducing cytokines production by Th1, IL-12 inducing IFN Y and IL-4	The concentration increases in TB patients when compared to control.	GC has a direct effect to the dendritic cells which increases IL-12 secretion, with less secretion of IL-12 and more secretion of IL-10. The effect of GC in Th2 is reducing the secretion of IL-13,IL-10
DHEA	Reducing the secretion of TGF-B and antagonizes Th2	DHEA concentration decreases by 50% in TB patients	DHEA is permissive to GC inhibition in the cellular immune response, but not in the process of inflammation.

Hormone	Profile and immune response	Hormone concentration during disease	Description
Estrogen	There is shift in Th2 and the secretion of Th1 is reduced, stimulating the synthesis of pro-inflammatory cytokines IL-1, I-6, TNF-a, and inhibit IL-4, IL-10, and IFN-Y	In patients with TB infection, the estrogen level will increase	This hormone will strengthen the humoral immune response and protect the immune cells to apoptosis.
Progesterone	Increasing the progesterone level will inhibit Th1 and produce anti-inflammatory IFNY	In TB patients, the progesterone level will increase	Prevent NFK-B activation & reducing the activity of NK cells
Testosterone	Reducing the expression of IL-4, macrophage, and shift toward Th2 and reducing the secretion of Th1	Testosterone concentration will decrease by 50% in TB patients	Activating the innate immunity. Testosterone will increase the susceptibility to TB infection.
Prolactin	Capable of stimulating the secretion of proinflammatory cytokines	Immune modulator. Increase in prolactin level will result in body weight decrease. It is found increase in prolactin level in TB patient	Stimulating and regulating phagocytosis
Thyroid (T3, T4)	Increasing TNF- α, IL-6, and decreasing TSH	T3, T4 increase in TB patients	Inflammatory cytokines inhibit the thyroid
Growth hormone	IFN- γ inhibit the monocytes phagocytosis	GH level decrease in Patients with TB infection	GH is a human macrophage activator

Table 3. The relation of immunoendocrine in tuberculosis infection and the role in pathogenesis.

8. Soft tissue and hard tissue recovery

M. tuberculosis infection results in hard and soft tissues damage. In this subchapter, the mechanism of the hard and the soft tissues damage in general and the process of recovery of the hard and soft tissues from tuberculosis infection will be discussed.

8.1. Hard tissue (bone)

In the process of infection, there will be struggle of the immune system resulting in tissue damage. In adult patients, osteomyelitis mostly occurs from the direct inoculation and from infection spread from other location. The source of infection may be from direct contamination, iatrogenic contamination during medical procedure, or transmission from contaminated soft tissues. Generally, the development of tuberculosis infection in osteomyelitis is in the form of bacterial invasion, vascular disruption, necrosis, and sequestration [23].

8.1.1. Hard tissue damage

Damage in the bone may be identified through the following:

(a) Bone cells death that will subsequently generate discontinuity or gap among the structure of bones. Immune reaction and *M. tuberculosis* infection will cause obstruction in micro-vascular and resulting in bone cells necrosis.

(b) Bone matrix lysis and denaturation of protein in the bone.

(c) After *M. tuberculosis* is ingested by the macrophage, there will be T cells recruitment. Subsequently, T cells will be activated and produce various cytokines, among others are IL-2 and IFN Y, and then macrophage change into epitheloid cell. The epitheloid cells will combine and form multinucleate giant cells and release lysosomal enzymes resulting in lysis of the surrounding structure of the infection.

(d) Bacterial and tissue debris. The battle between the immune cells and *M. tuberculosis* will generate debris that will be cleaned up by macrophages or join in the caseous necrosis.

(e) Sequester is bone cuts died of vascular disorder.

8.1.2. Hard tissue recovery

There are three items in bone recovery:

(a) The mechanism of the body eliminates the debris through macrophages and immune cells phagocytosis.

(b) The mechanism of sequester and dead tissue decomposition.

(c) The mechanism of debris release through sinus.

At the same time, new bone formation also happens in random order starting from periosteum (peripheral); recovery reaction in the form of hematoma formation also occurs from the middle. Growth factor produced by the stem cells in the periosteum will stimulate the formation of new vascular and nervus that will cover the new bone.

8.2. Soft tissue

Soft tissue is found in almost all over the body. This tissue functions to connect, support, and surround a structure or organ in the body. The types of soft tissue are:

(a) Fat tissue

(b) Muscle tissue

(c) Connective tissue (tendon and ligament)

(d) Synovial tissue

(e) Blood vessel

(f) Lymph tissue

(g) Peripheral nervus

8.2.1. Soft tissue damage

Infection could enter the soft tissue through a torn barrier. When bacteria are in the soft tissue, the macrophage will come and phagocyte the bacteria. Macrophage containing the bacteria will release degradation enzymes and induce the release of cytokines for poly-morphonuclear cells recruitment. Degrading enzymes produced by the macrophages will cause lysis of the cells around the infected soft tissue. Meanwhile, polymorphonuclear cells will trigger further immunity reaction that macroscopically the infected soft tissue will look swelled, suppurated (filled with inflammatory infiltration), and cause pain (due to proinflammatory cytokines release).

8.2.2. Soft tissue recovery

Soft tissue recovery consists of several phases:

(a) Phase of bleeding and inflammatory components recruitment

Inflammatory phase consists of two main phases, early inflammatory and advanced inflammatory phase.

- Early inflammatory phase

  In the early inflammatory phase, complement cascade components activation will occur and will be invaded by neutrophil granulocytes (polymorphonuclear) that will fill the wound area in 24–48 hours. The substance in charge of attracting the neutrophils is protein matrix, growth factor, complement, and peptide products from destructed bacteria. Soon the PMN will attach to the endothelium and migrate to the wound area. In the wound area, PMN will phagocytize the bacteria and other foreign substances. Further, PMN also releases enzyme that will lyse and free radicals from oxygen. During this period, the epidermis will increase mitotic activity. In the next 24–48 hours, the epithelial cells in both ends of the wound will migrate and proliferate along the dermis and fill the defect or void components. PMN activity will stop after a few days and the remaining cells will be cleaned up by the macrophages.

- Advanced inflammatory phase

  In advanced inflammatory phase, the monocyte cells will fill the wound area. The monocytes will then change into macrophages. The substance attracting the macrophage is the complement, blood coagulation components, immunoglobulin fragments, residual collagen and elastin, and cytokines. Macrophages play many roles in this phase. Besides cleaning the wound area from residual bacteria and tissue, macrophage also secretes growth factor that is functioned to trigger proliferation of extracellular

matrix by the fibroblast, smooth muscle cells, and endothelial cells to stimulate angiogenesis. In this phase, it is already seen the collagen fibers although the fibers have not yet interconnected to each other.

(b) Proliferation phase

This phase occurs after 3 days to 2 weeks. In this phase, fibroblast migration occurs that will produce collagen matrix, hyaluronan, collagen, and proteoglycan. This component is the constituent of extracellular matrix that will support cell growth therein. In this phase, the formation of granulation tissue also occurs. One of the signs of recovery is the formation of granulation tissue. The characteristic of this tissue is pink color, soft, and granulated on the surface. Histologically, this tissue is composed of fibroblast that still continues to proliferate and vascular loop in the collagen matrix that loses. This phase is marked with angiogenesis and new vascular formation (neo-vascularization). In the phase of neo-vascularization, some things will occur: "old" vascular basal membrane degradation to enable the formation of new capillary; endothelial cells migration due to angiogenic cells stimulation; and endothelial cells maturation. The newly formed blood vessels will still swell due to endothelial connection that has not yet been perfect. Granulation tissue can also become a standard to predict wound prognosis. Good tissues will be reddish in color, luminous, hyperemia, and look moist, while the tissue with bad recovery will look soft, brittle, and beefy. A thin layer of epithelium is also formed in this phase and called epiboly. Epithelialization needs humid condition, sufficient nutrition, and free from *Mycobacterium tuberculosis* disturbance.

(c) Remodeling phase

In this phase, collagen synthesis and breakdown happen continuously. Extracellular matrix will undergo remodeling. In this phase, there will also be contraction of wound due to fibroblast and the surrounding extracellular matrix interaction and this process is influenced by cytokines and growth factor such as TGF B, platelet-derived growth factor, and basic fibroblast growth factor. Fibroblast will also produce metalloproteinase that will degrade the collagen. Metalloproteinase depends on zinc to perform its activities.

(d) Wound maturation

This process is the final subphase of the remodeling phase. Fibronectin and hyaluronan will be degraded and the collagen bundle will thicken in line with the increase in tension in the wound. However, this newly formed collagen fibers will not equalize the strength of the previous collagen before the wound.

9. Healing process

If the immune system is not capable of killing the bacteria, the healing process is an additional effort the body does to reduce the *M. tuberculosis* infection. This effort could be done in three mechanisms: bactericide, bacteriostatic, and immunomodulatory by the stem cells.

9.1. Bactericide

Isoniazid and streptomycin have bactericide properties to *M. tuberculosis,* however, the activity of rifampicin is stronger compared to isoniazid and streptomycin both in the lag phase and the log phase [27].

9.1.1. Isoniazid

Isoniazid works by inhibiting the biosynthesis of nicolate (mycolic acid), which is the main component of the cell wall of *M. tuberculosis*. In low concentration, isoniazid will prevent fatty acid chain extension as the first form of mycolic acid. Isoniazid can also remove the acid-resistant property and reduce the number of fat extracted by methanol, by the drug, into the cells.

9.1.2. Rifampicin

Rifampicin is easily absorbed through the gastrointestinal tract. The ester is rapidly hydrolyzed in the bile and catalyzed by esterase in high pH. After 6 hours, all drug preparation will be deacetylated and in the deacetylated form, this drug is still a potent antibiotic. About 6% of the drug excreted through the urine will still be in its initial form, 60% of this drug will be excreted through feces. Rifampicin's half-life is 1.5–5 hours. If consumed with food, the absorption will be inhibited. Drug distribution reach all over the body, even the cerebrospinal liquid. Rifampicin becomes unique for the color make the urine, saliva, tears, and feces red. Rifampicin could be both bacteriostatic and bactericide depending on the concentration. The bactericide activity of rifampicin is obtained through the inhibition of nucleic acid synthesis by inhibiting DNA-dependent RNA polymerase in the subunit B.

9.1.3. Ethambutol

Ethambutol is a bacteriostatic agent that works through the obstruction of cell wall component, i.e., mycolic acid, formation. This drug also inhibits arabinosyl transferase involved in the cell wall biosynthesis. Resistance will easily occur if ethambutol is used alone without combination with other drugs. Ethambutol is well absorbed through the gastrointestinal tract, the bioavailability reach up to 80%, but the penetration to the cerebrospinal liquid is poor. This drug is eliminated through kidney.

9.1.4. Pyrazinamide

Pyrazinamide is an amide derivate from pyrazine-2-carboxylic acid and a nicotinamide analog, and is the third most important antituberculosis drug (OAT) after isoniazid and rifampicin. Pyrazinamide can kill *M. tuberculosis* in the cells in acid environment. The work mechanism is by disturbing the fatty acid synthesis and conversion into pyrazinamidase acid from the tuberculosis bacilli of a semidormant subpopulation in acid environment.

9.2. Bacteriostatic

Bacteriostatic works by preventing and inhibiting bacterial growth, but does not kill them so that bacterial eradication will depend largely on the body's immune system. Isoniazid, rifampicin, ethambutol, and pyrazinamide are first-line anti-tuberculosis drugs having bacteriostatic activity that is almost the same with *M. tuberculosis*, except that the bacteriostatic activity of isoniazid depends on the phase of growth. In the bacteriostatic condition, the host's self-defense mechanism, such as phagocytosis, and antibody production usually will impair the bacteria; in other words, the inhibition of bacterial growth is conducted by utilizing the immune system of the body [28].

9.3. Immunomodulatory by stem cells

Immunomodulatory properties of stem cells are reported to be in the T cells proliferation using a kind of stimuli, including mitogen, CD3/CD28, dan alloantigen. The relation of mesenchymal stem cells and proliferation inhibition of T cells is already known, among others, by reducing the expression of activator marker like CD25, CD38, CD69 in PHA lymphocyte, suppressing the proliferation of CD4 and CD8 [29, 30].

Immunomodulation capability of stem cells seems to rise before the secretion of IL-2 because the antiproliferation effect in mitogen induced by periphery lymphocytes may be repeated by adding IL-2. Further study showed that the mesenchymal stem cells supernatant does not have any role in inhibiting SPM proliferation, but in an *in vitro* experiment by using semipermeable membrane (in order that SPM and leucocytes separated) it is proven that there are soluble factors that can penetrate the membrane and have role in the proliferation suppression. Among the soluble factors produced by the mesenchymal stem cells are prostaglandin E2, IL-10, and hepatic growth factor. The factors proven could suppress the antigen response mediated by T cells. It is also proven that the induction of indolamine 2,3-dioxygenase by the mesenchymal stem cells will stimulate IFN Y. Therefore, the inhibition of mesenchymal stem cells to the proliferation of T cells could be due to tryptophan depletion [29].

The mesenchymal stem cells also have a role in molecular bond programming cell death (PD-1) and the ligand PD-L1 dan PD-L2 that resulted in the inhibition of T cells proliferation through direct contact between mesenchymal stem cells and target cells. The mesenchymal stem cells also increase CD4 and CD25 in cells and proved to have inhibition effect to proliferation and secretion of B cells IgG. When mesenchymal stem cells that are isolated from bone marrow and B cells extracted from periphery blood are cultured together, the result is inhibition of B cells proliferation and immunoglobulin formation due to soluble factors.

Mesenchymal stem cells also interact with dendritic cells by inhibiting the proliferation of monocytes into dendritic cells by also inhibiting the maturation of dendritic cells. Immature dendritic cells will alter the energy of T cells. Mesenchymal stem cells also proved to alter the cytokines secretion of the dendritic cells, such as IL-10, and reduce the regulation of inflammatory cytokines, such as IFN Y and IL-12 dan TNF alpha.

10. Conclusion: key results

The journey of *M. tuberculosis* to the microenvironment of the bone occurs through various environments, which tests the survival of *M. tuberculosis* itself. Generally, microenvironment may be classified as living environment, organic environment, and inorganic environment. *M. tuberculosis* has an extraordinary capability to survive, in responding to the environment threatening its life, by controlling the surrounding environment and adapting to the environment by transforming itself into dormant state or by inactivating all metabolisms.

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Chapter 4

Conventional and Molecular Diagnosis of Drug-Sensitive and Drug-Resistant Pulmonary Tuberculosis

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Additional information is available at the end of the chapter

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Abstract

Tuberculosis is a transmissible disease, which is primarily caused by the bacteria *Mycobacterium tuberculosis* and by other *Mycobacterium* species, forming the *Mycobacterium tuberculosis* complex. Until the end of the 20th Century, most cases of pulmonary tuberculosis were considered curable. Nevertheless, the rising of tuberculosis resistant to first- and second-line anti-tuberculous drugs is threatening the world's tuberculosis control programs. Due to this fact, the World Health Organization and other public health institutions recommended applying the conventional methods, affordable by low-incoming countries, to diagnose tuberculosis and to develop faster and more sensitive and specific methods to identify *M. tuberculosis* and determine their condition of anti-tuberculous drug resistance or drug sensitivity. In this chapter, we mention the most used conventional and molecular methods designed to identify *M. tuberculosis* and to determine their drug sensitivity or drug resistance. We also briefly describe the fundamentals of methods and its advantages and limitations.

Keywords: TB diagnosis, conventional and molecular methods, resistance detection

1. Introduction

Tuberculosis (TB) is a transmissible disease, which is mainly caused by the bacteria *Mycobacterium tuberculosis* (MTB) [1] and, in a minor grade, by other *Mycobacterium* species, which form the *Mycobacterium tuberculosis* complex (MTBC), where *M. canettii, M. tuberculosis, M. africanum, M. microti, M. pinnipedii, M. caprae, M. bovis and M. bovis Bacillus Calmette-Guérin* (BCG) are included [2, 3]. Until the last third of the 20th Century, most cases of active pulmonary tuberculosis (PTB)

were considered curable, while the patient stringently followed an appropriate treatment. Otherwise, eventually, patients could no longer respond to their treatment. Due to this circumstance, in 1993, the World Health Organization (WHO) recommended a global TB control strategy, called directly observed treatment, short-course (DOTS) [4]. According to the WHO, DOTS is "*the most cost-effective way to stop the spread of TB in communities with a high incidence is by curing it.*" This asseveration is correct if, and only if, the MTB strain is sensitive to the first-line anti-TB drugs (isoniazid, rifampin, ethambutol and pyrazinamide). The TB epidemiological problem arose when drug-resistant (DR) MTB strains appeared in the world scenario. Consequently, DOTS, in the cases of DR-TB, was no longer effective in a considerable number of TB patients, especially when the causal agents were resistant to rifampin and isoniazid (the most effective anti-TB drugs). This kind of DR-MTB strains is called multidrug-resistant MTB (MDR-MTB). Faced with that situation, the WHO recommended to all government countries applying the DOTS-plus strategy. The DOTS-plus regimen includes two or more drugs to which the isolate is susceptible, including one drug given parenterally for 6 months or more to patients having MDR-TB [5]. Over the years, the TB epidemiological situation has turned even worst. In 2006, the WHO declared a new modality of DR-TB as an emergency. This modality of TB was called extensive drug-resistant TB strains (XDR-TB), which was considered virtually untreatable with conventional drugs, 11 years ago. XDR-TB is an MDR-TB, which is also resistant to three or more second-line drugs [6]. Nevertheless, even for these extreme cases of DR-TB, there is a hope to find a cure, considering that, lastly, new drugs are under development [6]. Therefore, it is mandatory to identify the causal agent of TB and to determine if MTB bacteria are sensitive to first-line anti-tubercular drugs or resistant; and, in the second case, it is also necessary to define the drug resistance profile of each particular clinical MTB isolate.

In spite of the scientific advances—described below—to identify and characterize MTB isolates, a great challenge is facing, mainly by the developing countries, to train and hire appropriate personnel, as well as to implement adequate diagnosis laboratories. Many countries have joined the efforts of the WHO and other agencies to improve identification and treatment of cases of pulmonary TB, like the Stop Tuberculosis Partnership [7]. However, it is essential to increase the efforts of all involved, scientists, medical professionals, governments, altruist institutions and the general population to contain and control TB. The presence of XDR-TB is an uncontrolled threat, which could effectively become a pandemic with catastrophic consequences.

In this chapter, we describe those conventional and modern methods more often used to diagnose, isolate characterize and determine drug resistance profiles of MTB isolates and discuss the advantages and limitations of each of them.

2. Conventional TB diagnosis methods

2.1. Clinical diagnosis of active TB

A person is suspicious of having pulmonary tuberculosis (PTB) if shows at least 2- to 3-week duration of cough and had or has been in contact with PTB-infectious individuals [8].

Nevertheless, an MTB infection is usually asymptomatic in people who are well nourished and free of complications that compromise their immune system. This form of TB is known as latent TB infection (LTBI), which means that they do not have active TB disease, but could develop it in the future (a process known as TB reactivation). It is estimated that one in three people in the world has LTBI, and that, among these individuals, 5–10% present a risk of TB reactivation; and most of them will develop TB within the first 5 years after their MTB infection. Considerably, the risk of reactivation increases when predisposing factors are present. LTBI eventually progresses to active TB, when the infected individual experiences any condition which compromises his general health state, as HIV/AIDS or malnutrition [9]. On the other hand, PTB is considered, if the patient presents the following signs and symptoms: a frequent cough, usually throwing sputum (phlegm), chest pain, weakness, fever and evening or night sweats. In advanced cases, secretions are often accompanied by blood (hemoptysis), loss of weight, a pale skin and bright and sunken eyes [10]. The PTB diagnostic is reinforced by a positive Mantoux test (also known as tuberculin skin test [see below]). The presence of lung lesions (caverns), observed by a chest X-ray, denotes an advanced PTB [11, 12].

2.2. Mantoux test

The Mantoux test is performed to determine the sensitivity to tuberculin of each patient. This test consists of inoculating on the forearm, a small amount (5.0 units of tuberculin in 0.1 mL) intradermal (between the skin layers) of a protein extract of the mycobacteria dissolved in glycerol. This extract is known as PPD (purified protein derivative). Determine the diameter of the skin induration 45-72h after injection (thickened and hard skin), the halo (circle) of erythema (reddening of the skin) should not be taken into account. An intradermal reaction is considered positive when the induration diameter is 5.0–15.0 mm or greater. A positive test indicates that the person was in contact with MTB, but does not necessarily indicate that person is ill. A consideration of TB illness or not should be given if risk factors and medical history suggest this condition. For example, a person allergic to tuberculin or one who received the BCG vaccine against TB will give a positive reaction to tuberculin without being sick. These people are false positives. In contrast, an individual may have a Mantoux negative reaction and an PTB, if he also has AIDS (having his immune system compromised). Thus, this individual is anergic and a false negative [13].

2.3. Chest X-ray

Chest radiographs should be used to rule out the possibility of PTB in a person who has a positive reaction to a tuberculin skin test and no symptoms of the disease. An anteroposterior chest X-ray is one of the most important tests to be performed in a patient having TB or suspected TB. Chest X-ray findings include parenchymal infiltrates, hilar adenopathy, cavitation, nodules and pleural effusion [14]. Infiltrates or consolidations and cavities are often seen at the lung upper lobes, with or without mediastinal or hilar lymphadenopathy. However, lesions may appear anywhere in the lungs. In HIV/AIDS suffers or other immunosuppressed persons, any abnormality may indicate TB. In contrast, the chest X-ray may even appear entirely normal. On the other hand, longstanding healed TB lesions are usually presented as

pulmonary nodules in their hilar area or upper lobes, with or without fibrotic scars, volume loss and bronchiectasis (enlargement of parts of the airways within the lung). Furthermore, pleural scarring may be present. Nodules and fibrotic scars may contain slowly multiplying tubercle bacilli with the potential for future progression to active TB. Persons showing these lesions, if they have a positive tuberculin skin test reaction, should be considered high-priority candidates for the treatment of latent infection, regardless of age. Conversely, calcified nodular lesions (calcified granuloma) represent a very low risk for future progression to active TB [15].

2.4. Classical laboratory methods to identify PTB

Classical laboratory methods to identify MTB are used worldwide due to these are very useful and reliable.

TB-diagnostic methods are the detection of mycobacteria, by acid-fast bacilli (AFB) staining and fluorescence microscopy and isolation and cultivation of mycobacteria, allow establishing a most specific diagnostic of PTB than only above clinical diagnostic methods. Both identifying, isolating and characterizing MTB in a patient sputum along with a PTB clinical picture are necessary because to all signs and symptoms of PTB, described above, may be confused with other diseases, such as coccidiomycosis [16]. In other words, it is essential, in first instance, a clinical diagnostic of PTB and confirm or discard it by conventional laboratory procedures [11, 12].

3. Detection of AFB

3.1. Ziehl-Neelsen staining (ZN)

Mycobacteria are not well stained by Gram's method. Therefore, alternative methods were developed long time ago. The most commonly used technique to identify AFB is ZN. Notwithstanding, this method is not 100% specific or sensitive for MTB, since some bacteria species, like those of the *Nocardia* genus, are also AFB [17]. In addition, there are more than 120 species of mycobacteria, which are not causal agents of PTB and that also are AFB. On the contrary, some mycobacteria produce atypical pulmonary symptoms that are not MTB [18].

The ZN staining consists of dying, discoloring, counterstain and observing. The culture in suspension, or a liquid biological sample, is deposited onto a slide and dried and fixed with a flux of heat air. The slide is submerged into a phenol-carbol fuchsin solution; then, this smear is heated to enable the dye that penetrates the waxy mycobacterial cell wall and bind mycolic acids. Once the preparation was dyed, this is rinsed with tap water, and an acid decolorizing solution (1% solution of hydrochloric acid in isopropyl alcohol or methanol) is applied to remove the red dye from any non-AFB cell or material. Only AFBs (such as mycobacteria) will retain the phenol-carbol fuchsin dye, because of the protection provided by a waxy lipid layer, characteristic of AFB. After discoloration of the sputum smear, counterstaining is performed with malachite green or methylene blue, which will stain non-AFB material that was not able to retain the first dye. After that, a contrast between the red AFB, of the non-AFB material green or blue color will be observed with a microscope. The above procedure may vary due to structural differences between mycobacteria genera. For instance, *M. ulcerans* is strongly AFB,

whereas others, like *M. leprae*, are weakly AFB. *M. ulcerans* is decolorized with 3% ethanol, whereas it is needed 0.5–1% sulfuric acid to decolorize *M. leprae* [19]; in addition, the staining and discoloration times can also change from one mycobacterium genera to another.

The ZN is considered as the gold standard. This technique requires highly trained personnel in AFB staining and detection. According to the WHO [20], the AFB detection time and training of specialized personnel need to be improved, and so make TB diagnoses and start treatments for patients in a more opportune manner [20].

3.2. Fluorescence microscopy

An alternative for ZN staining is fluorescence microscopy (FM), which is currently used in many laboratories with available equipment. FM is faster than ZN, because FM allows visualize MTB bacilli in an easier manner than ZN. An FM study lasts 1.0 min and ZN requires 4.0 min. In addition, FM has been shown to be at least 10% more sensitive than classical light microscopy [21]. In high-volume laboratories, rapid laboratory turnaround times can prove to be crucial in the diagnosis of presumptive mycobacterial disease. In such settings, test characteristics such as staining time and background fluorescence contribute to overall laboratory efficiency in reporting results [22]. Also, FM is a semi-automated method and ZN must be carried out manually and to observe AFB directly under the microscope, by an experimented operator. On the other hand, ZN method is more affordable for low-resource institutions than FM, since fluorescence microscopes are noticeably more expensive than light-field microscopes, which are used to perform a sputum inspection.

Auramine and rhodamine are the main stains used in FM to detect mycobacteria in biological samples. These dyes are non-specific fluorochromes that bind mycolic acids of the mycobacterial wall. Once the dye has penetrated into the MTB wall, the mycolic acid-dye complex resists discoloration by alcohol-acid solutions. Counterstain helps to prevent fluorescence other than AFB, thus making the test more specific by reducing the possibility of artifacts. Counterstain is done with potassium permanganate. Once the staining is finished, AFBs are observed under an epifluorescence microscope. Under the UV light, AFBs gleam in yellow or bright orange over a dark background. The AFB-screening examination of smears is performed with a fluorescent microscope equipped with a 20X or 40X objective and a 100X oil immersion objective to observe the morphology of fluorescing organisms [23].

Nowadays, there are available new systems for identifying mycobacteria in clinical samples, which is based on light-emitting diodes (LEDs). This LED system costs considerably less than the original system and deserves to be evaluated in developing countries [24]. One of these methods describes an adaptation of a standard fluorescent microscope for illumination using a 'Royal Blue' Luxeon™ LED and demonstrates that this form of lighting is suitable for detection of auramine O-stained *Mycobacterium* spp. The authors claim that their method is of low cost, low power consumption, safety and that the reliability of LEDs makes them an attractive alternative for mercury vapor lamps [25].

In conclusion, in spite of the usefulness of AFB stain, using the ZN staining or FM, without a mycobacteria culture, AFB visualization has a poor negative predictive value. An AFB culture should be performed along with an AFB stain; this has a much higher negative predictive value.

4. Isolation and culture of MTB

4.1. Conventional procedure

All following procedures mandatorily are carried out in laboratories of level III microbiological contention and must be performed by trained personnel. A TB diagnostic must include an identification of MTB in appropriate biological samples. This is achieved by isolating the infectious agents and observing their colonies, developed in a solid culture medium (usually Lowenstein-Jensen's). Typically, PTB forms white, opaque, cauliflower-shaped colonies. Using a few of these colonies, the biological and biochemical characteristics of the isolates must be determined to identify MTB [26]. It must be kept in mind that a definitive TB diagnostic must be integrated by the correspondent clinical signs and symptoms and the bacteriological findings [27].

A standard laboratory procedure to identify MTB consists of decontaminate and liquefy freshly obtained sputum samples. One of the most used methods to liquefy and decontaminate sputum is Petroff's method, which has been modified several times [28]. One of the most important characteristics of method consists of the destruction, by sodium hydroxide, of many of the rapidly growing micro-organisms, which are contaminating to biological samples. After this decontamination, the viable bacteria are concentrated by centrifugation. The sediment is inoculated in a culture medium and incubated at 37°C in a 5% CO_2 atmosphere until typical MTB colonies are observed. The most used method to isolate MTB from sputum or other body fluids, like urine or cerebrospinal fluid, is the Lowenstein-Jensen solid medium, which usual composition is malachite green, glycerol, asparagine, hen's eggs and a salt solution (composed by potassium dihydro phosphate, magnesium sulfate and sodium citrate). The medium is aliquoted, put into glass screw-tapped bottom flat tubes and coagulated in an oven by heat. The sedimented bacteria are inoculated on the surface of a Lowenstein-Jensen medium slant. MTB is characterized by a slow growth, being its doubling time 10–20 h. It is required to incubate the sputum preparations for 5–8 weeks before typical colonies appear. Usually, the slow growth of mycobacteria in vitro is an obstacle to start an adequate treatment for TB opportunely. Other essential bacteriological characteristics of MTB are as follows: unlike other mycobacteria, MTB colonies are white (they do not produce pigments in the presence or absence of light) [29]. MTB is niacin- and catalase-positive and reduces nitrates to nitrites [30]. AFB detection by microscopy, plus culture, isolation of mycobacteria and their laboratory biochemical characterization are the gold standard for TB diagnosis. Even though the processes above have a low sensitivity (between 60 and 80%) [30], the generalized use of the theme is vital for preventing TB spreading worldwide, with an emphasis in DR-TB and XDR-TB [2].

4.2. Automated procedure

At a date, a totally automated system is available. This system is the BD BACTEC™ MGIT™ Automated Mycobacterial Detection System 960. MGIT means Mycobacteria Growth Indicator Tubes (MGIT). The BD BACTEC™ MGIT™ system offers several advantages over other tests and systems, among which can be emphasized an easy operation, an automated and a continuous quality checks, saving hours of work of the personnel, as well as of the equipment. By

showing positivity as it happens, time is optimized for patient care. It also uses barcode technology to facilitate data entry and processing, and patient and sample tracking.

The fundamentals of BD BACTEC™ MGIT™ system is as follows: at bottom of each MGIT unit is placed an oxygen-quenched fluorochrome (tris 4,7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate) embedded in silicone. As mycobacteria grow into the culture tube: the free oxygen is utilized and replaced by carbon dioxide. Because of depletion of free oxygen, into the culture tube, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube: under a source of UV light a fluorochrome is stimulated and emits visible light. The fluorescence is quantified by the instrument, as the intensity of fluorescence is directly proportional to the extent of oxygen depletion and, therefore, to the concentration of viable mycobacteria.

Procedure. Each MGIT unit contains 7.0 ml sterile modified Middlebrook 7H9 broth base, which must be added with MGIT PANTA—an antibiotic mixture designed for suppress contamination—OADC (oleic acid, albumin, dextrose and catalase) or, preferably, MGIT 960 growth supplement. According to the manufacturer, MGIT growth supplement is essential for growth of mycobacteria belonging to MTBC. MGIT tubes are incubated, inside the instrument, and monitored for increasing fluorescence every 60 min. Under the above conditions, a typical MTB culture reaches a concentration of mycobacteria equivalent to 10^5–10^6 colony forming units (CFU) per mL. On the other hand, when a preparation remains non-fluorescent after 6 weeks (42 days), it is considered as negative. The presence of contaminating bacteria can be detected, because these microorganisms generally produce a heavy turbidity [31].

5. Determination of MTB drug resistance

5.1. Conventional methods

Once MTB isolates are identified, it is necessary to determine their drug resistance profile. Conventional drug-resistant tests (DRTs) are first used to evaluate resistance to streptomycin, isoniazid, rifampin, ethambutol and pyrazinamide. This information is crucial for the treating physician knows if DOTS will be effective to cure a patient having TB. Nevertheless, if a particular isolate shows resistance to rifampin, isoniazid or both drugs, the resistance profile to second-line drugs [injectable aminoglycosides (amikacin or kanamycin), capreomycin and fluoroquinolones] must be determined [32]. This information will permit that the physician decides if the patient should be treated with a combination of first- and second-line drugs, following the current recommendations of the WHO for MD-TB. Three of the most used DRTs are the classical agar proportion method, the BACTEC 960 fluorometric method and microplate Alamar blue assay (MABA).

5.2. Agar proportion method

The method of proportion using Middlebrook 7H10 agar has been considered the "gold standard" method for several decades. This method allows separate the resistant bacilli from

the susceptible bacilli and establishes their proportions in the culture (% of resistant bacilli and % of sensible bacilli to a particular drug). This information is obtained by culturing the isolated strain in duplicate. One slant or plate has only Middlebrook 7H10 agar, and the duplicate is cultured with the same medium, added with the anti-tuberculous drug to be evaluated. Then, the percentage of colony forming units (CFU) present in the treated culture is calculated with respect to the total number of CFU found in the untreated culture [33].

5.3. Fluorometric method: BACTEC 960 system

The BACTEC MGIT 960 SIRE (streptomycin, isoniazid, rifampin and ethambutol) and PZA (pyrazinamide) susceptibility test for MTB are used extensively in the world to identify the presence of mycobacteria in biological samples and to determine the sensitivity to first-line drugs [34]. The drug susceptibility test, using this system, is based on the same principle than the aforementioned BACTEC 960 system for mycobacteria detection, and the same instrument is used, but following the particularities that request this drug-sensibility system. The procedure is as follows: two MGIT units are needed to perform the mycobacteria DRTs for each first-line anti-MTB drug. Each tube is inoculated with the test mycobacteria culture. One MGIT unit is added with a known concentration of one of the test anti-MTB drugs, and the other one is a non-treated growth control, which is inoculated and incubated under the same conditions than the test culture. Mycobacteria growth is monitored by the BACTEC 960 instrument, which automatically interprets if a particular culture is susceptible or resistant to the drug of interest. In other words, when the control culture reaches a mycobacteria concentration equivalent to 10^5–10^6 CFU, in about 7–8 days, the growth in the test tube is compared with the control. If the test drug is active against the isolated mycobacteria, its fluorescence will be significantly lower than controls.

5.4. Microplate Alamar Blue Assay (MABA)

MABA is a micromethod, which takes advantage of the dichromatism of resazurin: when the medium is reduced, resazurin changes irreversibly from blue to an intense pink and also intensely fluoresces under UV light. In addition, resazurin is soluble in water. These remarkable properties made possible that Alamar blue™ or resazurin has been widely used to determine cell viability and growth in MTB and many other cells and microorganisms [35]. MABA has been specifically applied to determine drug resistance of MTB [36] in synthetic, semisynthetic [37] and natural products [38]. MABA is also used to determine the minimal inhibitory concentrations (MIC) of isoniazid, rifampin, streptomycin and ethambutol in MTB. In general, the procedure followed with MABA consists of using bacterial suspensions having approximately 6×10^6 (CFU)/mL. The product to be evaluated for its anti-MTB activity is previously sterilized—by filtration or dissolved in 100% dimethyl sulfoxide (DMSO)—and then diluted in fresh Middlebrook 7H9 broth supplemented with an oleic acid dextrose catalase supplement (OADC). Then, 200 μL of this mycobacterial suspension are put into the first well of 96-well microplates. From this well, a twofold diluted series is formed, using the remaining wells, placed in the same lane than the first.

The MIC of the evaluated product is equivalent to its concentration being in the first pink-colored well [38].

5.5. Tools for molecular diagnosis and drug resistance determination

During the last decades, the number of tests to identify MTB has been growing rapidly. Today, the molecular diagnostic tools are faster, more sensitive and more specific than the conventional tools. Nevertheless, the conventional microbiological methods remain being the gold standards. Furthermore, the isolation, culture, identification of MTB and determination of first- and second-line anti-TB drug profile have not been replaced, with advantage, by any molecular tool. In other words, currently, molecular diagnosis tools are only complimentary for traditional diagnosis methods.

5.6. Molecular diagnosis tools

Currently, many molecular tools are available. In general, these methods analyze MTB-DNA or -RNA by polymerase chain reaction (PCR)-amplification, with very few exceptions. There are three basic molecular procedures for amplifying MTB nucleic acids: PCR final point, RT-PCR and quantitative or real-time PCR (qPCR). In addition, multiple variants have been described for the above nucleic acid amplification methods (see below). In general, the above methods detect polymorphisms, mutations or deletions to discriminate between MTB and other bacteria species and different MTB clusters. In addition, molecular methods offer the possibility of rapidly and accurately identifying MTB, genotyping and determining resistance to rifampin or rifampin and isoniazid [30, 39, 40].

5.7. Main genetic markers to identify MTB

The genetic markers currently used to identify and classify MTB are as follows: the IS*6110* insertion element, the gene encoding the 65-kDa heat shock protein, named hsp65, a polymorphic guanine-cytosine-rich sequence (PGRS-RFLP), mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR), direct repeats (DRs) or clustered regularly interspersed short palindromic repeats (CRISPR), single nucleotide polymorphisms (SNPs) and large sequence polymorphisms (LSPs) [41]. Nevertheless, not all the above markers are used in identifying MTB with clinical purposes, but in epidemiological and basic studies. Thus, in the following paragraphs, we discuss only the markers and methods useful to identify MTB with TB-diagnosis purposes, although some of them are also using to perform epidemiological studies.

IS*6110* considered as an ideal target to identify MTB and classify clinical isolates in clusters, according to their random fragment length polymorphism (RFLP [see below]). IS*6110* is randomly distributed throughout the MTB genome and its number of copies ranges from 0 to 26 [42]. A limitation of RFLP-based detection of IS*6110* is the inability of this method to discriminate strains with less than six copies of IS*6110*. This situation is especially important in India. Narayanan et al. in 2002 reported that 41% of clinical isolates contained a single copy of IS*6110* [43]. In addition, Sankar et al. specifically pointed IS*6110* as a molecular target that is not

sensitive enough to encompass all TB cases, especially PTB. In fact, it is well known that some MTB strains lack the element IS*6110* [44]. However, a group of investigators reported up to 94.1% MTB in clinical isolates using IS*6110* as the molecular target and nested PCR—see below —as the method of choice. Until relatively recently, IS*6110* was considered exclusive to the MTBC. However, Coros et al. [45] reported that *M. smegmatis* also has the IS*6110* element. This latter finding could negatively affect the specificity of IS*6110*.

Gene hsp65. The second most widely used molecular target is hsp65. This gene encodes for a heat shock protein of 65 kda, and the MTB genome contains a single copy of that gene. Variations in the primary sequence of hsp65 serve to identify species of rapidly and slowly growing mycobacteria [46]. The limitation of this target for the molecular diagnosis of TB is precise that the MTB genome has a single copy of the hsp65 gene.

Direct repeats. The direct repeat (DR) region in MTBC strains (do not confuse DR region) with drug resistance, see above]) is a fragment of DNA located in a hot spot for the integration of IS elements. This region is composed of multiple repeat sequences of direct variants (DVRS). Each DR replicate [47] is composed of 36 bp repetitive sequences separated by a non-repetitive sequence of 35–41 bp in size called spacers [48]. Generally, each spacer is found only once in the DR region. During the MTB evolution, some spacers could be deleted from the MTB-genome, due to a high mutation rate in the DR region [49]. Genome DR region of MTBC strains is extensively used for genotyping mycobacteria, due to DR spoligotyping-based patterns allow distinguishing MTB from *M. bovis* and other members of the MTBC [47].

6. Methods to identify MTB in biological samples

6.1. Method to determine RFLP

This method is an exception for others based on nucleic acid amplification. Therefore, it is necessary purifying a considerably greater quantity of DNA from MTB isolates than the DNA quantity needed for those methods based on nucleic acids amplification. RFLP consists in isolating, propagating MTB, purifying the DNA, cutting up the DNA with a specific restriction enzyme, separating the fragments by gel-electrophoresis, hybridizing the DNA fragments with an IS*6110* labeled probe and visualizing the RFLP pattern and analyzing it [50].

6.2. Methods to identify MTB by amplifying its genomic DNA

6.2.1. Polymerase chain reaction (PCR)

PCR provides a very useful tool for diagnosing TB. The endpoint PCR has two clinical applications: the identification of MTB and the determination of drug resistance of the clinical isolates.

6.2.2. End-point PCR

A typical analysis of end-point PCR consists in a separation of the PCR product by gel-electrophoresis and its visualization and certification of the expected PCR product. Originally,

end-point PCR was described by Kary Banks Mullis, who received the Nobel Prize in chemistry in 1993, for his invention of this procedure. The process, which Mullis conceptualized in 1983, is hailed as one of the monumental scientific techniques of the twentieth century [51]. PCR allows obtaining millions of DNA or RNA —see below—sequences from few original copies of the sequences of interest, called amplicons [2]. The success of molecular detection of MTB depends on the quality of the biological samples, the methods of extraction of the nucleic acids and the amplification and detection techniques. In addition, it is of fundamental importance to select the appropriate amplicons and to design and synthesize the complementary oligonucleotides (primers) to generate genuine DNA copies of the amplicons [52].

MTB identification by PCR can be done starting from sputum samples, MTB isolates or MTB laboratory strains (as H37Rv). In general, the PCR procedure, starting from sputum, is as follows: the sputum sample is decontaminated and concentrated usually using Petroff's method and centrifugation (see above). Mycobacteria are washed with a saline pH buffer and lysed by incubating them with a solution of lysozyme, proteinase K and sodium dodecyl sulfate (SDS). The DNA is extracted with chloroform-cetyl trimethyl ammonium bromide (chloroform-CTAB) and precipitated with isopropanol. The amplification of specific regions of DNA needs a very pure and non-degraded DNA, a heat-resistant DNA polymerase (usually Taq polymerase, which is obtained from the thermophile bacteria *Thermus aquaticus*), a mixture of the four deoxyribonucleoside triphosphates [deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP)], a pH buffer solution, salts of Mg^{2+} or Mn^{2+} and K^{+} and a pair of DNA primers, which sequences are complementary to the 3′ ends of the two DNA strands, embracing the region to be amplified. Typically, PCR consists of a series of 20–40 cycles. Each cycle consist of three steps: (1) denaturation of DNA at >90°C (separation of the two strands of DNA), (2) annealing (hybridization of primers with each DNA strand) and (3) extension (synthesis of the two new DNA strands) [53].

The analysis of DNA fragments produced by PCR consists of separate them according to their size (number of base-pairs [bp]). DNA band separation is achieved by applying an electrophoresis run in gels of agarose. After the electrophoresis is complete, DNA appears ordered in the gel as bands, from the heaviest (top) to the lightest (bottom). DNA bands in the gel must be stained to make them visible. DNA may be visualized using ethidium bromide, which, when intercalated into DNA, fluoresces under ultraviolet light. This stain must be managed carefully, using adequate protective equipment, because ethidium bromide is carcinogenic. A good alternative is using fluorescent-specific DNA stains, like GelStar® and SYBR® Green. Both strains are very sensitive and offer the advantage, over ethidium bromide, of being non-carcinogenic. In addition, it is not necessary washing the gels after staining for increasing the specificity of analyses. Therefore, both stains are excellent for detecting nucleic acids in agarose gels. GelStar is used to detect both single-stranded and double-stranded DNA and RNA, whereas SYBR Green is preferred for visualizing bands of double-stranded DNA in gel electrophoresis [54]. Then, the PCR product is sized, with the aid of a DNA molecular weight ladder, which must be placed in the same gel of experimental DNA, before running the electrophoresis. Usually, the first lane at left of the gel is chosen to put the molecular weight ladder. This DNA-size ladder is formed by a set of double-stranded DNA fragments having known and different sizes. The size of PCR products is estimated by interpolation or simply

comparing the position of the DNA marker, having a size equivalent to the size expected for the PCR product of interest. The identification on the gel of the amplified product with the expected molecular weight indicates the presence of an MTBC member in the sample studied [55, 56].

6.3. Variations of PCR-final point of DNA

The main variations of the basic point final PCR are nested PCR, multiplex PCR, spoligotyping [57, 58] and RFLP-PCR. These variants were designed to improve the sensitivity and specificity of basic RFLP and PCR.

6.3.1. Nested PCR

Nested PCR is widely used, because this method significantly improves the sensitivity and specificity of basic PCR. The nested PCR consists of using two sets of primers, both directed against the same target DNA sequence, which may be a gene or some other region characteristic of a mycobacterial species [59]. The first set of primers is designed to hybridize to the most distal regions of the target sequence, the first amplification run is made and the resulting amplicons are used, in turn, to amplify a slightly inner region of the used original target sequence. Once performed the first series of PCR cycles, the PCR is used to carry out the second amplification procedure. Prasad et al. designed a nested PCR-based assay using the hupB gene of MTB (Rv2986c) and *M. bovis* (Mb3010c) as a method to differentiate these two closely related species [60]. Our research group developed a method for the diagnosis of TB based on nested PCR that targets the gene coding for the β-subunit of MTB RNA polymerase. This method is noticeably more specific and sensitive than all that have been published to date or have been approved by the FDA. Most current methods require cultivating mycobacteria for a few days and do not work with certain types of samples. In contrast, our method can work well with the genome of a single bacterium and samples of sputum [61] or cerebrospinal fluid [62], without the need to culture the biological samples. Currently, the patent is in a process.

6.3.2. Multiplex PCR

Multiplex PCR refers to the use of PCR to amplify, simultaneously, several different DNA sequences. That is to say, multiplex PCR allows performing many separate PCR reactions, all together, carrying out only one procedure. Otherwise, amplification by PCR of several DNA sequences would require several runs, as well as more reagents and longer time to perform them. On the other hand, if additional information is required from a particular DNA sequence, this may be gained by accomplishing a single, conventional PCR test.

PCR multiplex has enormous value in infectious disease that can be caused by a variety of microorganisms, for instance, meningitis. Meningitis can be caused by MTB [63], several other bacterial species (*Streptococcus pneumoniae, Haemophilus influenzae* type b, *N. meningitidis*, group b *Streptococcus* and *Listeria monocytogenes)* [64] and fungi [65]. Meningitis is a medical emergency, and immediate steps must be taken to establish the specific cause and initiate effective

therapy [66]. PCR multiplex is a rapid, sensitive and specific diagnostic test for acute bacterial meningitis. PCR is particularly useful for analyzing cerebrospinal fluid of patients who have been treated with antibiotics before lumbar puncture [64]. Furthermore, in 2002, Shah et al. [67] reported a multiplex PCR-based assay performing a rapid and specific differentiation of *M. bovis* and MTB. This procedure can be beneficial for medical and veterinary microbiological laboratories. The designed multiplex PCR to identify MTB or *M. bovis* is based on the differential amplification of pncA gene. This test is highly specific and sensitive. Only 20 pg of pure mycobacteria DNA are needed, and none PCR amplification product has been obtained from any atypical mycobacterial isolate.

There is a series of requirements to be met to perform a good multiplex PCR analysis. These are as follows: (1) Accomplish the standard requirements for primer design. (2) Design one pair of primers for each expected amplicon. (3) Apply suitable temperatures, allowing DNA polymerase works accurately in every multiplex PCR cycle. (4) The primer design must be optimized; so that, all primer pairs work properly, at the same time and in a single reaction. (5) Apply similar alignment temperatures for all primers. (6) Choose the size of those DNA regions to be amplified so that be possible obtaining amplicons having clearly different sizes among them. Allowing, in this way, to distinguish the bands between them in the electrophoresis gel, (7) in case of needing to differentiate amplicons having the same size, the primers must be labeled with fluorescent dyes having distinctive colors. For certain multiplex analyses, commercial multiplexing kits are available. It has been informed that multiplex PCR has a sensitivity and specificity of 95% [68].

6.3.3. Random fragment length polymorphism (RFLP)-PCR

RFLP-PCR is a fast and reliable method for identifying non-TB mycobacteria [69]. This method is based on amplifying fragments of diverse genes; for instance, hsp65, the gene for histone-like protein hupB and pncA. Gene hsp65 are present in all species of mycobacteria. The selected target of hsp65 gene is a 439 bp fragment. Using hsp65 can be distinguished between MTB, *M. avium* and *M. intracellulare*, but not between MTB and *M. bovis* [70]. Gene hupB encodes for an histone-like protein. RFLP-PCR of hupB allows differentiation of MTB and *M. bovis*. The amplicons generated have 645 bp and 618 bp, respectively. pncA PCR assays were found specific in detecting MTB and *M. bovis*, as well as the *M. avium* complex in human sputum [71].

6.3.4. Real-time PCR

Real-time PCR, also known as quantitative PCR (qPCR), offers several advantages over end-point PCR, which are as follows: (1) as its name indicates, it is quantitative; (2) reduces the risk of cross-contamination because it minimizes the need to manipulate samples after performing PCR; (3) the manual processing time is reduced; (4) the run preparation times are also reduced and (5) offers high sensitivity and specificity [72]. Since the last decade, qPCR for the detection of MTB target genes in clinical specimens has contributed to improved diagnosis and epidemiological studies of TB. This is because qPCR offers the advantage of combining amplification and detection of molecular targets in a single step; and there is no need to use nested PCR [73].

Therefore, qPCR is rendering obsolete those techniques based on end-point PCR, which are much less sensitive. The qPCR technique involves the use of fluorescent chromophores (fluorophores) covalently attached to the primers. The fluorophore is placed at the 5′end of the primer (probe) and at the 3′ end or internally, a fluorescence quencher is inserted covalently. During the PCR extension phase, the exonuclease activity of the Taq polymerase cuts the initiator in the 5′-3′direction, and the fluorophore and quencher are separated, which results in a detectable fluorescence that is proportional with the number of accumulated amplicons. A spectrofluorometer integrated with the thermocycler reads the emerging fluorescence. The PCR occurs in a dynamic way, and the results are read continuously. A computer integrated with the equipment, with the help of specialized software that accumulates and analyzes the data, generates a DNA amplification curve under study. The computer program allows monitoring the amplification curve on a screen. Usually, an analysis of qPCR is carried out in 1.5 h. The analysis described above can be performed with different platforms. SYBR green (cyanine colorant) is widely used for qPCR because it is very sensitive and high cost/benefit in uniplex formats. Besides of SYBR green, a number of options are available, such as TaqMan probes, molecular beacons, scorpion primers, fluorescence resonance energy transfer probes (FRETS) and primer-probe energy transfer. There are also different fluorescent chromophores, e.g. 6-carboxyfluorescein-aminohexyl amidite (6-FAM), carboxy-X-rhodamine (ROX) and cyanine (Cy5) as reporters at the 5′end and a quencher at the 3′ end. Considering the advantages and disadvantages of IS*6110* and of hsp65 discussed above, some authors have proposed using them in a multiplex qPCR format, which would presumably offer a more specific and inclusive diagnosis [2].

6.3.5. Real-time (RT) PCR

RT-PCR allows to amplify specific regions of the MTB genome or to obtain DNA starting from RNA by using a reverse transcriptase. This form of PCR is called RT-PCR (reverse transcriptase-PCR). The nested RT-PCR is a variation of the nested-PCR. Nested RT-PCR offers the same advantages than nested PCR, discussed above; with the difference that nested RT-PCR allows obtaining a cDNA strand, using the original mRNA sequence as template. A reverse transcriptase is used instead of a DNA polymerase to synthesize the cDNA. Then, like in nested PCR, the first set of primers is designed to hybridize to the most distal regions of the target sequence, the first amplification run is made and the resulting amplicons are used, in turn, to amplify a slightly inner region of the used original target sequence [59].

6.3.6. Spoligotyping

Besides of PCR multiplex, spoligotyping offers the possibility of distinguishing between *M. bovis* and MTB. Forty-three types of mycobacterial spacers are known. Of these, 37 are typical of MTB and other 6 of *M. bovis* BCG. An edition of the international spoligotyping database namely SpolDB4/SITVIT was introduced containing 1939 different spoligotypes (ST) identified. Furthermore, spoligotyping allows grouping MTB isolates according to the presence or absence of intermediate regions of the DR locus (direct repeats) in each MTB isolate, forming

specific patterns. The DR locus is a member of the CRISPR (clustered regularly interspaced short palindromic repeats) family of the MTBC.

Spoligotyping is used to analyze genetic diversity of DR locus. It is useful for clinical, molecular epidemiology, evolution and population genetics laboratories [74]. This method is relatively easy to use, robust and allows numerical analysis. Spoligotyping was designed to detect biotinylated, amplified products from MTB and *M. bovis* from the DR locus. This method is based on reverse hybridization to the spacer sequences (43 synthetic oligonucleotides), which are covalently attached as lines to a nylon membrane. The hybridization is performed by turning 90° the amplicon template concerning the immobilized synthetic oligonucleotides. To immobilize the perpendicular samples, an acrylic device is used. So that, each homology will give rise to a tiny square of hybridization. Non-hybridizing products are eliminated with successive washes and will leave a blank space where the correspondent synthetic oligonucleotide is placed. On the other hand, those hybridized products will be detected using the streptavidin-peroxidase system, which binds to the biotin present in the amplified products. Results are visualized by incubating the hybridization template with luminol, in such a way that peroxidase catalyzes the oxidation of the reagent, resulting in light emission, which is developed by exposing the membrane to an X-ray film. The patterns obtained to reveal absence or presence of the spacers is read as a binary code, which can be easily interpreted and computerized. A commercial kit for MTB spoligotyping is available. In addition, a spoligotyping databases from MTB and *M. bovis* isolates from worldwide are available online.

6.4. Methods to determine MTB-drug resistance

6.4.1. End-point PCR to determine MTB-MDR

Most PCR-based tests described above were designed exclusively to detect the presence of MTB in biological samples. Fortunately, there are other PCR-based tests designed to identify MTB in biological samples and to know whether these microorganisms are resistant to rifampicin or rifampicin and isoniazid. The most important tests are described below.

The methods for detecting resistance to anti-TB drugs are based on the detection of mutations in the genes that are associated with that resistance condition and this is achieved by the application of sequencing or hybridization techniques. Recent advances in the rapid and direct detection of mycobacteria, with an emphasis on MTB, are based on the analysis of 16S rRNA gene sequence or oligonucleotide hybridization (oligohybridization), typing of strains and detection of patterns of drug susceptibility. Semi-automated systems for culture have greatly increased sensitivity and reduced the time required to perform the detection and identification of mycobacteria in clinical specimens. However, further research is still needed to assess the impact and cost/benefit of new diagnosis methods. In addition, well-designed clinical trials are still required to evaluate new diagnosis methods and thus enable medical staff to have methods to help them respond quickly [75]. PCR-based tests to identify mutations in the katG and rpoB genes that are associated with resistance to rifampicin and isoniazid, respectively, may assist in the early identification of resistance to these drugs in mycobacteria [40]. The WHO Stop TB Partnership's New Diagnosis Working Group and the Foundation for

Innovative New Diagnosis (FIND) ranked tools for diagnosing active and drug-resistant TB in three categories: (1) tools approved by WHO; (2) tools that are in the last phase of development or evaluation and (3) tools that are in the early stages of development [76]. Tools approved by WHO include LPAs for the diagnosis of MDR-TB, by GenoType® MTBDR plus, Hian Lifescience, Nehren, Germany; INNO-LiPA Rif. TB, produced by Innogenetics, Ghent, Belgium, an assay for rapid detection and speciation, whose manufacturer is Capilia TB-Neo, TAUNUS, Numazu, Japan and the GeneXpert MTB/RIF system, produced by Cepheid, CA, USA, a nucleic acid amplification test (NAAT) to screen for MTB-MDR. In the third group, tools that are in the early stages of development include an assay based on the detection of lipoarabinomannan, a breath analyzer, a loop-mediated isothermal amplification technology, called TB-LAMP, produced by Eiken Chemical Co Ltd, Tokyo, Japan, and a phage-based bioassay for the rapid diagnosis of MDR-TB [77]. Another very promising development is the one belonging to the company Hain Life-science, Nehren, Germany. This technique is used to screen resistance of MTB strains to second-line injectable drugs: amikacin, kanamycin and capreomycin.

6.4.2. RT-PCR and line probe assay (LPA) for detecting MDR

The LPA is based on the principle of reverse hybridization, in which the 16S-23S spacer region of the rRNA (ribosomal RNA) is amplified by PCR. The amplicons are hybridized with oligonucleotide probes which are placed on nitrocellulose strips and are detected by a colorimetric system (usually by the biotin/avidin system). The probe is biotinylated and the avidin is covalently attached to a chromophore. There is currently a system for an LPA called Inno-LiPA Mycobacteria, produced by Innogenetics, Belgium. Inno-LiPA Mycobacteria allows identification of CMTB species, *M. kansasii, M. xenopi, M. gordonae, M. avium* complex, *M. intracellulare, M. scrofulaceum* and *M. chelonae-M abscessus* complex species [78]. This system has demonstrated an accuracy of 99.2%. INNO-LiPA Rif has also proven its usefulness in examining the resistance/susceptibility of rifabutin in mycobacteria [79]. The LPAs allow obtaining results in 24 h, with a very high sensitivity (99%), as already mentioned above. This system is the only one under development that will allow detection of MTB resistant to second-line drugs. The limitation of LPAs is that in most resource-poor countries, the facilities required to avoid contamination of amplicons, laboratory supplies and the equipment necessary to perform amplification of genetic material by PCR are not available [80].

6.4.3. GeneXpert MTB/RIF

The method called GeneXpert MTB/RIF is based on real-time PCR and RT-PCR. The target is the rpoB gene (encoding the beta subunit of RNA polymerase). This gene has in its sequence a hot zone, which is very susceptible to mutations associated with resistance to rifampicin. GeneXpert MTB/RIF is the fastest and safest system known until now, being able to produce reliable results in less than 2 h. Therefore, GeneXpert MTB/RIF allows physicians making accurate diagnoses and prescribing appropriate treatments, practically during the patient's visit to his office. GeneXpert MTB/RIF is fully automated. The system consists of an instrument, a personal computer, a bar code reader and specialized preloaded software to interpret the results. The system requires disposable GeneXpert cartridges that contain everything

needed to perform nucleic acid amplification. As the cartridges are individual and watertight, the possibility of cross-contamination and false results is eliminated. Concerning to usefulness and availability of GeneXpert MTB/RIF, van Rie et al. [77] stated that is the first system that has a true point of care [POC] tool in regions with limited resources. The automation and simplicity of the GeneXpert MTB/RIF system allow non-mycobacteriology personnel to obtain reliable results. According to WHO, GeneXpert MTB/RIF should be used as an initial test for the diagnosis of TB and in symptomatic patients having a high risk of be sick of MDR-TB. On the other hand, one of the main limitations that GeneXpertMTB/RIF has is the fragility of the equipment, especially in places where the power supply fails continuously. On the other hand, it is doubtful whether the detection of resistance only to rifampicin is sufficient to guide a suitable treatment in areas where there is a high incidence of polyresistance [81].

6.4.4. Proteomic molecular tools

Proteomics is one of the most recent technological advances, which will surely bring enormous benefits in the diagnosis of fast and simple PTB. Significant progress has already been made in this regard. The sources of MTB proteins are easier to obtain and analyze the serum or plasma of patients with TB and to control the serum of healthy controls. Several focused papers on the above-mentioned sense have already been published: (1) Deng et al. in 2011 [82]; (2) Liu et al. in 2010 [83]; (3) Zhang et al. in 2012 [84] and (4) Liu et al. in 2011 [85]. However, this approach is neither accessible nor suitable for microbiological laboratories. The reasons are as follows: currently, the instruments required to perform a TB diagnosis are costly, very sophisticated systems and software and a high degree of specialization are required, not only for the correct handling of the equipment [mass spectrometers, generally associated with high-performance liquid chromatography (HPLC) equipment]. In addition, once the main proteins are known, other specialized databases must be identified and consulted to determine their function.

7. Immune molecular tools

Among molecular diagnosis methods, there are tools based on the detection of antibodies, for instance, the Enzyme Linkage Immuno Assay (ELISA). That is, these techniques are based on the use of serological tests [86]. The vast majority of patients with TB (90%) live in low- or middle-income countries. In these countries, the diagnosis of TB is based on the identification of AFB in unprocessed sputum samples and using conventional microscopes. Mycobacterial culture methods partially alleviate the low sensitivity of the ZN microscopy method. Nevertheless, we have already discussed the limitations of this method and those of techniques based on nucleic acids amplification.

7.1. Methods based on immunochromatography

Methods based on immunochromatography are suitable for use in low-income areas, because these tests can be done without specialized equipment and with minimal training. The most commonly used MTB detection tests are based on the detection of anti-MTB antibodies. One of

the aspects to be considered in the evaluation of commercial methods or the design of new methods based on the detection of anti-TB antibodies is that the profile of antigenic MTB proteins that are recognized by the antibodies generated in the immune response of the host. A possible limitation of these methods consists of that the set of antibodies used as biomarkers varies according to the progression of TB [87]. Therefore, a reliable diagnostic tool based on an adequate combination of antigens is still required. Currently, there are about 40 commercial serological tests for rapid TB diagnosis. These systems use various antigen compositions to detect patients with anti-MTB antibodies. However, there are insufficient data on its reliability in patients with HIV/AIDS and uninfected persons.

7.2. Immunoprecipitation in cellulose acetate strips

A usual format for rapid TB diagnosis is to use cellulose acetate strips with one line for the test to which MTB antigens have been prefixed and another line as a positive control having predefined MTB antigens and human antibodies anti-MTB antigens. At the end of the strip, a section is left where the patient's blood or serum sample is placed, plus a suitable volume of some saline buffer, and then, a conjugate of anti-human antibodies labeled with colloidal gold or some chromophore is placed. A positive reaction is observed when the antibodies from the patient and the conjugate migrate on the cellulose acetate strip, and immunoprecipitation occurs, and consequently, binding of the conjugate on the line with the preset MTB antigens. On the other hand, the human chromophore/anti-antibody conjugate also binds to the positive control line with the MTB antibody and the preselected human anti-MTB anti-antibody. These tests take only a few minutes. Due to its usefulness and simplicity, it is currently considered a high priority to rigorously evaluate these tests to take advantage of them with safety and advantage as soon as possible. These tests are conducted by the National TB Programs of the United States of America [86]. Even though immunoprecipitation in cellulose acetate strips is considered as a promising tool, the WHO disapproves the use of such immunological methods for the diagnosis of TB, especially when it is intended to replace the search for AFB with microscopy [88].

7.3. Enzyme Linkage Immuno Assay (ELISA)

ELISA serves as a presumptive test in cases of suspected TB. The ELISA plates are sensitized with a complete soluble extract of *M. bovis*, strain BCG. The test serum is diluted, and after addition of the immunoenzymatic conjugate and its substrate, the spectrophotometric reading is made and it is determined whether it is positive or negative, according to the cutoff value determined in 100 sera from apparent healthy subjects coming from an endemic area of TB. The result is reported as positive or negative. Given the individual differences in response and cross-reactions with antibodies induced by other mycobacteria, the negative result should be interpreted with caution as it does not necessarily rule out TB [55]. Therefore, commercial ELISA tests are considered to have limited sensitivity and inconsistent specificity for the diagnosis of TB. Nevertheless, this method is the basis for interferon-gamma release assays (IGRAs), which are useful to detect LTBI (see below), using interferon-γ detection, as we discuss below.

7.4. Interferon (INF)-γ

The basic utility of IFN-γ is the diagnosis of LTBI, although IFN-γ detection is also used intensively for the diagnosis of active TB. Tests based on the measurement of IFN-γ release for the diagnosis of TB are generically called IGRAs. The infection usually ends by infiltration into the lung tissue of $CD4^+$ T lymphocytes, which release IFN-γ. In turn, IFN-γ activates macrophages [89]. Identification of LTBI is a difficult issue. Therefore, the current diagnostic methods are based on markers of infection. Because of this, the intradermal reaction described above has long been used. Currently, kits are available to measure the release of IFN-γ as an immunodiagnosis alternative.

7.5. Interferon-gamma release assays (IGRAs)

IGRAs are designed to diagnose LTBI. These detect a cellular immune response to MTB. A remarkable characteristic of IGRAs consists in that results produced by these methods are not affected by the status of vaccination with BCG. Thus, in contrast with Mantoux method, IGRAs are useful for evaluation of LTBI in BCG-vaccinated individuals, particularly in settings where BCG vaccination was administered after infancy or multiple times. On the other hand, the IGRA's limitations are as follows: (1) These are unable to distinguish between an LTBI and an active-TB disease. (2) A positive IGRA result may not necessarily indicate an MTB infection, because it can also be caused by a non-tuberculous mycobacteria infection. (3) A negative IGRA does not rule out an active TB disease; many researchers have shown that up to a quarter of patients with active TB have negative IGRA results.

The IGRAs mainly used in the world are base ELISPOT and Quanti-FERON Gold In-tube.

7.6. Enzyme-linked immunospot (ELISPOT) assay

One of the best-known IGRA is ELISPOT, produced by Abcam. This system is used to determine the number of IFN-γ-producing T cells. ELISPOT employs monoclonal or polyclonal antibodies—preferably monoclonal antibodies because of their greater specificity than polyclonal antibodies. The antibodies are immobilized under aseptic conditions in the bottom of polyvinylidene fluoride (PVDF) microplates. The microplates are blocked, usually with serum albumin, which does not react with any antibody in the assay. Patient's cells are deposited on the microtiter plates at different densities in the presence of an antigen (in this case, the ESAT-6 MTB protein or the CPF10 protein filtrate). Both targets are strongly specific for TH1 cells in an MTB infection and are absent in BCG [90], whereas *M. kansasii, M. szulgai, M.marinum, M. flavensens* and *M. gastric* react with MTB-ESAT-6 and MTB-CPF-10. Microplates are put in a wet chamber at 37°C in an incubator with 5% CO_2 atmosphere for the time indicated by each manufacturer [91]. The specificity in HIV-infected individuals is 90%, while the specificity of the intradermal reaction is 57% [92].

7.7. Quanti-FERON Gold In-tube™ (QFT-GIT)

QuantiFERON Gold In tube (QFT) was specifically designed to LTBI. QFT-GIT is produced by QIAGEN. The QFT-GIT assay is an ELISA-based method. The test uses whole blood samples

from LTBI-suspicious patients and peptides from three TB antigens (ESAT-6, CFP-10 and TB7.7) in an in-tube format. The result is reported as quantification of IFN-γ in international units (IU)/mL of blood. An individual is considered positive for LTBI if the concentration of IFN-gamma concentration secreted by the responsive cells to TB antigens is higher than the test cut-off. These estimations are made after subtracting the IFN-γ concentration found **in the negative control** [93]. Several authors have reported that QFT-GIT has a sensitivity of 64–93% with an average of 70% [94]. Diel et al. found an average of 88% sensitivity of QFT-GIT with a range of 85–90% [95].

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Chapter 5

Diagnosis of Tuberculosis among Children and Adolescents

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Additional information is available at the end of the chapter

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Abstract

The authors discuss the challenging aspects of the diagnosis of tuberculosis in children and adolescents, since there is no gold standard for its diagnosis. The different clinical and radiological presentations and the low bacteriological positivity of tuberculosis in childhood are grounds for confrontation to the present. Immunological tests called interferon gamma release assays (IGRAs) failed to overcome the tuberculin skin test in practice. Advances with nucleic acid amplification tests, on the other hand, have contributed to the diagnosis of tuberculosis among adolescents. Standardized systems for diagnosis can be useful as tools for screening or for decision-making in childhood tuberculosis.

Keywords: tuberculosis, diagnosis, child, adolescents

1. Introduction

It is estimated that one-third of the world population is infected by *Mycobacterium tuberculosis* and that each year about nine million people develop the disease, out of which 11% are children. This percentage can be higher in countries with high burden of tuberculosis (TB). At least one million children are sick with TB every year. In 2015, as many as 210,000 children died from TB, out of which 40,000 were patients coinfected with HIV [1, 2].

It is believed that genetic predisposition influences the resistance of certain individuals who, even in contact with patients with baciliferous TB, are not infected with *M. tuberculosis* [3].

In childhood, the distinction between infection and disease is often difficult. Some authors avoid the term latent TB (or latent TB infection) in children, preferring to use the term TB

infection. The progression of infection to disease can be subtle and go unnoticed. This difficulty becomes more remarkable, often in extrapulmonary TB [2, 3].

From a practical point of view, the diagnosis of TB infection occurs when the child is asymptomatic with normal chest radiography and a reactive TB skin test (TST) or interferon gamma release assays (IGRA). On the other hand, the diagnosis of TB disease or active TB is a challenge. It occurs when the child is symptomatic (with symptoms consistent with TB) and chest or other X-ray is abnormal, compatible with TB. TST or IGRA can be reactive or not.

2. Differences between pulmonary tuberculosis in children and adolescents

The WHO has adopted standards for TB management in childhood that emphasize the importance of clinical, radiological, and epidemiological data consistent with TB and bacteriological confirmation, where possible, to diagnose the disease. Anti-HIV serology is also useful, especially in endemic areas of both diseases [4]. Even today these elements are essential for the diagnosis, since bacteriological confirmation is difficult in general, in children. Children mostly develop primary TB and are non-bacilliferous or paucibacillary (in 80% of cases the diagnosis is made without bacteriological confirmation). Up to 7 or 8 years of age, children do not know how to expectorate properly to take samples for examination and, in addition, TB in this age group is usually paucibacillary, that is, with sputum smear negative. Recently, the incorporation of molecular diagnosis of TB allowed positive results in a higher percentage than those observed with the smear. Yet TB in children differs fundamentally from the disease in adults, as in childhood forms of negative TB prevail [4].

It is important to divide TB in childhood into two aspects. TB in children up to 10 years of age (TB in children) and from 10 years of age (TB in adolescents). In the first case, most of the forms are *primary TB*. In adolescents, most are *adult-type TB*. That is, it is similar in clinical and radiological presentation to the pattern seen in adults. Adolescents are often bacilliferous and may transmit TB, develop excavated pulmonary forms, and, moreover, are able to cough and eliminate bacilli, similar to what occurs in adults [5, 6]. To collect specimens for bacteriological examination of children is only possible, most of the times, with the use of gastric lavage and induced sputum, since children under 5 or 6 years of age are almost unable to expectorate. The sputum smear or other specimen is processed by the traditional Ziehl-Neelsen method. The culture can be performed by Loewstein-Jensen method or liquid media—Ogawa or Middlebrook. More recently, molecular methods of nucleic acid amplification (NAATs) have been advocated. Among these methods, the *Gene-Xpert MTB/RIF* method (Cepheid, California) was recommended by the WHO in 2013 to diagnose TB in childhood [5, 7, 8].

Note that in childhood, compared to a case with high clinical suspicion, although the bacteriological and molecular tests are negative, the diagnosis of TB can and should be done.

Serological diagnosis of childhood TB was insistently sought, as it does not depend on the bacterial burden in the lesions. This is an advantage in patients whose disease progresses with

small number of bacilli. However, a study with commercially available serum tests in 2011 supported the WHO position of not recommending them for routine use. Thus, serological methods are no longer used as a diagnostic tool in children [3].

In adolescents, the diagnosis of TB may be bacteriologically proven in most cases. Patients from this age group with suspected TB are, most of the times, able to expectorate and are bacilliferous [5, 6, 9]. Soon, sputum smear microscopy can be a useful method, associated with clinical and radiological findings.

Table 1 shows differences in pulmonary TB in children and adolescents.

Children (primary TB)	**Adolescents (≥10 years old) (adult-type TB)**
Signs and symptoms: persistent fever, weight loss, interruption or loss in height and weight percentile, cough> 2 weeks, fatigue, night sweats	Signs and symptoms: persistent fever, cough >2 weeks, night sweats, weight loss, blood spitting
Contact with adult with TB	There may be no history of contact with people with TB
Radiography: enlarged mediastinal lymph nodes, miliary pattern, pneumonia with no response to antibiotics	Radiography: infiltration in the upper third, excavations, pneumonia
Slow evolution pneumonia	Slow evolution pneumonia
Reactive tuberculin skin test	Reactive tuberculin skin test
Negative bacteriological tests	Positive bacteriological tests (AFB and culture)
Positive Xpert * in a few cases (~10%)	Positive Xpert in most cases

**Gene-Xpert MTB/RIF.*

Table 1. Differences in pulmonary tuberculosis in children and adolescents.

3. Basis for the clinico-radiological diagnosis

Some risk factors for active TB in childhood continue to be valued for the diagnosis of active TB [4]:

- Intradomiciliary contact with a bacilliferous case recently diagnosed
- Under 3 years of age
- HIV infection
- Severe malnutrition

The clinical manifestations of TB, especially in young children, are subtle and may go unnoticed. Discrete changes such as weight loss or lack of weight gain, fatigue, and decreased interest in playing and decreased physical activity can be frequent and sometimes are not noticed by the family. Unexplained fever, irritability, or nystagmus can be subtle findings in the early forms of meningoencephalitis caused by TB, for example. TB meningitis is a case of

clear cerebrospinal fluid (CSF), the laboratory test of which shows a predominance of monocytes in the early stages and lymphocyte stage, in evolution, lower glucose blood sugar, and high levels of protein [4].

Pulmonary TB is taken into account in some situations, such as slow evolution pneumonia. Cases where the child is being treated with antibiotics for acute pneumonia and shows no good performance should be valued as suggestive of TB. Most relevant clinical data such as evening fever, weight loss, profuse night sweats, and coughing or combinations of these signs and symptoms may facilitate suspected diagnoses of TB, especially if it has remained for over two weeks.

Some aspects remain as challenges for the diagnosis of TB in children. Young infants may present a subtle case of TB, with signs and symptoms that are confused with other infectious and noninfectious diseases. In such cases the diagnosis may be delayed by a longer or shorter time, depending on the diagnostic suspicion capacity of the health team, and its consequences can be disastrous. TB externalized as severe and acute pneumonia (patients admitted to ICU on ventilatory failure) is rare and difficult to diagnose. The suspected clinical appearance of TB in HIV-infected patients or with severe malnutrition or the discovery of HIV in patients with TB almost always confuses doctors who are attending such patients. An extensive differential diagnosis of diseases related to HIV immunosuppression, which are confused with TB is necessary. It is no less difficult to diagnose multidrug-resistant TB (MDR-TB) in children. In general, contact with adults with MDR-TB is the key to establish such a diagnostic suspicion [2, 10]. The performance of susceptibility testing of patient specimens with suspected MDR-TB is of limited use in childhood, because most cases are negative (no bacillus). When bacteriological examinations of the child cannot be done, but information is available on the sensitivity to adult drugs with which the child comes into contact, it can be assumed that his/her treatment is done with the same regimen of adult MDR-TB. When the child's clinical specimen is available and it is possible to perform Xpert, rifampicin resistance may be a proxy resistance to isoniazid. That is, there is high probability of being a case of MDR-TB. More recently, *Line-probe assays* have become available, which can simultaneously detect resistance to rifampicin and isoniazid. Another promising method is *MTBDR-sl*, which detects resistance to fluoroquinolones, aminoglycosides, and ethambutol simultaneously. These latest tests are rarely used in childhood (Heemskerk, Guidance).

Although there is more radiological and clinical data suggestive of TB than others, the epidemiological context and the clinical picture have to be taken into account to achieve the diagnosis of TB. Since there is no gold standard for the diagnosis of TB in childhood, it is necessary to consider the epidemiological reality of the region where the work is being done. Is it an endemic area for TB? Is the patient followed up in a basic health unit? Or in a reference center? Has the child with suspected TB had contact with an adult with TB or suspected TB? Was this a sporadic or lasting contact? Has the child suspected of intrathoracic TB been treated with antibiotics for common germs recently, with no successful result? All these questions may help contextualize the highest probability or not of diagnosing the disease in the child.

The possible contact of the child with an adult (especially if bacilliferous) is of great interest especially in young children. Infants and preschool children tend to have closer contact with adults than children of school age and adolescents. Thus, the history of contact with a person with TB can be epidemiologically valuable data on suspected TB diagnosis in childhood.

Household contact is more important than an external or sporadic one. However, in certain locations, the difference between household and external contact is subtle. In poor communities, it is common for people to live together in neighboring houses; children are cared for by people in these communities and sleep in their relatives' houses. So it is worth asking if the child has contact with other people supposedly with TB (cough> 3 weeks) in or out of his/her home. In nurseries or schools of endemic regions of TB, cases are sometimes discovered where employees or teachers with TB who were diagnosed late and may have infected children who interacted with them. A situation of extreme gravity is the occurrence of TB cases in pediatric intensive care units (PICU) or nurseries. Newborns in condition of extreme vulnerability can be infected by *M. tuberculosis* and can develop the disease, the diagnosis of which is often difficult.

It is known that the period of greatest risk of developing TB is the first 2 years following the primary infection by *M. tuberculosis*. This interval of 2 years is the most appropriate time to initiate preventive measures such as treatment of latent TB infection (isoniazid preventive therapy, IPT) [10].

4. Radiological diagnosis

The most common radiological patterns of pulmonary TB in children are hilar lymphadenopathy identified on front X-ray and more visible in profile incidence (**Figure 1a** and **1b**); miliary

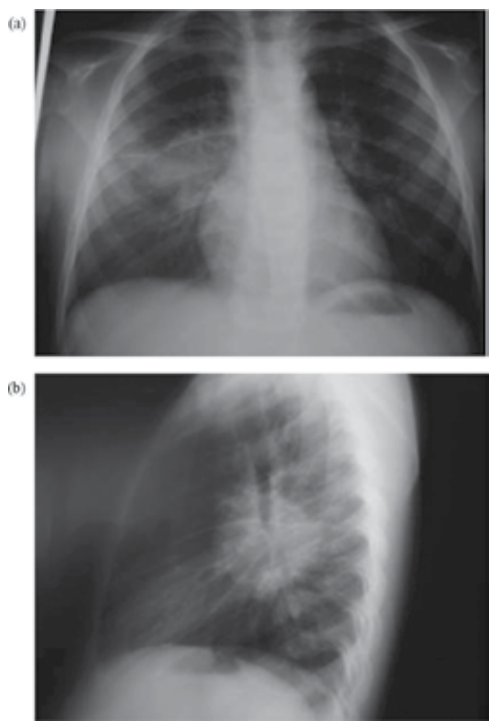

Figure 1. (a and b) Enlarged hilar lymph glands: front and profile incidences (Primary TB).

aspect—diffuse micronodular pattern in both hemithoraces, corresponding to hematogenous spread of TB (**Figure 2**) and appearance of alveolar pneumonia, that is, pattern of bacterial pneumonia caused by common germs, like pneumococcus, which may only be a case of TB when it becomes a case of slow evolution, not responsive to antibiotics (**Figure 3**) [4, 10]. It is often emphasized that many times we observe clinical and radiological dissociation in TB in children. The clinical evaluation of the child is disproportionately favorable in relation to the extent of radiation injury. That is, the radiological image suggests a serious pneumonia but the patient does not show signs of severity that would be expected in a pneumonia caused by common germs.

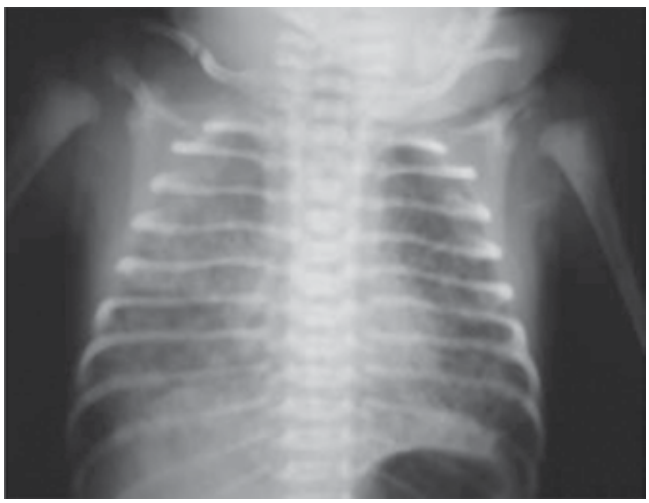

Figure 2. Micronodules in both lungs; miliary TB.

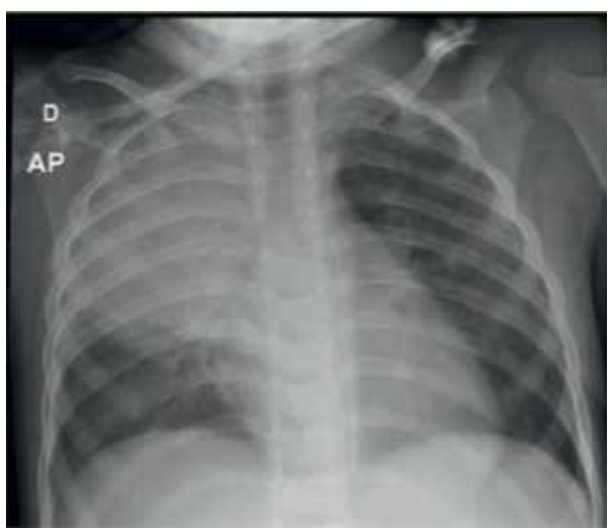

Figure 3. Slow evolution pneumonia; radiological aspect of a Pneumococcus pneumonia. Infiltrate on the left lung.

Some of these radiological findings in suspected cases of TB in children always require some differential diagnoses. Thus, the miliary pattern may appear on acute histoplasmosis or lung metastases of neoplasm (**Figure 4**); the unilateral or bilateral adenomegalies may arise in the course of Hodgkin's disease or sarcoidosis, and slow progression of pneumonia may also occur in atypical pneumonia (for Mycoplasma, for example), pneumonia caused by common germs, actinomycosis, nocardiosis, and other noninfectious agents in immunocompromised patients.

In adolescents, radiological aspects are identical to the pattern seen in adults: adult-type TB (**Figure 5**).

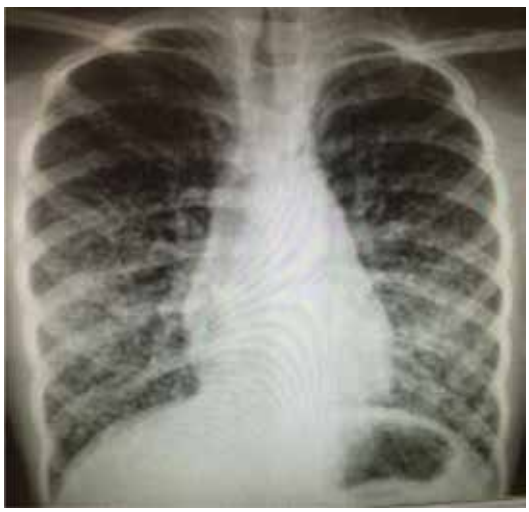

Figure 4. Micronodules in both lungs. Aspect of miliary TB. In fact, it was a thyroid carcinoma with metastases.

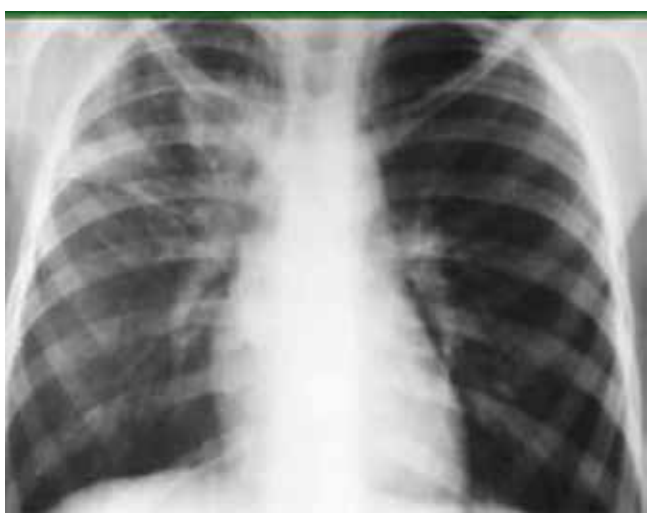

Figure 5. Pulmonary infiltrate in upper zone of the right lung. Aspect of TB *adult type*.

5. Immunological diagnosis

The main methods of immunological diagnosis of TB are the tuberculin skin test (TST) and *interferon gamma release assay* or IGRA.

The TST with PPD, conducted by the Mantoux technique, introduced in 1907, is the intradermal injection of 5 tuberculin units (TU) of purified protein derivative PPD-S (PPD) or 2 TU PPD RT23 (are equivalent). In the event of cellular immunity to such tuberculin antigens, a delayed hypersensitivity reaction will occur within 48–72 h [11].

The reaction will cause skin induration at the injection site, and its transverse diameter must be measured (induration mm) by a trained individual and interpreted using stratified cutoffs: 0–4 mm = no reaction; 5 mm or more = reaction.

Immunity measured by TST may also reflect exposure to other environmental mycobacteria, vaccination by BCG (Bacillus Calmette–Guérin-*Mycobacterium bovis*), or a previous TB infection. This type of reactivity can lead to false-positive results. On the other hand, false negative results may occur especially in immunosuppressed people, for example, with advanced HIV infection or using immunosuppressive medication [3].

The IGRA is an *in vitro* test which detects the production of IFN-γ in peripheral blood by T cells of the host infected by *M. tuberculosis*. The identification of immunogenic proteins of the mycobacteria, antigens ESAT-6, CFP-10, and TB 7, which are specifically expressed by pathogenic strains of *M. tuberculosis* complex, allowed the development of IGRA. These antigens are encoded in the region of difference 1 (RD1) of the genome of *M. tuberculosis*. RD1 contains genes encoding the secretive system of the bacillus, known as ESX-1. Such antigens are very specific of *M. tuberculosis* although there is some evidence of cross-reactivity with *Mycobacterium leprae*, not yet proven; positive results in individuals infected with *Mycobacterium marinum* and *Mycobacterium kansasii* have also been evidenced [3, 11].

There are two types of IGRA. The test conducted by QuantiFERON TB Gold methodology allows the measurement of *in vitro* level of IFN-γ produced by T cells that have been stimulated by the said antigens. The result is reported as number of IFN-γ in international units (IU) per milliliter. An individual is considered positive if the amount of IFN-γ is above the cutoff point of the test (considering the negative control). Another method may be used: T-SPOT.TB. It is an immunosorbent assay (ELISPOT) performed in separate and counted mononuclear cells from peripheral blood which are incubated with ESAT-6 and CFP-10 antigens. The result is reported as the number of T-cells producing IFN-γ (spot-forming cells). An individual is considered positive if the points count in the antigens exceeds the negative control points. Indeterminate IGRA results may occur due to a low IFN-γ response of the positive control (mitogen) or a high IFN-γ response of the negative control. Further studies are needed on this test in children [7, 11].

6. IGRA and TST in tuberculosis diagnosis in children

The purpose of carrying out the TST or IGRA in children is to determine whether there is or not *M. tuberculosis* infection while other factors such as contact history and clinical and

radiological picture will allow diagnosis. Thus, the negative result of these tests does not exclude TB disease, nor does its positive result confirm it. Both tests have limitations inherent in specific methodologies and rationales. In addition, IGRAs are still poorly studied in children living in regions with high TB prevalence or HIV-infected children [7]. Some studies suggest that in children above 5 years of age vaccinated with BCG, IGRAs may be preferred to the TST, whereas in children who have not been vaccinated with BCG, the IGRAs and TST are equally indicated. In Brazil, to consider a child to be infected with TB, the cutoff point of the TST ≥ 5mm was adopted in children vaccinated more than 2 years or in immunosuppressed children; in children vaccinated with BCG less than 2 years, the cutoff point is ≥ 10 mm. These cutoff points were decided as above, since during the 2 years after the application of BCG vaccine, the TST reaction will gradually decline [6].

IGRAs and TST alone do not allow the diagnosis of TB disease; its role is to indicate TB infection. Both tests have a similar sensitivity for detecting infection by *M. tuberculosis.* More studies are needed in children, because IGRAs still yield indeterminate results, which are justified by the low capacity of producing immunoglobulins in children in early childhood. In patients infected with HIV, the sensitivity of IGRAs is low, similar to what occurs with the TST [11].

The cost of IGRAs is much higher and is also technically more complex than the TST. Furthermore, it involves collecting blood, which is a limitation in the case of children. On the other hand, the TST requires two visits to the clinic in order to get the result within 48–72 h. The trend of international standards is to keep the TST as a standard for identifying cases of TB infection, at the expense of IGRAs, due to the advantages of the TST in terms of cost, more reliable results in small children, and lack of need for laboratory resources [4].

Table 2 lists the main differences between IGRAs and TST.

Characteristic	TST	IGRA
Antigens used	Many, PPD	3 (QFT) or 2 (T-SPOT)
Sample	Intradermal injection	Blood draw
Patient visits required	2	1
Distinguish between Latent TB and TB disease	No	No
Cross-reactivity with BCG	Yes	No
Cross-reactivity with non-TB mycobacteria	Yes	Only rare species *
Differing positive values by risk	Yes (5-10-15)	No
Causes boosting	Yes	No
Subject to boosting by previous TST	Yes	Possible
Durability over time (stays positive with or without treatment)	Yes	Unknown
Difficulties with test reproducibility	Yes	Yes
Relative cost	Lower	Higher
Location of need for trained staff	"Bedside"	Laboratory

Characteristic	TST	IGRA
Estimated specificity in BCG-unvaccinated children	95–100%	90–95%
Estimated specificity in BCG-vaccinated children	49–65%	89–100%
Estimated sensitivity (confirmed TB disease)	75–85%	80–85%
Estimated sensitivity (clinical TB disease)	50–70%	60–80%

**M. marinum, M. kansasii, Mycobacterium szugai, Mycobacterium flavescens.*

Source: Ref. [11].

Table 2. Comparison of the TST and IGRA.

7. Molecular diagnosis

The Xpert MTB-Rif system (Xpert) is a test based on nucleic acid amplification used to detect *M. tuberculosis* and for the screening of drug-resistant strains. It uses real-time polymerase chain reaction (PCR) technique, on the Gene Xpert platform, which allows to integrate three processes: purification, concentration, and amplification of nucleic acids by polymerase chain reaction (PCR) and detection of nucleic acid sequences in the genome of *M. tuberculosis*, specifically in *rpoβ* gene. This technique does not require mycobacterial DNA manipulation after amplification and thus reduces the complexity and risk of cross reactivity by DNA product amplification [12].

Not only does it allow, in a period of 2 h in the laboratory, to identify *M. tuberculosis* but also to detect bacterial resistance to rifampicin (RIF) by amplification, using PCR, of five overlapping probes which are supplemental to the region determining RIF resistance, consisting of 81 base pairs of rpoβ gene *in M. tuberculosis*. Then, this region is examined in order to identify mutations associated with resistance [3].

It is a quick, highly automated test and does not depend upon the machine operator. The only manual step involves adding the correct dose of the reagent into the specimen under analysis, and it is subsequently homogenized for 15 min and transferred to the Xpert cartridge.

The system consists of a Gene Xpert instrument, a computer, a barcode reader, and a preinstalled software to perform tests on samples taken and to view the results generated on the screen and to report as negative or positive *M. tuberculosis*, as well as to report whether sensitive or not to RIF. However, a positive result does not necessarily indicate the presence of viable bacilli, since the method can identify the DNA of live or dead microorganisms [7, 12].

Various specimens (organic liquids, aspirated peripheral ganglia) can be processed, but its main application is in sputum samples, and its wider applicability is in adults with suspected pulmonary tuberculosis [7]. Its use in extrapulmonary samples still has a variation between different studies, in terms of sensitivity and specificity according to the analyzed material, as well as considerations related to cost-effectiveness in these samples [13].

The following samples should not be processed: those composed exclusively of saliva; containing food particles; consisting solely of pus (no mucus and greenish); consisting exclusively of blood; with quantity less than 1 ml in the case of pulmonary and extrapulmonary samples, except cerebrospinal fluid (CSF); CSF samples amounting to less than 0.1 ml.

The sensitivity and specificity values for a single Xpert in adult sputum approach 88% (95% CI: 83–92) and 98% (95% CI: 97–99), respectively. Among those with positive sputum smear, the sensitivity is around 98% (95% CI: 97–99) and among those with negative sputum smears, it is around 68% (95% CI: 59–75) [14].

Xpert has still limited use in childhood, since its excellent performance is seen in bacteriologically confirmed TB, which is the minority of cases in children [7]. It has been shown that, by using culture as the gold standard, the sensitivity of Xpert on a single test in children under 15 years of age is from 42.9 to 90%, both in sputum and in induced sputum [13, 15, 16]. For the gastric lavage samples, sensitivity is 68.8% [15] and for nasopharyngeal aspirate it is 48% [17]. Specificity is similar to the adult (over 98%). A second test in children with negative sputum smears may increase the sensitivity of Xpert in 27.8% [13].

The prospect of broadening the diagnosis of TB in children with the use of Xpert was the subject of some studies carried out in South Africa and other African countries. There was higher positivity of Xpert in cases of PT in relation to culture for *M. tuberculosis* on specimens, such as gastric lavage, nasopharyngeal aspirate, and sputum induction. It became clear that Xpert contributes to increased diagnostic capacity of pulmonary TB in microbiologically confirmed TB [12, 17].

In Detjen and colleagues meta-analysis with studies using Xpert for the diagnosis of pulmonary TB in children and adolescents, the pooled sensitivities and specificities of Xpert for TB detection, when compared to culture: 62% (IC 95%: 51–73) and 98% (IC954%: 97–99) in sputum and induced sputum, respectively, and for samples from gastric lavage: 62% (95% CI: 51–73) and 98% (95% CI: 96–99), respectively. When compared with microscopy, the Xpert sensitivity was better [18].

In New Delhi, India, in a study investigating suspected cases of pediatric TB in public services, it was observed that Xpert was able to detect twice as many cases of pulmonary TB compared to smear and to increase the capacity to detect resistance to RIF, which is a quick and promising test for pediatric patients [19].

8. The role of clinical examination

The clinical examination does not always contribute significantly to the diagnosis of TB in children. It is recommended to evaluate the child's weight and development and to identify possible growth retardation attributable to the chronic nature of TB. In adolescents the clinical examination can be more informative: weight loss, poor general condition and presence of cough, and sometimes, blood spitting, call the doctor's attention to the possibility of the disease. Physical examination changes in the respiratory tract, such as bronchial or *cavernous breath*, have been reported for decades in patients with excavation (caverns) in the lungs, but they are currently little researched or recognized. Occasionally, wheezing in the chest can be

located by auscultation, due to extrinsic compression of the bronchi by mediastinal lymphadenopathy [10].

Extrapulmonary manifestations of TB may be evident on clinical examination, such as peripheral lymphadenopathy. Peripheral lymph node TB can present with cervical lymphadenopathy with progressive worsening, sometimes fistulated (scrofuloderma). Osteoarticular tuberculosis affecting the column (Pott disease) can present, insidiously, with back pain and night crying, deformity in the column, and difficulty in walking. Meningoencephalitis in the early stages evolves with discrete findings such as conjugate deviation of the eyes, irritability, vomiting, and behavioral changes, reaching to severe cases of neck stiffness. Pleural TB, most common in children and adolescents, may manifest as a pleural syndrome without apparent cause, with afebrile patient, or low fever and with no difficulty breathing even in cases of large spills. Cutaneous TB in general is difficult to diagnose by multiple lesions that it can determine. An experienced dermatologist in general needs to be contacted.

The manifestation of skin that most commonly can relate to TB is erythema nodosum. This transient injury, usually in the anterior tibial crest, may arise in TB primary infection. However, causes such as streptococcal infections, drug reactions, and leprosy, among others, should always be considered in these cases [4, 8].

Remember that extrapulmonary manifestations associated with pulmonary TB can be seen with some frequency. Thus, when one is investigating a suspected case of extrapulmonary TB, such as meningoencephalitis, bone, or other location, chest X-ray is recommended concurrently. Although the patient is asymptomatic for lung, pulmonary radiological damage may occur silently, which will ultimately assist in the diagnosis of TB.

Cases of pulmonary TB with severe course, resulting in ventilatory insufficiency, are uncommon but require the experience of an intensive care team to reach the diagnosis. Severe acute pneumonia of rapid course, similar to those caused by common germs, was described in infants and children with HIV. Clinical findings such as oral candidiasis, parotid hypertrophy, and digital clubbing can be seen in HIV-infected patients, in which TB-HIV coinfection can be considered. Lymphocytic interstitial pneumonia, currently uncommon, is a chronic process associated with the HIV virus that mimics the miliary TB. It is pneumonia with diffuse micronodular interstitial pattern, similar to *Pneumocystis jirovecci*, which can affect patients with HIV/AIDS and whose differential diagnosis of TB is a challenge [10].

The diagnosis of TB should also be sought in child living with an adult with TB, even if it is asymptomatic. This is the case of asymptomatic contacts. The contact control is one of the most valued preventive measures of TB in childhood, because it allows diagnosing TB cases at an early stage and taking preventive measures, such as the IPT, provided that the existence of active TB can be ruled out. Asymptomatic contact should preferably be submitted to chest radiography and TST or IGRA. If the X-ray is abnormal, compatible with TB, regardless of the result of the TST/IGRA, the child must be considered as a case of TB and justifies starting treatment of the disease. If the X-ray is normal and the TST/IGRA is reactive, IPT is indicated [4, 10].

9. Clinical classification of intrathoracic tuberculosis in children

The international consensus proposed in 2015 aimed at clinical standardization of intrathoracic TB cases in childhood called for, among others, the following clinical findings suggestive of TB: persistent cough for ≥ 2 weeks, growth arrest in the last 3 months (documented), and fatigue [20].

Standardizing sought to establish parameters for research in TB area, so that the terms used are easy to understand and even among researchers. It also facilitates the clinical approach and can be used in routine care.

Based on the clinical, radiological, and epidemiological findings, in immunological confirmation of *M. tuberculosis* infection and bacteriological confirmation, the following rating for intrathoracic TB in childhood was proposed [20]:

1. **TB confirmed**—Finding of *M. tuberculosis* (Culture or Xpert MTB/RIF) in at least one respiratory specimen.
2. **TB not confirmed**—No bacteriological confirmation and at least 2 of the following findings: signs and symptoms suggestive of TB; chest radiograph compatible with TB; close contact or evidence of immune infection by *M. tuberculosis*; Positive response to treatment.

Infected by *M. tuberculosis*—immunological evidence of infection by *M. tuberculosis* (positive TST or IGRA).

Not infected by *M. tuberculosis*—with no immunological evidence of infection.

3. **TB unlikely**—No bacteriological confirmation of TB; no criteria of unconfirmed TB, and no evidence of infection by *M. tuberculosis*.

10. Algorithms and scoring systems for diagnosis

For more than four decades, algorithms and scoring system have been published for diagnosis of TB in childhood. Some were validated and others were not. In Brazil, a scoring system is used for the diagnosis of pulmonary TB (intrathoracic) since 2002, which has been validated in HIV-infected and HIV-uninfected children [9, 21] and already tested in other countries [22, 23]. In HIV-infected patients, the system scored better sensitivity than specificity, becoming therefore more suitable as a screening test [24].

In HIV-uninfected children, the system provided sensitivity and specificity values greater than 86%. One of the advantages of this system is that it requires no bacteriological confirmation, which is admittedly useful in small children unable to expectorate (**Table 3**).

Characteristic	Points
Clinical manifestations	
Fever or cough, lost energy, sputum, weight loss, or night sweats for >2 weeks	+15
No symptoms or symptoms for <2 weeks	+0
Respiratory infection improving with or without antibiotic treatment for common bacteria	−10
Thoracic X-ray	
Hilar adenomegaly or miliary pattern	+15
Exudate or patch shadow (with or without cavitation) unaltered/worsening after > 2 weeks with antibiotic treatment for common bacteria	+15
Exudate or patch shadow (with or without cavitation) <2 weeks	+5
Normal	−5
Adult TB contact	
Regular contact	+10
None or occasional contact	0
BCG vaccination and TST	
BCG ≥2 years ago or no BCG, TST >5 mm	+15
BCG <2 years ago, TST >10 mm	+15
TST ≤5 mm, regardless of BCG	0
Nutritional status	
Severe malnutrition (grade III)	+5
Eutrophic or non-severe malnutrition	0

Table 3. Guide for Brazilian ministry of health diagnostic system.

11. Conclusion

The diagnosis of TB in children is based on clinical and radiological features adding other data as epidemiological and immunological (TST or IGRAs) information. In children (<10 years old) the bacteriological confirmation is difficult because in these individuals the TB is negative. There are few cases in which we can establish the disease confirmation due to the etiopathogenesis of primary TB. Primary TB means a disease of a small quantity of bacillus (*M. tuberculosis*). However, in adolescents (>10 years old) the TB assumes other characteristics: it is an adult type TB. In this form of TB the majority of the patients are bacilliferous (positive), so the bacteriological confirmation is possible. So, the molecular diagnosis using *Gene-Xpert MTB/RIF* test is adopted in several endemic countries with good results.

12. Projections and applications

As the diagnosis of pediatric TB is a challenge, we consider that the professional capacitating is necessary for enhancing the despistage of cases in childhood. Till today, the only diagnostic tool for childhood TB is the *Gene Xpert TB* test. However, this test is almost only useful in adolescents who have a bacteriological disease. The majority of cases in children depend on the health professional capacity of interpreting clinico-radiological and epidemiological aspects aiming to establish the correct diagnosis of TB.

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Chapter 6

Drug Resistance in *Mycobacterium tuberculosis*

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Additional information is available at the end of the chapter

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Abstract

Tuberculosis (TB) remains to be a serious health problem worldwide. There is an increased transmission of *Mycobacterium tuberculosis* strains with drug resistance, hence complicating TB control. The deciphering of the *M. tuberculosis* genome, together with the implementation of new molecular biology tools, has allowed the identification of changes in nucleic acid sequences with a functional impact. These mutations have become important in the design of early-diagnostic kits to identify the resistance profile of *M. tuberculosis*. Since the conventional methods to determine the identity of *M. tuberculosis* strains based in cultures are laborious, time-consuming and performed by specialized technicians, the result is generated until 4 months after receiving the samples. During this time, patients with TB are not adequately treated, and resistant strains may be transmitted to the rest of the population. In this chapter, we describe the most relevant mutations in genes associated with drug resistance in *M. tuberculosis*, the analysis of gene expression to identify new markers of drug resistance strains, and the development of new antituberculosis drugs against drug-resistant strains.

Keywords: *Mycobacterium tuberculosis*, drug resistance, mutations, gene expression, antituberculosis drugs

1. Introduction

Tuberculosis (TB) has remained a serious health problem since *Mycobacterium tuberculosis*, the main agent of this disease, infects about one third population. In 2015, 10.4 million cases of TB were estimated and, although only a small percentage (5–10%) develops the illness, its control has complicated due to the emergence of drug resistance strains [1]. Tuberculosis regiment treatment includes the first line drugs rifampicine, isoniazide, ethambutol and pyrazinamide and strains that develop resistance to the two more effective antituberculosis drugs, isoniazide and rifampicine, named

multidrug resistance strains [2, 3]. The resistance phenomenon in *M. tuberculosis* has been highly related to mutations in specific genes [4], and this association has been the base for the implementation of rapid diagnostics kits [5] but unfortunately mutations do not explain completely the resistance in all cases [6, 7], suggesting that other mechanisms could be involved. New approaches to search new markers and mechanisms of resistance have been performed. One of these is the evaluation of changes in gene expression [8]. Together with the diagnostics of TB, the implementation of new schemes of treatments is necessary to restrict the transmission. The development of new drugs against drug resistance *M. tuberculosis* has resulted promissory to control TB [9].

2. Mutations that confer resistance in *Mycobacterium tuberculosis*

Although the rate of resistance to first and second line drugs in *M. tuberculosis* varies among countries, the resistance phenomenon has complicated the tuberculosis control worldwide. There two types of observed resistance in *M. tuberculosis*, one is the genetic resistance where mutations in genomic regions, on target genes, confer the capacity to avoid the drug effect; the second is the phenotypic resistance, where epigenomic modifications, including alteration of protein structures, generate resistance to drugs without mutation on DNA. Although the current knowledge of the molecular genetic basis of resistance to antituberculosis drugs has advanced rapidly the last years [10], there are unknown mechanisms in how bacilli is able to resist to drugs. Identification of clinical isolates with resistance to antituberculosis drugs would facilitate the timely and accurate diagnosis to initiate an appropriate treatment.

Many works have revealed, using microbiological and clinical data, mutational events in clinical isolates from patients with tuberculosis. Multidrug resistance appears to result from the sequential acquisition of mutations. Possible reasons for the acquisition of mutations include inadequate prescription and delivery of chemotherapy, poor compliance, or an insufficient number of active drugs in the treatment regimen [11]. Mutations or combinations of mutations have been found in strains displaying single or multidrug resistance. Here, we summarized the most common mutation found in clinical isolates that confer resistance to the first and second line antituberculosis drugs (**Table 1**).

2.1. Isoniazid

Due to its properties as a bactericidal drug, isoniazid has been widely used as the first line drug in the treatment against *M. tuberculosis* complex members. Mutations on *katG* and *mabA-InhA* genes have repeatedly been associated with isoniazid resistance [10] (**Table 1**). In the case of *katG*, the most common of mutation is S315, which is present in 50–95% of isoniazid resistant clinical isolates [12]. The occurrence of mutations is also observed in the promoter region of *mabA*/*inhA*. Mutations in the *inhA* promoter can also confer cross-resistance to ethionamide [13]. There are other genes as *ahpC*, *kasA,* and *ndh*, encoding for alkyl hydroperoxidase reductase, β-ketoacyl-ACP synthase, and NADH dehydrogenase, respectively, which have also been associated with isoniazid resistance.

Drugs	MIC (μg/mL)	Drug mode of action	Gene	Target enzyme	Frequency of mutations (%) associated with resistance
Isoniazide	0.02–0.2	Inhibits mycolic acid synthesis and other multiple effects	*katG*	Catalase peroxidase	30–60
			InhA	Fatty acid enoyl acyl carrier protein reductase A	70–80
			ahpC	Alkyl hydroperoxidase reductase	Not known
			kasA	β-ketoacyl-ACP synthase	Not known
			ndh	NADH dehydrogenase	9.5
Rifampicin	0.05–1	Inhibits RNA synthesis	*rpoB*	β subunit of RNA polymerase	95
Streptomycin	2–8	Inhibits protein synthesis	*rpsL*	Ribosomal protein S12	65–67
			rrs	16S rRNA	
			gidB	7-Methylguanosine methyltransferase	33
Ethambutol	1–5	Inhibits arabinogalactan synthesis	*embCAB*	Arabinosyl transferase	70–90
Pyrazinamide	16–100	Disrupts plasmamembrane and energy metabolism (inhibits pantothenate and CoA synthesis)	*pncA*	Pyrazinamidase	>70
			IS6110 insertion		Not known
Fluoroquinolones	0.5–2.5	Introduces negative supercoils in DNA molecules	*gyrA*	DNA gyrase	42-85
			gyrB		
Kanamycin/ Amikacin	2–4	Inhibits protein synthesis	*rrs*	16S rRNA	>60
Capreomycin/ Viomycin	2–4	Inhibits protein synthesis	*rrs*	16S rRNA	40-100
			tlyA	rRNA methyltransferase	80
Ethionamide	2.5–10	Disrupts cell wall biosynthesis by inhibition of mycolic acid synthesis	*InhA*	Fatty acid enoyl acyl carrier protein reductase A	>60
			ethA	Flavin monooxygenase	>60
			ethR	Transcriptional repressor	Not known

Data taken and modified from [16] and [17].

Table 1. Genes associated with resistance to various anti-TB drugs.

2.2. Rifampicin

Rifampicin is highly bactericidal for *M.tuberculosis*, where this drug is able to bind to the β subunit of RNA polymerase, and this event induces hydroxyl radical formation in susceptible mycobacteria [14]. Resistance to rifampicin is acquired by mutations in a region of the 81-bp region of the *rpoB* gene, encoding the β subunit of RNA polymerase, and these mutations have been found in ~96% of rifampicin-resistant clinical isolates. The most frequent mutations are located in positions 516, 526, and 531. Also, there is evidence that mutations in the *rpoB* gene generate cross-resistance to rifamycins [15]. It is important to mention that not all mutations in *rpoB* are associated with rifampicin resistance [16].

2.3. Streptomycin

It has been considered as a second line antituberculosis drug, which binds to the 30S subunit of the ribosome and blocks protein synthesis. The resistance is provoked by mutations in the *rpsL* gene, which encoded the S12 protein, and the *rrs* gene, which encoded the 16S rRNA. The mutations in both genes are the main mechanism of streptomycin resistance, and it has been found in 65–67% of resistant clinical isolates [16]. There is another gene, *gidB*, involved in streptomycin resistance. This gene encoded a 7-methylguanosine (m(7)G) methyltransferase, and mutations have been found in 33% of clinical isolates resistant to streptomycin.

2.4. Ethambutol

Ethambutol has a target, the inhibition of the enzyme arabinosyl transferase, which is involved in the biosynthesis of cell wall arabinogalactan. The enzyme is encoded by the embB gene, harboured in the embCAB operon, and mutation in this gene is related to ethambutol resistance. The most frequent mutation found in the *embB* gene is located in codon 306. More than 68% of resistant clinical isolates have mutations in the *embB* gene [16].

2.5. Pyrazinamide

This pro-drug is converted to its active form, pyrazinoic acid, and it only kills non-growing persistent bacteria. The mutations of the *pncA* gene are scattered along this genomic region, and it is the main mechanism of pyrazinamide resistance. The majority of pyrazinamide-resistant clinical isolates (72–99%) have showed mutations on the *pncA* gene sequence. Due to a high correlation between mutations and pyrazinamide resistance, it has been suggested that the use of mutations to predict the resistance profile to pyrazinamide; however, there are silent mutations that do not confer resistance [16].

2.6. Fluoroquinolones

Fluoroquinolones are able to inhibit the activity of DNA gyrase. When the activity of DNA gyrase is affected, the chromosomal DNA acquires a supercoiled conformation [17]. Then, mutations on *gyrA*, encoding DNA gyrase, are strongly associated with fluoroquinolone

resistance. There are many reports where mutations located in the *gyrA* region are present in 42–85% of clinical isolates resistant to fluoroquinolones (Louw et al., 2009). Also, mutations on the *gyrB* gene have also been associated with fluoroquinolone resistance, where 3% of clinical isolates harbor the mutation in this gene. The most common mutations of the *gyrA* gene are located in codons 90, 91 and 94 [16].

2.7. Amikacin/kanamycin

Amikacin and kanamycin are considered as second-line antituberculosis drugs. It has been identified that the *rrs* gene as the target of action of these drugs; however, the molecular mechanisms that confer resistance are focused to inhibition of protein synthesis [18]. About 60% of the clinical isolates resistant to amikacin/kanamycin have mutations on the *rrs* gene [17]. The most common mutations are located at the position 1400 of the *rrs* gene, which causes high-level resistance these drugs.

2.8. Ethionamide

This prodrug requires to be activated by the mono-oxygenase EtaA/EthA. It has been described as the only bactericidal agent against *M. tuberculosis*. Ethionamide inhibits mycolic acid synthesis. Mutations in *inhA* also confer resistance to ethionamide. Frequency of mutations on *etaA*/*ethA*, *ethR,* and *inhA* genes in clinical isolates resistant to ethionamide reaches 60% [16].

Mutations described in *M. tuberculosis* have led to the implementation of rapid molecular diagnostic kits with the aim to diagnose TB and detect drug resistance in a shorter period compared to drug susceptibility testing based on the culture of the microorganism [19].

Within the rapid methods approved by the WHO, there are real-time PCR-based assays, as Xpert MTB/RIF, the line probe assays Genotype MTBDR*plus* and Genotype MTBDR*sl.* The XpertMTB/RIF tests allow *M. tuberculosis* detection as well as resistance to rifampicine. A multicenter study in which 6648 patients were evaluated, the Xpert MTB/RIF test allowed the detection of 90.3% of the TB confirmed cases based on culture, as well as 67.1% of the TB cases diagnosed by microscopy. For detection of rifampicine resistance, a sensitivity of 94.4% and specificity of 98.3% were reported, and an indeterminate rate of 2.4%, which was lower than that of culture diagnose with 4.6% [20]. On the other hand, the Genotype MTBDR*plus* allows detection of resistance for the first line drugs, while Genotype MTBDR*sl* detects resistance to the second line drugs. A meta-analysis of Genotype MTBDR*plus* reported a pooled sensitivity of 0.91, 0.96, and 0.91 and a pooled specificity of 0.99, 0.98 and 0.99 for the detection of isoniazide-, rifampicine-, and multidrug-resistance, respectively. Both, sensitivity and specificity settings have 95% confidence intervals [21]. Finally, in a multicenter study realized in 2012, the accuracy of the Genotype MTBDR*sl* was evaluated in 200 *M. Tuberculosis* isolates; in this study, the sensitivity reported was between 77.3 and 92.3% for the detection of resistance to fluoroquinolones, ethambutol, amikacin, and capreomycin while for kanamycin was 42.7 and 22.6% for XDR detection; the specificity was 82% for all drugs [22].

3. Searching for new markers to identify drug resistance of *Mycobacterium tuberculosis*

In the understanding and linking-up of genetic associations with the drug resistance phenotype in *M. tuberculosis*, mutations in specific genes have been the most common association as previously described; nevertheless, not all resistant *M. tuberculosis* strains have the related mutations previously reported suggesting that other mechanisms could be involved in this phenomenon [6, 23, 24]. For this purpose, the expression level of some genes has been studied. One of them is efflux pump genes, these are important elements that play a role in the extrusion of drugs out of the cells conferring *M. tuberculosis* resistance to drugs [25]. The efflux pumps have been classified in super families: ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR), and multidrug and toxic-compound extrusion (MATE) [23]. In *M. tuberculosis*, the efflux pumps consist of (a) 12 mycobacterial large membrane proteins (MmpL) belonging to RND-type transporters [26], (b) 37 ABC transporters (26 complete and 11 incomplete) from which 21 are putative exporters which include antibiotic transporters that represent the 2.5% of the genome [27, 28] and (c) 16 putative MFS drug efflux pumps [29]. Some findings have reported efflux pump genes to be overexpressed in drug resistance *M. tuberculosis* strains (**Table 2**) [25, 30-32]. The importance of efflux pumps involved in drug resistance has led to suggest the analysis of the implementations of a combined therapy of antituberculosis drugs together with efflux pump inhibitors [23].

Locus	Symbol[a]	Gene name[a]	Drug-resistant phenotype	References
Rv2688c		Antibiotic-transport ATP-binding protein ABC transporter	XDR	[32]
Rv1634		Drug efflux membrane protein	XDR	[32]
Rv2936	*drrA*	Daunorubicin-dim-transport ATP-binding protein ABC transporter drrA	XDR	[32] [30]
Rv2937	*drrB*	Daunorubicin-dim-transport membrane protein ABC transporter drrB	XDR	[32] [30]
Rv0820	*phoT*	Phosphate-transport ATP-binding protein ABC transporter phoT	XDR	[31]
Rv2136c	*uppP*	Conserved membrane protein	MDR	[25]
Rv2846c	*efpA*	Membrane efflux protein efpA	MDR	[30]
Rv0849		Conserved membrane transport protein	MDR	[30]
Rv1250			MDR	[30]
Rv1410		Aminoglycosides/tetracycline-transport membrane protein	MDR	[30]
Rv1634		Drug efflux membrane protein	MDR	[30]
Rv2994		Conserved membrane protein	MDR	[30]

Locus	Symbol[a]	Gene name[a]	Drug-resistant phenotype	References
Rv2333c	*stp*	Involved in transport of drug across the membrane (export)	MDR	[30]
Rv2459		Conserved membrane transport protein	MDR	[30]

[a]Data obtained from TB database and/or TubercuList, XDR: extensively drug-resistant.

Table 2. Overexpressed efflux pump genes in drug-resistance *Mycobacterium tuberculosis* strains.

Furthermore, studies in drug resistance strains have reported other genes as differentially expressed between sensitive and drug-resistant strains. Functional categories of these genes are among others, stress response and translation (**Table 3**). On the other hand, expression of intergenic regions (IGs) has also been associated with a drug resistance phenomenon in *M. tuberculosis* [8, 25, 31], suggesting that an additional analysis is necessary to evaluate and confirm the contribution of these regions in drug resistance.

With the aim to demonstrate the resistance association between the level of expression of some genes and drug resistance, assays using recombinant strains of *M. tuberculosis* as well as other *Mycobacterium* strains treated with different drugs and/or overexpressing genes of interest have been analyzed [25, 33-35]. The new findings related to differences of gene basal expression between susceptible and resistant *M. tuberculosis* strains can contribute to identify newly genetic drug-resistant markers that could contribute in the early diagnosis of drug-resistant tuberculosis, which could be applied in the establishment of a more efficient drug therapy [8, 30].

Locus	Symbol[a]	Gene name[a]	Expression level modification	Drug-resistant phenotype	References
Rv1181	*pks4*	Polyketide beta-ketoacyl synthase pks4	I	XDR	[31]
Rv1182	*ppA3*	Polyketide synthase associated protein papA3	I	XDR	[31]
Rv1184c		Hypothetical exported protein	I	XDR	[31]
Rv0826		Conserved hypothetical protein	I	XDR	[31]
Rv1483	*fabG1*	3-oxoacyl-[acyl-carrier protein] reductase fabG1	I	XDR	[31]
Rv1592c		Conserved hypothetical protein	I	XDR	[31]
Rv1623c	*cydA*	Membrane cytochrome D ubiquinol oxidase subunit I cydA	I	XDR	[31]
Rv2585c		Conserved lipoprotein	I	XDR	[31]
Rv2621		Transcriptional regulator	I	XDR	[31]
Rv3269		Conserved hypothetical protein	I	XDR	[31]
Rv0287	*esxG*	Esat-6 like protein esxG	I	MDR	[8]
Rv0288	*esxH*	Low molecular weight protein antigen 7 esxH	I	MDR	[8]

Locus	Symbol[a]	Gene name[a]	Expression level modification	Drug-resistant phenotype	References
Rv1037c	*esxI*	Esat-6 like protein esxI	I	MDR	[8]
Rv1642	*rpmI*	50S ribosomal protein L35 rpmI	I	MDR	[8]
Rv1630	*rpsA*	30S ribosomal protein S1 rpsA	I	MDR	[8]
Rv3487c	*lipF*	Esterase/lipase lipF	R	MDR	[8]
Rv3418c	*groES*	10 kda chaperonin groES	R	MDR	[8]
Rv1161	*narG*	Respiratory nitrate reductase alpha chain narG	R	MDR	[8]
Rv1819c		Drugs-transport transmembrane ATP-binding protein ABC transporter	R	MDR	[25]

[a]Data obtained from TB database. XDR: extensively drug-resistant, MDR: multidrug-resistant, I: gene with induced expression in the resistant strain analyzed, R: gene with repressed expression in the resistant strain analyzed.

Table 3. Differential expressed genes in drug-resistance *Mycobacterium tuberculosis* strains.

4. Development of new drugs against *Mycobacterium tuberculosis* drug resistance strains

Even though tuberculosis antibiotic treatment therapy is described, drug resistance in *M. tuberculosis* complicates the TB control. In 2015, 480,000 MDR tuberculosis cases were estimated and, in addition, 100,000 more cases were added which had resistance to rifampicin [1], these cases are more likely to develop multi-drug resistance. Drug-resistant TB has led to the implementation of new therapeutic regimens involving second line drugs, once drug susceptibility testing results are available [36].

Drug therapy for a patient infected with a susceptible *M. tuberculosis* strain lasts 6 months with diverse combinations of the first-line drugs rifampicine, isoniazid, ethambuthol, and pyrazinamide, while treatment therapy for a patient with DR tuberculosis can last up to 20 months and include a fluoroquinolone, an injectable aminoglycoside plus an oral bacteriostatic second line drug and a first line drug (for details consult D'Ambrosio et al. [36]).

Because the problem of resistant tuberculosis is increasing, searching for new drugs continues with the aim of improving the therapeutic regimens currently used, shorten treatment duration in addition to find more effective drugs for latent TB and drug-resistant strains. The development of new antituberculosis drugs implicates the following stages: basic research, discovery of new antituberculosis compounds or drugs, preclinical and clinical studies conformed by phases I, II, and III to finally get to the technology transfer; all these processes entail long periods of time [37]. In this continuous search for better antituberculosis drugs, many natural, semi-synthetic, and synthetic compounds have been evaluated *in vitro* and *in vivo*. We will mention some new drugs that are based on the structure of first line drugs, among which some analogues have been described with activity against sensitive and drug resistant *M. tuberculosis* strains. Thereby based on ethambutol, some of the novel described

compounds comprise SQ109 and analogues based on carbamate prodrugs [38], S2824 and analogues with a homopiperazine ring [39], 1,2 diamines [40], ferrocenyl compounds [41] and dihydrosphingosine-ethambutol analogues [9]. Within pyrazinamide analogues, it has been described POEs (pyrazinoic acid esters) and 5-Cl-substituted pyrazinoic acid derivatives [42]. However, it is necessary to consider the adverse effects of these compounds. For isoniazide-based compounds, there has been reported aromatic and heterocyclic aldehydes containing electron-withdrawing or donating groups [43], and rifampicin has been described within the rifamycins as well as among others as rifabutin, rifapnetine, rifalazil, and rifametane [44].

As general conclusion, although mutations are commonly associated with drug resistance in *M. tuberculosis*, other studies are necessary to discover genetic markers that support the early diagnostic of drug resistance in strains that enable the establishment of optimized therapeutic schemes limiting their transmission.

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Chapter 7

The Physiology of *Mycobacterium tuberculosis* in the Context of Drug Resistance: A System Biology Perspective

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Additional information is available at the end of the chapter

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Abstract

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (*Mtb*), is the main cause of death due to an infectious disease. After more than 100 years of the discovery of *Mtb*, clinicians still face difficulties finding an effective treatment for the increasing number of drug-resistant cases. The difficulties in the clinical setting can be related to the slow pace at which the understanding of the physiology of this bacterium has occurred. *Mtb* is distinct from other microorganisms not only due to its slow growth and difficulties to study in the laboratory, but also due to its inherent physiology such as its complex cell envelope and its metabolic pathways. Understanding the physiology of drug susceptible and resistant *Mtb* strains is crucial for the design of an effective chemotherapy against TB. This chapter will review the mycobacterial cell envelope and major physiological pathways together with recent discoveries in *Mtb* drug resistance through different "omics" disciplines.

Keywords: drug resistance, physiology, systems biology, proteomics, genomics, lipidomics

1. Introduction

The history of tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (*Mtb*), has a remarkable involvement in human history; particularly in the evolution of human society and in the development of many scientific disciplines. TB has a negative role in many pages of human history, taking lives of many renowned artists, politicians, as well as poor, wealthy, young, or adult individuals. After its apparent "resurgence" at the end of the last century with the concomitant arises of HIV/AIDS cases, TB has been mainly associated with poverty and

immunosuppression. *Mtb* was the model microorganism that inspired Koch to develop his postulates which are a cornerstone of sciences such as Microbiology and Immunology. What Robert Koch never probably imagined was that over a century later, this disease was going to continue as the leading cause of death, mostly because of the increasing number of drug-resistant TB cases. Regarding TB treatment, it is discouraging to see how this lethal disease only started to be cured and controlled during 1950s with the discovery of the first chemotherapy. It is hard to imagine the labor of a clinician taking care of TB patients before 1950s without an available treatment option. However, this same scenario is similar to what many health providers face nowadays with the current spreading of drug-resistant cases, particularly multidrug resistant (MDR) and extensively drug resistant (XDR) TB cases. The majority of drug-resistant *Mtb* strains found in clinical settings emerge due to mutations in genes that are involved in the antibiotic mode of action (drug activator, drug target, etc.). These bacterial genes have important roles in bacterial metabolism and pathogenicity. Therefore, the study of drug-resistant *Mtb* strains has been evolving from the exploration of the associated genotype (i.e. specific resistance-conferring and compensatory mutation(s)) to the entire phenotypic impact that mutation events confer to the bacteria beyond the drug resistance feature. The study of the global drug-resistant phenotype in *Mtb* thorough comprehensive system biology approaches (such as genomics, proteomics, and lipidomics) is expected to reveal important aspects that will help TB researchers in the development of new anti-TB chemotherapies and overcome the current global challenges toward an effective TB control. This chapter will describe an overview of *Mtb* physiology and metabolic pathways as an important scaffold to understand the physiological changes that some *Mtb* strains (specific genotypes) undergo after acquiring resistance to the major anti-TB drugs: isoniazid (INH) and rifampicin (RIF) from a biochemical perspective.

2. Review of *Mtb* major metabolic pathways and cell envelope

Mtb physiology is a broad subject comprising *the study of the function and activities of this bacterium and its parts*. In this chapter, we will narrow the study of *Mtb* physiology to its major metabolic pathways and cell envelope, particularly in the context of drug resistance.

2.1. Major central metabolic pathways in *Mtb*

Mtb has the ability to use very variable carbon sources *in vitro* such as carbohydrates, alcohols, and lipids (including cholesterol and fatty acids) (reviewed in Ref. [1]). Similar to other representative species of the Actinomycetales order, *Mtb* possesses a predominant aerobic metabolism, with the genes encoding for enzymes of the main energetic metabolic pathways such as glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway. Despite the genetic evidence of a complete TCA cycle in *Mtb* [2], there is no sufficient biochemical evidence to show the presence of all enzymatic reactions of the TCA cycle in *Mtb*. In fact, some researchers propose that TCA cycle in *Mtb* is not complete because this organism lacks the alpha ketoglutarate dehydrogenase (α-KDH) enzyme [3–5]. Instead, alpha ketoglutarate decarboxylase (α-KGD and Rv1248c) and succinic semialdehyde dehydrogenase (GabD1/2, Rv0234, and Rv1731, respectively) are proposed as the enzymes that catalyze the step from alpha ketoglutarate (α-KG) to succinate in *Mtb* TCA cycle under normoxic conditions.

Particularly, α-KGD catalyzes the production of succinic semialdehyde, which can then be converted to succinate by GabD1/2 [5]. Also, experimental evidence suggests that *Mtb* operates a reversed TCA cycle with the reduction of fumarate to succinate to maintain the membrane potential in the absence of oxygen [6].

Mtb also has the glyoxylate shunt which allows the bacteria to bypass some enzymes of the regular TCA cycle under specific metabolic conditions (hypoxia or growth on fatty acids as carbon source) [2]. Under anaerobic conditions, the enzyme isocitrate lyase (Icl) (which is proposed to be required for virulence [7]), together with the α-KG ferredoxin oxidoreductase are believed to complete the cycle effectively bypassing the conversions of α-ketoglutarate to succinate to fumarate [8].

Mtb has the ability to use enzymes in multiple metabolic pathways to prolong its survival, a feature that is known as metabolic plasticity. For instance, Icl not only participates in the glyoxylate shunt and the methyl citrate cycle but also protects *Mtb* from the oxidative stress generated by the treatment with isoniazid (INH), rifampicin (RIF), and streptomycin [9]. Another example is the dihydrolipoamide dehydrogenase (Lpd) that can act as the E3 component of the pyruvate dehydrogenase or can provide electrons to the dihydrolipoamide succinyltransferase (DlaT, previously known as SucB) or be part of the branched-chain keto acid dehydrogenase complex to metabolize branched-chain amino acids [9]. On the other hand, *Mtb* has pathways with redundant enzymes (that include a variety of isozymes) that can catalyze the same reaction, which guarantees that vital processes occur despite possible external or internal stresses. A good example of this is the fatty acid degradation or β-oxidation pathway, which suggest that *Mtb* not only has a high lipid catabolism activity, but also that this is crucial part of its own metabolism [1].

2.2. Lipid metabolism: β-oxidation and fatty acid synthesis

Lipid metabolism is a highly relevant physiologic process in *Mtb*, with more than 6% of the genome devoted to these reactions and almost 20% of the genome encoding for genes related to cell wall processes. Lipid metabolism is an important part of this chapter as some enzymes of the lipid biosynthetic pathway are the target of anti-TB drugs such as INH and ethionamide. Compared to *Escherichia coli*, *Mtb* possess five times more enzymes dedicated to lipid metabolism. *Mtb* lipid metabolism is more lipolytic than lipogenic, probably as a result of the wide variety and amount of lipid sources in the human host as well as in the bacterial envelope [2]. For this reason, the first part of this chapter will focus on fatty acid degradation with a subsequent description of recent findings regarding fatty acid synthesis. Fatty acid degradation is a key process in *Mtb* metabolism and can explain some of its metabolic plasticity, while fatty acid synthesis is crucial in the understanding mechanism of action of the previously mentioned anti-TB drugs.

2.2.1. Fatty acid degradation

Fatty acid catabolism in *Mtb* is a process of successive oxidations where the β-carbon of the fatty acid is oxidized to a carbonyl group (**Figure 1**). In this process, the main goal is the synthesis of acetyl-CoA and reduced cofactors (such as NADH, $FADH_2$) that can fulfill energy requirements in the cell and also intermediates that can serve as substrate for anabolic processes [2]. Specifically, odd-chain fatty acids produce acetyl-CoA while even-chain fatty acids

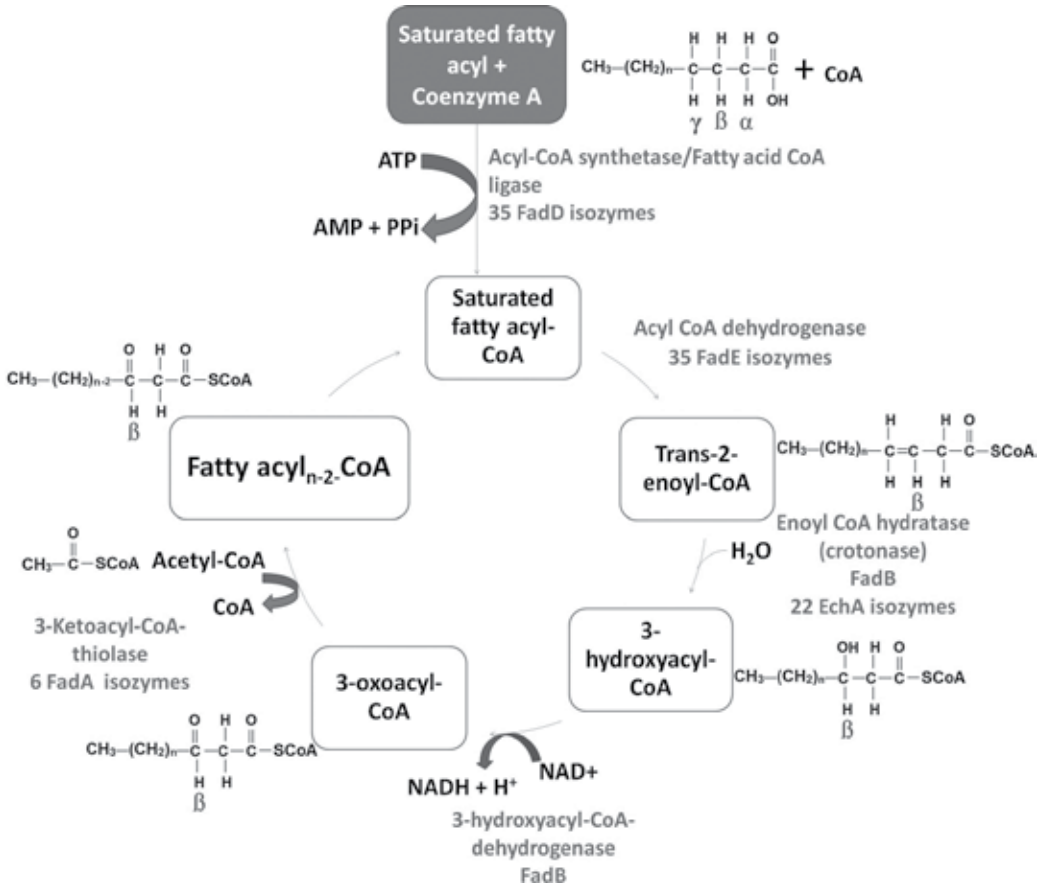

Figure 1. β-Oxidation of fatty acids in *Mtb*. Greek nomenclature indicates the different oxidations that take place in the β-carbon. The enzymes show the different number of identified isozymes that participate in this cycle.

produce acetyl-CoA and propionyl-CoA in addition to acyl-CoA derivatives missing two carbon units [2, 10]. By studying the *Mtb* genome, Cole et al. found at least 35 genes encoding for enzymes that catalyze the first step of fatty acid degradation only. As shown in **Figure 1**, most reactions in this pathway can be carried out by several isozymes. Of these, EchA5 and FadB3 are essential for *Mtb* growth and considered possible drug targets [11].

2.2.2. Fatty acid synthesis

The complexity of *Mtb* lipids can be partially explained by the fact that *Mtb* has both fatty acid synthases (FAS), type I and II. Cole et al. described the main enzymes of FAS I and II at the genetic level and recent reviews have compiled previous biochemical work, all of which have generated a better understanding of the complex pathways responsible for mycolic acid synthesis in *Mtb* [12–14]. The characterized enzymes that participate in FAS I and FAS II are shown in **Table 1**. FAS I is found mainly in eukaryotes and all the reactions are performed by a single multidomain homodimeric enzyme Fas (Rv2524) that has a mass higher than 300 kDa [2, 14]. This enzyme

Description	Gene	Rv number	Enzyme
FAS I	*fas*	2524	Fatty acid synthetase
Transition FAS I to FAS II	*fabD*	2243	Malonyl-CoA ACP transacylase
	accD6	2247	Acetyl/propionyl-CoA carboxylase (beta subunit)
	acpM	2244	Acyl carrier protein
	fabH	0533	β-Ketoacyl-ACP synthase III
FAS II	*kasA/B*	2245/2246	β-Ketoacyl-ACP synthase
	fab1 or MabA	1483	β-Ketoacyl-ACP reductase
	hadA/B/C	0635/0636/0637	(3)-hydroxyacyl-ACP dehydratase subunit A/B/C
	htdX	0241	3-hydroxyacyl-thioester dehydratase
	echA10/11	1142/1141	Currently annotated as a enoyl-CoA hydratase, but proposed to be 2-trans-enoyl-ACP isomerase
	inhA	1484	2-trans-enoyl-ACP reductase
Modifications			
Desaturases	*desA1/2/3*	0824/1094/3229	Acyl carrier protein desaturase
Methyltransferases (methylation, oxygen function introduction and cyclopropanation)	*mmaA1*	0645c	Methoxymycolic acid synthase 1
	mmaA2	0644c	Methoxymycolic acid synthase 2 (distal cyclopropane in α-MA, proximal cis-cyclopropane in keto-MA)
	mmaA3	0643c	Methoxymycolic acid synthase 3 (oxygenated MA)
	mmaA4	0642c	Methoxy mycolic acid synthase 4 (oxygenated MA)
	cmaA1	3392c	Cyclopropane-fatty-acyl-phospholipid synthase 1 (distal position)
	cmaA2	0503c	Cyclopropane-fatty-acyl-phospholipid synthase 2 (proximal position-specific in methoxy-MA)
Mycolic acid modification	*pcaA* (*umaA2*)	0470c	Mycolic acid synthase (proximal cyclopropanation function α-MA)
	umaA	0469	Mycolic acid synthase
Clainsen-type condensation	*accD4*	3799c	Acyl-CoA carboxylase
	accD5	3280	Acyl-CoA carboxylase
	fadD32	3801	Fatty-acid-AMP ligase
	pks13	3800	Polyketide synthase-13
Mycolic acid processing	*mmpL3*	0206	Transmembrane transport protein-3
	Rv3802	3802	Proposed to be a Mycolyltransferase I, recently shown to have phospholipase and thioesterase activity
	cmrA	2509	Reductase
	fbpA/fbpB/ fbpC2	3804c/1886c/0129c	Fibronectin-binding protein ABC or antigen 85 complex

Table 1. Enzymes that participate in the FAS I and II pathways in *Mtb*.

has seven catalytic domains: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein, β ketoacyl reductase, and β ketoacyl synthase [12]. Fas (Rv2524) uses acetyl-CoA and malonyl-CoA as substrates for the synthesis of acyl-CoA derivatives of 16 and 18 carbon units which are in turn used for the synthesis of membrane phospholipids. FAS I route also produces an acyl-CoA derivative with 26 carbon units that becomes the short α-alkyl chain or α-branch of the mycolic acids. FAS I and II are connected by the synthesis of acyl-CoA derivatives with 20 carbon atoms that are used in the FAS II pathway as the starting molecule for the elongation of mycolic acids (reviewed in Ref. [14]).

There are important aspects to highlight regarding FAS I and II in *Mtb*. First, proteins FabD, AcpM, and FabH act in the transition between FAS I and FAS II, generating ACP derivatives (the substrate required for the FAS II pathway). Second, there are two known Claisen-type reactions occurring: one before the FAS II starts (responsible for the condensation of malonyl-ACP with acyl-CoA and catalyzed by FabH) and one shared with the polyketide synthase system (catalyzed by Pks13). The latter reaction generates a carbon-carbon bond between two activated fatty acids at the end of the mycolic acids synthesis. This second condensation takes the α-branch (produced through FAS I) and the longer meromycolate chain (produced through FAS I and II) to form a "pre-mature mycolic acid" [13, 14].

Regarding FAS II specifically, this pathway is involved in fatty acid elongation instead of *de novo* synthesis (contrary to what occurs in most bacteria, where FAS II has *de novo* synthesis capacity) [12]. *Mtb* needs to use both FAS I and II to generate its characteristic mycolic acids [13, 14]. Therefore, the study of mycolic acids synthesis is in fact a study of both FAS pathways in *Mtb*. In FAS II, there is one different enzyme for each specific step, allowing for various levels of regulation. Most of the core enzymes of FAS II are NADPH or NADH dependent and organized in different clusters distributed through the genome (**Figure 2** and **Table 1**). FAS II can be further divided into type I and type II elongation (E1 FAS II and E2 FAS II, respectively). Here, both types are catalyzed by the core proteins InhA, MabA, HadABC, and FabD, and elongation can be done by either KasA (E1) or KasB (E2). Despite the sequence homology between the condensases KasA and KasB, they are predicted to participate in two different stages during the FAS II pathway: KasA may catalyze the first elongation steps (E1-FAS II) while KasB might be involved in the later steps (E2-FAS II), ultimately producing full-length mycolates with more than 40 carbon units [12, 13]. A representation of matured α-mycolic acid is depicted in **Figure 2B**.

The meromycolate chain resulting from FAS II cycle can be "decorated" with chemical modifications such as cyclopropanations and methylations that are introduced before the second Claisen-type reaction occurs. These modifications can be at distal or proximal positions and are carried out by S-adenosyl-methionine (SAM)-dependent methyl transferases (**Table 1**). Unsaturations on the other hand, are proposed to occur differently under aerobic or anaerobic conditions. The method of double bond introduction in mycolic acid in *Mtb*, however, remains unclear. Under aerobic conditions, desaturases encoded by *desA1*, *2*, and *3* and other candidates such as Rv1371 are believed to complete the double bond introductions at the distal position, before the Claisen-type condensation take place. Under anaerobic conditions, unsaturations are believed to take place during the FAS II cycle in

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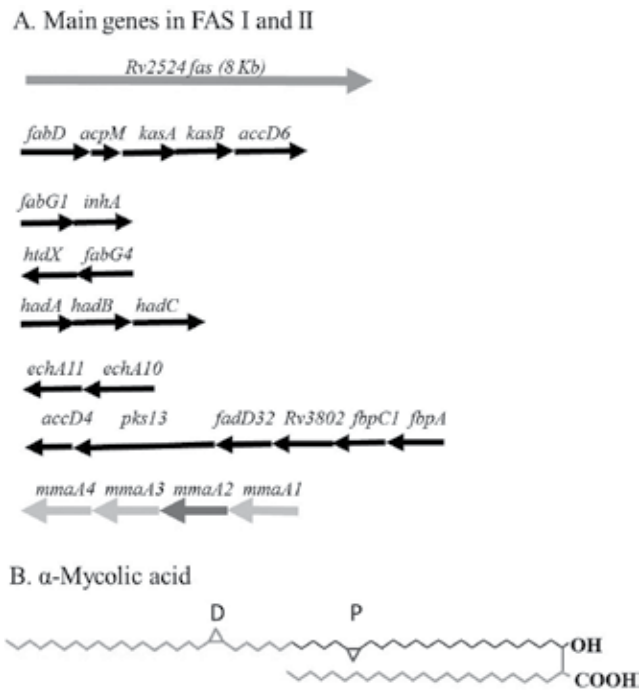

Figure 2. (A) Major operons involved in mycolic acid synthesis in *Mtb*. (B) Structure of an alpha-MA, the color represent the source of the carbon chain by either FAS I (light gray) or FAS II (black). P, proximal; D, distal.

the transition of the trans 2-enoyl intermediate to its 3-cis isomer in the distal position, resembling what FabM does in *Streptococcus pneumoniae* [13]. By sequence homology, this enzymatic reaction could be mediated by EchA10 and EchA11 in *Mtb*; however, there is not enough experimental evidence to support this hypothesis (**Table 1**). Finally, the oxygenated mycolic acids (keto and methoxymycolic acids) have a common precursor (hydroxymycolate) that is synthesized by the action of the SAM-dependent methoxymycolic acid synthase 4 (MmaA). The synthesis of methoxymycolic acid is additionally driven by the MmaA3 enzyme (**Table 1**) [12, 14].

After the modification in the meromycolate chain and the last condensation reaction occur, a mycolic acid (either α-, keto, or methoxymycolic acid) molecule is formed and can be attached to a trehalose molecule by the action of the Corynebacterineae mycolate reductase A, encoded by Rv2509 (also known CmrA) [15]. Once the mycolic acid is covalently linked with trehalose to form trehalose monomycolate (TMM), it is transported to the cell wall by the protein MmpL3 [16]. TMM is then the source of the mycolyl group for arabinogalactan and for other TMMs in the cell wall, generating trehalose dimycolate (TDM); in a reaction catalyzed by the fibronectin-binding proteins (Fbp) ABC ([17], reviewed in Refs. [14, 18]). Much of the understanding of the FAS I and II routes has been based on sequence homology with reference bacterial strains and mutation analysis using model organism such as *Mycobacterium smegmatis* and *Mycobacterium phlei*. Despite the vast knowledge about the mycolic acid synthesis pathway,

many unanswered questions remain regarding components of the FAS II pathway that are under current research [13].

2.3. Redox metabolism

In general, reduction-oxidation (i.e. redox) reactions are highly relevant for *Mtb*, since they not only comprise the necessary defence mechanisms developed to combat the host response during the infection, but they are also part of its own bacterial metabolism [19]. Redox reactions could generate endogenous or exogenous stress for the bacteria. The endogenous redox stress is generated during aerobic or anaerobic respiration, where *Mtb* is exposed to reactive oxygen (ROI) and reactive nitrogen intermediates (RNI), generated when the bacterium uses oxygen and nitrogen as the final electron acceptor in the electron transport chain, respectively [20, 21]. RNI can be also generated when *Mtb* relies on glutamate metabolism for survival. During host-infection, *Mtb* can experience a wide range of oxygen levels that can drastically alter its metabolism going from hyperoxic stress (when is in aerosol droplets) to low oxygen tension (during the intracellular phase in alveolar macrophages) to finally hypoxic to anoxic stress (in granulomas). Additionally, inside the macrophage, *Mtb* is exposed to both ROI and RNI. Hydrogen peroxide (H_2O_2) and the superoxide radical (O^{-2}) are the two most common ROI forms that are produced by macrophages and neutrophils to eliminate *Mtb* [22]. During hypoxic conditions, the alteration in redox homeostasis leads to a higher NADH/NAD^+ ratio which generate superoxide radicals that disrupt the redox balance in the cell. Consequently, enzymes with heme and sulfur complexes (i.e. cytochrome C, aconitase) can be severely affected. Therefore, the ability of *Mtb* to survive the redox stress from the host determines its success during the infection process. This stress has an impact on the bacterial metabolic pathways as well as on the expression of virulence factors [20, 21].

Intracellular or exogenously originated reactive oxygen species (ROS) and RNI have the potential to damage lipids, DNA, and proteins by oxidation, peroxidation, and nitration reactions [23], which can result in protein inactivation, and alteration of both cell organization and signal transduction. Therefore, it is crucial to successfully maintain redox homeostasis to keep the integrity of the cell. Intracellularly, the changes in the redox and nutrient levels are sensed by WhiB proteins (WhiB1-7) while extracellularly different molecules such as nitric oxide (NO), carbon monoxide (CO), and H_2O_2. The reduced and oxidized forms of the nicotinamide adenine dinucleotide (NADH/NAD^+) can work as sensors that induce a direct transcriptional response or indirectly alter transcription through a two-component regulatory system such as DosRS-DosRT [2, 21]. Moreover, different bacterial enzymes participate in the neutralization of the host-induced ROI and NOI such as superoxide dismutase (SodA), catalase-peroxidase (KatG), and the antioxidant complex formed by alkyl-hydroperoxidases (AhpC and AhpD), dihydrolipoamide acyltransferase (DlaT), and dehydrogenase (LpdC). Other enzymes in the redox metabolism include the peroxiredoxins (AhpE, TPx, Bcp, and BcpB) and thioredoxins (TrxA, B, and C).

Of these, KatG also plays a central role in *Mtb* resistance to INH. *Mtb* has only one single copy of *katG* with a coding sequence of 2223 base pairs (bp) generating a 704 amino acid protein with a molecular weight of approximately 80.6 kDa. KatG is presented as a dimeric haemoprotein that belongs to class I peroxidase superfamily, because of its high homology with yeast cytochrome C peroxidase [24]. KatG activates the prodrug INH, however its functions extends

beyond this activation. This enzyme is in fact one of the most important catalase-peroxidases that help the bacterium overcome external and internal redox stress. KatG possesses a monofunctional catalase, broad-spectrum peroxidase, and peroxynitritase activity [25, 26]. The catalase-peroxidase activity is in the N-terminal domain of the protein that contains a heme-binding motif, however, the C-terminus is also required for its catalytic function [24, 27]. KatG activity has also been associated with virulent *Mtb* strains, which are able to infect for longer periods and cause increased pathology in the host [28, 29].

As discussed above, redox reactions play an important role in bacterial respiration. In the next section, details about the cellular respiration process in *Mtb* are discussed. The relation with this topic and this chapter is based on the association of drug resistance mutations in important genes such as *katG*. The mutations in these redox homeostasis genes possibly generate an alteration in the respiration complexes in *Mtb* as well.

2.4. Respiration in *Mtb*

Given the dynamic *Mtb* lifestyle, respiration in the bacterium should be highly adaptable. Specifically, during respiration, *Mtb* uses oxygen and other compounds (such as fumarate or nitrate) as the final electron acceptor depending on the specific bacterial metabolic status and the surrounding environment [30, 31]. The respiratory apparatus is responsible for generating ATP and reduced coenzymes (NADH and/or $FADH_2$). Respiration is made possible by selected membrane-associated asymmetric complexes that allow for generation of proton motive force (PMF) and ATP, which are the major sources of energy in the cell. Different from other model organisms such as *E. coli* or *Bacillus subtilis*, *Mtb* obtains the majority of its ATP by the electron transport chain and the F_1F_0-ATP synthase machinery, with very little contributions from substrate level phosphorylation [31]. In fact, the ATP synthase is a recently successfully exploited target for developing anti-TB drugs of the drug class diarylquinolines such as the recently described clinical drug bedaquiline [31, 32]. Specifically, diarylquinolines interact with the transmembrane subunit C of the ATP synthase machinery [33]. This again emphasizes the importance of ATP synthase machinery in the respiration process in *Mtb*.

Most of the *Mtb* enzymes/complexes involved in aerobic respiration have been identified and are composed primarily of two NADH dehydrogenases (NDH-1 and NDH-2) and two terminal cytochrome oxidases (aa3-type cytochrome C oxidase and bd-type cytochrome oxidase). These enzymes participate in oxygen reduction and are coupled to generate the PMF that is used by the ATP synthase for the production of ATP. NDH-1 is encoded by the *nuo* operon (*nuoA-N*) and NDH-2 is present in two copies encoded by *ndh* and *ndhA*. Previous studies demonstrated that NDH-2 does not have a proton-translocating-function and is the main dehydrogenase in *Mtb*. NDH-2 reduces menaquinone to menaquinol that in turn can be oxidized by one of the terminal aa3-type cytochrome C oxidase and bd-type cytochrome oxidase complexes. Because the bd-type cytochrome oxidase (CytA-B) is not coupled to proton pumping, the direct oxidation of menaquinol by this oxidase is less energetically efficient compared to the aa3-type (CtaC-F). Instead, the oxidation of menaquinol can happen in a two-step process with the participation of the cytochrome bc1 complex (QcrA-C) and the terminal aa3-type cytochrome oxidase (CtaC-F) with a higher energy yield [31, 32].

Contrary to aerobic respiration, mediators in *Mtb* anaerobic respiration are poorly defined. However, *in vitro* hypoxic studies have allowed the identification of some important enzymes involved in this process. In a reduced-oxygen environment, the nitrate reductase (NarG-I), the nitrate transporter (NarK-2), and the NDH-2 dehydrogenase are upregulated. On the other hand, the ATP synthase subunits and the aa3-type cytochrome oxidase are downregulated. During a low oxygen tension, the bd-type cytochrome oxidase is believed to be more utilized since it has a higher affinity for oxygen. ATP synthase is still active although at a lower membrane potential not commonly seen in other organisms, underlining the importance of PMF in keeping the bacterium alive during this metabolic state. This could be a regular scenario for *Mtb* inside the granuloma driving low metabolic activity with low or no *Mtb* growth (dormancy) [22]. Also, in the absence of oxygen, *Mtb* uses a set of reductases (such as succinate/fumarate reductase and nitrate reductase), hydrogenases (coupling H_2 oxidation to respiration, encoded by Rv0082 and Rv0087), and ferredoxins (such as the encoded by *fdxA*) that preserve the PMF for bacterial survival [30, 31]. Other changes have been detected in anaerobic adaptation, for instance, the E1 subunit of the pyruvate dehydrogenase is upregulated. Under anaerobic conditions, *Mtb* can stay alive but its growth is strongly reduced [31]. This theme is relevant because as it was previously described, there is a wide variety of oxygen tension in the *Mtb* interaction with the host.

2.5. *Mtb* envelope

Moving to another important aspect of *Mtb* physiology, the cell envelope of this bacterium has been the focus of research for many decades because of its distinct features, importance in bacterial pathogenicity, and the generation of the host immune response. The mycobacterial cell envelope is complex such that nutrients penetrate 10,000 times slower than they can do in the *E. coli* outer membrane [34]. Components of the cell envelope, particularly the enzymes that participate in their synthesis, have been recognized as possible drug targets. The understanding of the cell envelope is also required to design drugs that will be able to cross this impermeable barrier efficiently [35].

The *Mtb* envelope forms the interface between pathogen and host. From the outside to the inside, the *Mtb* cell envelope is composed of a layer of non-covalently linked glycolipids, proteins, carbohydrates, and some lipids (the capsule), a covalently linked peptidoglycan layer that contains carbohydrates and lipids (the cell wall), and a plasmatic membrane (phospholipid bilayer). In 1991, Minnikin proposed visualizing the lipid material in the *Mtb* envelope as two distinct membranes, analogous to a Gram-negative bacterium [36].

The most external layer of *Mtb* has been described as a "capsule" by some scientists. This layer contains mainly polysaccharides and a small amount of lipids (2–3%). The major capsular component in slow growing mycobacteria, including *Mtb* is a glucan composed of repeating units of ->4-(-D-glucosyl residues substituted at position 6 with a mono- or oligoglucosyl residues). The capsular material also contains the heteropolysaccharide D-arabino-D-mannan and a mannan chain composed of ->6-(-D-mannosyl-1-> core with substitutions at some positions 2 with a (-D-mannosyl unit. Finally, the arabinomannan found in this extracellular material is "decorated" by other oligomannosides, which can be also secreted to the extracellular space [35], reviewed in

Ref. [37]. Glycolipids such as trehalose monomycolate (TMM) and trehalose dimycolate (TDM); phenolic lipids and glycopeptidolipids can be found in the outer part of the capsule and some of them are also cell wall-associated. TDM is also known as cord factor since it causes grow in "cords" *in vitro*. This particular glycolipid has been associated with the pathogenesis and immunogenicity of *Mtb* strains [38]. Lipoproteins such as LpqH (Rv3763), proteins such as Psts1 (Rv0934) and the Ag85 complex (FbpA, Rv3804; FbpB, Rv1886; and FbpC, Rv0129) are also commonly found in the capsular material [35].

The *Mtb* cell wall has a covalently linked backbone with a collection of cell wall-associated lipids and polypeptides. The covalently linked molecules include peptidoglycan, arabinogalactan, and mycolic acids. In addition to the presence of the last two biomolecules, there are two important hallmarks of the *Mtb* cell wall. First, the muramic acid is N-acylated, instead of N-acetylated as regularly observed in most eubacteria. Second, there are unusual cross-links between two chains of peptidoglycan that include bonds of two residues of diaminopimelic acid in addition to the usual D-alanyl-diaminopimelate linkage. Furthermore, mycolic acids represent about 40% of the cell wall [39, 40].

Mtb has a great variety of lipids that can be clustered into at least six lipid categories with around 2512 lipid groups [41]. Mycolic acids are the major constituent of the cell envelope. They were first named by Stodola and colleagues in 1938, who also depicted essential groups of their chemical structure. Mycolic acid structure was further defined by Asselineau in 1950 [42]. These are α-alkyl, β-hydroxyl, long-chain fatty acids that can be primarily covalently attached as esters of arabinogalactan in the cell wall or as "free lipids" in the capsule associated to trehalose in the TMM or TDM structures [12, 43]. Specifically, mycolic acids form an ester bound to the 5-position of the arabinose residue of the arabinogalactan [41]. Mycolic acids can also bind to glucose [44]. The covalently attached mycolic acids can be obtained by saponification or methanolysis of the cell wall of the delipidated *Mtb* cells. Because mycolic acids are not soluble in methanol, they can be separated from moderately long-chain fatty acids with ether or chloroform solutions. Mycolic acids have one carbon chain bound to the hydroxyl group called the meromycolic chain and another (shorter) carbon chain that is bound to the α-carbon [35]. The synthesis of these molecules was previously discussed in Section 2.2.2 of this chapter.

Mycolic acids are not unique structures of the *Mycobacterium* genera, they can be present in *Corynebacterium*, *Nocardia*, and *Rhodococcus*. Mycolic acids from *Mycobacterium* are longer in carbon units (C70–C90) and have the largest meromycolic chain [39]. Additional modifications such as the introduction of cyclopropane rings in the meromycolate chain, unsaturations, ethylenic groups, and methyl branches are also observed. Both *cis* and *trans* double bounds as well as cyclopropane rings can be found in the same type of mycolate. Some mycolic acids have additional oxygen functionality that is one feature used to classify them. These functionalities are keto, methoxy, carboxy, and epoxy. Other types of mycolic acids lack of these oxygen groups, they are called α-mycolic acids and α'-mycolic acids. α and α'-mycolic acids differ in their chain length, α'-mycolic acids are shorter (usually of 60 carbon units) whereas α-mycolic acids contains more than 70 carbon units. α-mycolic acids represent more than 70% of the total mycolic acids found in *Mtb*, followed by keto and methoxy variants (15 and 10%).

The cyclopropane structures in this fatty acids contribute not only to its cell wall structure, but also protect the bacteria from oxidizing agents such as H_2O_2 (reviewed in Ref. [14]).

Finally, the plasmatic membrane includes different types of phospholipids such as phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides (PIMs). PIMs are mainly located in the outer leaflet. Other important components are the highly immunogenic lipoglycan lipoarabinomannan (LAM) and lipomannan [39]. Due to the high abundance of LAM in the *Mtb* envelope, it has been tested as a biomarker for a point of care test with a wide range of sensitivity and specificity results in HIV-positive patients [45].

3. Mechanisms of drug resistance in *Mtb*

As is the case in other microorganisms, drug resistance in *Mtb* can be either intrinsic or acquired. *Mtb* cell wall structure and its low permeability are the major factors accounting for the high degree of intrinsic or natural tolerance to many antibiotics and other chemotherapeutic agents. Highly abundant mycolic acids in the cell wall reduce the cell permeability and create a crystalline-like structure after the cytosolic membrane. As seen in other mycobacterial species (especially in saprophytic species such as *Mycobacterium chelonae*), the more impermeable the cell wall, the more antimicrobial agents the mycobacteria can resist. Drugs such as sulphonamides, penicillin, tetracycline, and vancomycin are ineffective against *Mtb*. For vancomycin, this can explained because of its size and structure that do not allow its effective penetration through the *Mtb* "pseudo-outer membrane" [34]. However, recent findings demonstrated that *Mycobacterium bovis* and *Mtb* mutants lacking phthiocerol dimycocerosates are susceptible to glycopeptides such as vancomycin [46]. Additionally, the reduced number of porins in the *Mtb* "pseudo-outer membrane" possibly contributes to the intrinsic *Mtb* resistance against hydrophilic compounds. Among other intrinsic factors, *Mtb* possess β-lactamase enzymes (encoded by *blaC* and *blaS*) that make this bacterium naturally resistant to β-lactams [2, 47]. For acquired drug resistance, spontaneous mutations in chromosomal genes during a suboptimal drug therapy are the most common cause for drug resistance in *Mtb*. Efflux mechanisms are less common but also present in these bacteria [47]. These intrinsic and acquired mechanisms have synergistic effects and make TB treatment particularly cumbersome.

Although a combined therapy for TB is normally effective for most cases, TB cases resistant to a subsection or all anti-TB drugs have been reported in clinical settings. Because INH and RIF are the most widely anti-TB drugs used, there is a higher frequency of mono-resistance to any of these drugs or to both drugs (INH and RIF, known as multidrug-resistance TB or MDR-TB) among drug-resistant *Mtb* strains. The study of drug-resistant TB has been an ongoing process, mainly because the understanding of the mechanism of action of several first line drugs (such as INH and pyrazinamide) has been subject of intensive research and controversies [48]. The cumulative exposure of *Mtb* strains to suboptimal concentrations of anti-TB drugs in an intermittent manner creates most of the acquired drug-resistant TB cases. In this way, many TB patients lose the best options for effective treatment from a disease that was initially curable.

3.1. INH resistance

In 1951, the anti-TB properties of a new drug, INH, were reported. This was a critical event in TB history that was optimistically described as the "new treatment for the white scourge." Unfortunately, the appearance of INH-resistant (INHr) cases emerged the same year INH was introduced in medical practice [49]. INH resistance is one of the most common forms of drug-resistant TB. The resistance mechanism to this drug is multigenic and can be divided into three categories: prevention of drug activation, alteration of the target, and differential expression of the target. In the first group, mutations in *katG* that prevent the activation of INH are present in the majority of resistant cases to this drug [50, 51]. *Mtb* strains with a full deletion of *katG* also fall into this category. KatG function was first correlated to INH resistance in 1953, when Middlebrook et al. discovered that INHr *Mtb* strains lacked catalase-peroxidase activity and were less virulent in guinea pigs [52]. The molecular validation of this observation was completed later by Zhang et al., restoring the sensitivity to INH in some *Mtb* resistant strains after the introduction of the *katG* gene from *E. coli* [53]. More than 60 years of chemotherapy with INH in TB cases has allowed the development of different *Mtb* genotypes of INH-resistant (INHr) profile and their associated phenotypes. Currently, there are more than 300 known mutations in the *katG* gene alone associated with a wide range of minimum inhibitory concentrations (0.2–256 mg/L) [51]. These mutations include missense mutations, insertions, deletions, truncations, and full gene deletion. Depending on the position and nature of the mutation, *katG* mutants have different degrees of catalase-peroxidase activity [47, 51]. The mutation rate for the generation of INH-resistant strains is around 3.2×10^{-7} mutations/cell division (after exposure to 1 mg/L INH) *in vitro* [54–56] and presumably one in 10^{8-9} organisms *in vivo* [57].

In the category of alteration of the target and increased expression of the target, mutations in the *inhA* gene or its promoter are accounted. InhA is the most commonly validated target for INH [49, 58]. Currently, around 15 mutations in the *inhA* gene have been identified in *Mtb* strains with low-level resistance to INH. *inhA* mutations also drive resistance to ethionamide (ETH), since INH and ETH share this enzyme as target [51]. The most studied mutation is the S94A that results in the reduction of the enzyme affinity for NADH and a reduced ability of INH-NAD adduct to inhibit the enzyme. Additionally, mutations in the *inhA* promoter that increase InhA levels have been also identified. Therefore, both the reduction in enzymatic activity, specifically KatG and the overexpression of the target (InhA) serve as resistance mechanisms against INH. Other mechanisms of INH resistance include the accumulation of NADH (by redox alteration) that binds InhA and protects it from the inhibitory effect of the INH-NAD adduct. An additional resistance mechanism includes acetylation of the drug by the *nat* encoded arylamine N-acetyl-transferase which prevents INH activation by KatG [59, 60]. Finally, the drug efflux mechanisms include the participation of the protein EfpA, which is induced upon INH treatment [49]. It is important to describe that INH resistance in *Mtb* can be either low- or high-level when there is >1% of bacterial growth in the presence of 0.2 or 1 μg/mL of INH, respectively. Regularly, mutations in the *inhA* promoter are linked to low-level of INH resistance while mutations in *katG* are associated with high-level of INH resistance in *Mtb* [61].

3.2. RIF resistance

Followed the discovery of INH, rifampicin (RIF) was discovered in 1963 and reduced the anti-TB treatment from 18 to 9 months [62–64]. Currently, a shorter combined therapy with higher doses of rifampicin or isoniazid is being evaluated [65]. The rationale behind the increase dose of rifampicin is that the currently used dose of RIF was proposed in 1971 with the basis of generating a cost-effective treatment that was non-toxic for TB patients, albeit a study of the maximum dose of the drug tolerated in human has never been performed [66]. Recent studies in animal models have shown that higher doses of this drug could be effective even in shorter regimes, reducing also the probability to generate resistant microorganisms to the drug [66–68].

RIF resistance in *Mtb* is simpler than INH resistance. Up to date, mutations in one gene, *rpoB*, that encodes for the RNA polymerase β subunit and the target of the drug, are present in most of the RIF resistant (RIFr) cases. There are only four in the *rpoB* gene (N-terminus, and clusters I–III) where most of these mutations are found. In fact, mutations in an 81 base pair (27 codons) in the central region of cluster I, also known as the RIF resistance-determining region (RRDR), harbors more than 96% of all mutations associated with RIF resistance. Similarly to what is described for INH resistance, these mutations can be single amino acid substitutions, deletions, and insertions [62]. These mutations mainly affect the binding pocket where the drug interacts with the subunit of the polymerase. The most common amino acid substitutions observed in clinical RIFr strains include S531L and H526Y [69].

4. Impact of drug resistance in *Mtb* physiology as seen through proteomics perspective

Since there is a wider repertoire of INH resistance-conferring mutations compared with RIF resistance-conferring mutations (see Sections 3.1.1 and 3.1.2), a more variable phenotype in INHr strains compared to RIFr strains is expected. Additionally, the genetic lineage and background of each strain play an important role in the phenotype resultant after drug-resistance is acquired [69–71]. This is explained by the fact that compensatory mutations associated with some genetic backgrounds but not others may results in different competitive phenotypes. Our laboratory recently demonstrated that the same mutation causing INH resistance in two *Mtb* strains from different genetic lineages can result in different virulent phenotypes. Furthermore, these differences were associated with differences in protein levels of AhpC without any detectable mutation in the *ahpC* gene or its promoter. These *Mtb* strains were from different genetic lineages and exhibited a strongly different virulent profile in the mouse model of infection [72]. Therefore, following a "conservative approach," comparing clinically relevant clonal or isogenic *Mtb* strains is crucial to understand the changes in *Mtb* physiology caused by drug resistance events. However, obtaining pure clonal pairs of *Mtb* derived from clinical settings is quite challenging.

Clonal *Mtb* pairs conceptually defines a pair or group of bacterial strains that share the same progenitor, but are generated after successive replication events with the possibility to develop

one or more single nucleotide polymorphisms (SNPs) each time, possibly due to external pressure such as drug exposure, oxygen tension among other factors [73]. The development of more discriminative and high-throughput genetic tools has allowed a more accurate characterization of these clonal and isogenic strains. Isogenic and clonal strains are difficult to obtain from clinical cases due to the possibility of infection with different clones of *Mtb*, especially in high burden TB countries such as India and South Africa [74]. Furthermore, most settings with high burdens of TB do not routinely perform whole genome sequencing and are not equipped to carry a biobank of *Mtb* isolates. In the next sections, we will explore specific examples of *Mtb* strains that experienced compensatory physiological events after acquiring INH and/or RIF resistance comparing them to their clonal or isogenic parental strain.

We have used comparative shotgun proteomics of different *Mtb* cellular fractions to describe different aspects of the *Mtb* physiology *in vitro* and *in vivo*, including the effects of drug resistance-conferring mutations in the new bacterial phenotype [75–82]. The advantage of evaluating differences in protein abundance at each cellular fraction allows confirming if any differences seen are due to a global redistribution of protein levels or if changes in protein abundance are instead associated with a specific compartmentalization of the protein. After the elucidation of the genome of many organisms, proteomics emerged as a powerful methodology that not only describes the sequence, structure, and function of the proteins, but also extends to the analysis of complex mixture of proteins using high-throughput techniques [83, 84]. Proteomics analyze mature proteins considering all the complex post-translational events that occur in the cell and that finally represent the bacterial phenotype. As it was stated by LaBaer in 2011, "proteins provide the verbs to biology" [85, 86], and proteomics allow for naming different biological events [87]. As the proteome of the cell variate parallel to internal metabolic variation and external cues, proteomics is considered the most direct scaffold to measure cell activity [86]. Mass spectrometry (MS)-based technologies are central components of the protein analysis. These methods include shotgun and targeted proteomics that have different modes for acquiring mass spectra. Shotgun proteomics, a term coined by John Yates III and his laboratory, offers an indirect measurement of proteins through peptides derived from their enzymatic digestion [84]. Shotgun proteomics, also known as discovery proteomics, uses liquid chromatography (LC) connected to tandem MS (MS/MS) for the identification of the protein components in the sample. The protein identification is based on the determination of the amino acid sequence which is achieved by comparing the experimental tandem mass spectra with the theoretical tandem mass spectra generated from an *in silico* digestion of a protein database.

4.1. "The isoniazid resistance case": findings from *katG* mutant *Mtb* strain of the Beijing lineage through proteomics

Given the high frequency of *katG* mutations among INHr *Mtb* strains, this section will focus on the proteomics findings that were revealed in the study of an isogenic pair of the Beijing lineage after acquisition of drug resistance due to a *katG* mutation [80]. As the starting point, it should be noted that early studies revealed that INHr *Mtb* strains with *katG* mutations have different levels of the enzyme and a different degree of alteration of its catalase or peroxidase

activities [88]. These mutations have also different impact in the virulence and fitness of the INHr bacterium. However, to our knowledge, the study described here is unique as it used clinical isogenic pairs of *Mtb* strains resulting from *katG* mutations and associated with an INHr profile.

Consistent with previous studies, the global proteomics study of the Beijing clinical pair through LC-MS/MS demonstrated that the INHr strain had significantly reduced levels of KatG in three of the four subcellular fractions evaluated compared with its isogenic INHs progenitor. The fact that the levels of this protein were reduced in the soluble fractions (cytosol and secreted proteins) and the bacterial membrane is a clear indication that this INHr strain lacks its ability to activate INH. An additional 45 proteins were found with altered abundance; these protein changes may be a potential compensatory mechanisms related to the reduced KatG levels and its consequent impact on mycobacterial physiology and fitness [80].

Among the 45 proteins identified, proteins related to intermediary metabolism and respiration represented majority of differentially abundant between INHr and INHs strains. Among them, enzymes from the tricarboxylic acid (TCA) cycle (SucC, SucD, Mdh, Acn, and AceE) were all decreased in the INHr strain. Proteins related to lipid biosynthesis and degradation pathways also represented important differences between the strains, with mainly higher levels in the INHr strain. The proteins Fas, FabG4, and FbpD of the lipid biosynthetic pathway were increased. In the β-oxidation pathway, the dehydrogenases FadE22 and FadE32 and the acetyl-CoA acyltransferase FadA2 were increased, but the crotonases EchA9 and EchA21 were decreased in the INHr strain. Proteins in the virulence and detoxification category such DnaK and GroES were also increased in the INHr strain as well as the hypothetical protein Rv2204c. Finally, the transcription regulation proteins Crp and PrrA were also higher in the INHr strain compared to the INHs parental strain [80].

Interestingly, the INHr Beijing strain had the *katG* mutation L101R (identified in the INHr by whole genome sequencing) [89]. However, this *katG* mutation was not very stable for the Beijing INHr strain, which after successive passes reversed to the wild type genotype and INHs phenotype. A previous report of an INHr reversion in *Mtb* was observed in a *katG* mutant in the absence of the drug pressure [90]. Based on these reports, it is possible that not only the resistant-conferring mutations can result in a distinctive phenotype but also that these mutations are not easily conserved in the *Mtb* genome after removing the pressure that originates them.

A previous proteomic analysis using non-clonal *Mtb* strains found five proteins overexpressed in the INHr strains comparing whole cell lysates. These proteins were found through two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption ionization time of flight-MS (MALDI-TOF) and include OpcA, FixB, RegX3, a probable oxidoreductase (Rv2971), and Wag31. Most of these proteins were involved in cellular metabolism, including redox metabolism (such as OpcA, Rv2971, and FixB) and there was one transcriptional regulatory protein (RegX3). These proteins are not related to any of the known INH-resistance mechanisms and were not observed in the previous clonal study. However, they still confirm the alteration of proteins involved in redox stress and energetic metabolism [91].

A recent virulence study of laboratory and clinical clonal pairs of *Mtb* from the T lineage and with different susceptibility profiles to INH also showed an important reduction of the KatG protein in the INHr strains. Associated with this KatG reduction, this study revealed a variable alkyl-peroxidase C (AhpC) response in the INHr strains which was dependant on the genetic background. Although both clinically and laboratory-derived INHr *Mtb* strains had reduced levels of KatG, western blot analysis with anti-AhpC demonstrated that the laboratory INHr strain had increased levels of AhpC while the clinical INHr strain had reduced levels of AhpC compared to their clonal parental strain, respectively. The difference observed in the AhpC levels was also translated in a non-significant reduction of the virulence in the laboratory INHr contrasting the strongly significantly reduced virulent profile for the clinical INHr strain [72]. A more robust proteomics study trough LC-MS/MS is being developed to reveal more insights about the proteomics differences among this clinical and laboratory-derived clonal pairs.

4.2. Acquisition of rifampicin (RIF) resistance in isogenic *Mtb* strains of the Beijing and Haarlem lineage

Phenotypic consequences of mutations in the *rpoB* gene associated with RIF resistance are understudied in *Mtb*. However in recent years, this theme has gained interest given the association of *rpoB* mutations with a variety of phenotypes in other microorganisms. For instance, in *E. coli*, *rpoB* mutations mimic the "stringent" response that is usually driven by ppGpp under stress conditions [92]. In *B. subtilis* and *Streptomyces coelicolor*, *rpoB* mutations are associated with an increased antibiotic production and increased production of other metabolites [93–95]. In *Neisseria meningitidis* and *Staphylococcus aureus*, *rpoB* mutations lead to a decrease permeability of the cell wall, which can be related to a subsequent increase in tolerance to certain antibiotics such as vancomycin [96–98]. Interestingly, after exposure to RIF, *Mtb* also appears to have an increased tolerance to ofloxacin, probably because of an increase activity of efflux pumps [99], although recent findings from our laboratory as well as others also suggest a potential role for cell permeability [75, 100]. In our study, isogenic *Mtb* pairs with two different *rpoB* mutations and representing two different genetic lineages (Beijing and Haarlem) showed an increased abundance of proteins involved polyketide synthesis. Proteomics findings were confirmed by an independent transcriptomics analysis of the strains grown intracellularly in *in vitro* macrophages. Both RIFr *rpoB* mutants revealed significant increased expression of multifunctional enzymes of the phenolpthiocerol synthesis type I polyketide synthase PpsE and C, which are involved in the biosynthesis of phthiocerol dimycocerosate (PDIM) and other lipids in *Mtb* [75]. We also observed a significantly increased abundance of the ABC transporter *drrA*, which has homology with other daunorubicin efflux pumps, but it is also implicated in export of PDIM across the cell membrane [101, 102]. Both increased abundance in lipids, as well as potential increase in efflux pump activity may result in accumulative reduction of cell permeability and may have important implications in subsequent acquisition of drug resistance.

4.3. Study of multidrug resistance in *Mtb* trough proteomics

A handful of proteomic studies focused on the comparison of drug susceptible (DS) versus multidrug-resistant strains (MDR) *Mtb* strains are available in the literature [70, 103–107].

Although these studies analyze clinical DS and MDR *Mtb* strains, the majority of them were comparing either non-related strains or strains with specific different genetic lineage. For instance, one study included H37Rv and H37Ra in the comparison and other compared DS Central-Asian (CAS)-2 with MDR East-African Indian (EAI)-3 strains [104]. The latter did not allow the study of the MDR phenotype under the same genetic background. There was one study that evaluated *Mtb* strains isolated from one single patient after many treatment failure episodes. Here, we will explore the findings specifically related to the DS and the first MDR strain isolated, since the next *Mtb* strains were also resistant to kanamycin. The MDR strain of the CAS 1-Delhi genotype had increased levels of 10 proteins through 2D electrophoresis and MALDI-TOF compared to its DS clonal pair. These proteins include chaperonin Hsp70, bacterioferritin BfrA, mycolyl-transferase FbpD, a component of the translational apparatus GatA, the phosphoserine aminotransferase SerC, Wag31, and the hypothetical proteins Rv1827, Rv2204c, Rv0543c, and Rv2004c [103]. Interestingly increased levels of FbpD and Rv2204c were also found in the INHr study of Beijing lineage [80]. Similarly, protein Wag31 was increased in a previous proteomic study monoresistant *Mtb* strains.

The proteomic analysis of non-genetically related *Mtb* strains revealed commonly increased levels of GroEL2, DlaT, ESAT-6, and conserved protein Rv3699 in the MDR strains compared to DS strains [70, 105–107]. Similar to the previous INHr proteomics studies mentioned above, some studies showed increased levels of FadA2, FabG4, BfrA, GroES, FixB, Rv2971, OpcA as well as lower levels of Mpt63 in MDR versus DS *Mtb* strains. However, there were contrasting levels of the proteins Mdh and SahH among the MDR studies and also discrepant tendencies of Fas in MDR strains compared to the INHr study of the Beijing genotype. On the other hand, there was one study that found increased levels of PpsC in a MDR strain compared to H37Rv as it was described in the RIF resistance proteomics study of isogenic pairs of Beijing and Haarlem genotype.

The analysis using non-genetically related strains provide valuable insights about the protein dynamics among DS and MDR *Mtb* strains. However, the fact that proteins such as the catalase-peroxidase KatG are increased in MDR strains without establishing the INH-resistance mechanism [104, 107], generates some questions such as: Is this protein increase because some genotypes express constitutively more KatG? According to this, it is not possible to conclude that there are actually INHr strains that have increased levels of KatG and highlight the necessity of study the drug resistance event under the same genetic background.

5. Lipidomics studies in *Mtb* drug-resistant strains

5.1. Lipidomics in INHr *Mtb* strains

Among the different scientific disciplines supporting biological research, metabolomics is the study of chemically diverse groups of biomolecules including sugars, nucleotides, peptides, lipids, among others; using technologies such as MS and nuclear magnetic resonance (NMR). Lipidomics is a branch of metabolomics that specializes on the water-insoluble metabolites—lipids. These are diverse metabolites that are part of the major molecules in the cell (particularly, in the cell membrane) [108]. In *Mtb*, lipids are a very relevant group of molecules, since

at it has been previously discussed, they are responsible for the intrinsic-drug-resistant nature against some antibiotics, and its synthesis has been the target of some anti-TB drugs (INH and ETH). Consistent with this idea, it is plausible to think that the study of the *Mtb* lipid is an important part of the description of drug-resistant *Mtb* strains.

Thus far, only one metabolomics study has been reported comparing *katG* mutant-INHr strains derived from a drug susceptible parental strain of the Haarlem genotype. Through 2D-gas chromatography-TOF MS, this study showed increased levels of saturated fatty acids (FA) in the INHr strains; particularly saturated FA with 16–20-carbon chain compared with its wild type that could be as a result of the oxidative stress which makes the bacteria rely on the β-oxidation of fatty acids as a carbon and energy source [109].

5.2. Lipidomics in RIFr *Mtb* strains

The lipidomics studies in RIF resistant *Mtb* have been focused on the RIF resistant strains that are both laboratory and clinically isolated. The laboratory-derived W-Beijing and CDC1551 were used as the parental DS strains and were exposed to 2 ug/mL RIF to select for the RIFr strains. In this way, three different *rpoB* mutants (S531L, Q513E, and H526Y for each *Mtb* strain) were studied. The analysis revealed reduced levels of di-acylated sulfoglycolipid (Ac2SGL) and mycobactins (including carboxymycobactins), while increased levels of PDIM compared to their DS parental strain. These compounds were identified among 172 features in the W-Beijing group and 102 features in the CDC1551 group analyzed by high performance liquid chromatography (HPLC) mass quadrupole-time-of-flight (QTOF) MS and suggesting a global remodeling of the cell wall after acquisition of RIF resistance [100]. This study supports previous findings from our group, which included a significant increase of diacylglycerol phosphocholines and PDIM precursors as observed by ultra-performance liquid chromatography (UPLC)-QTOF [75].

6. Closing remarks

The systematic study of *Mtb* phenotype, its proteome and metabolome (including, but not limited to lipidome) permits a functional description of how *Mtb* adapts, and sometimes thrives, under intrinsic (i.e. host response) and extrinsic pressure (i.e. exposure to drugs). These types of studies help to resolve not only the features of drug-resistant strains, but also contribute to the discovery of the facile and specific detection of biomarkers of drug resistance and ultimately contribute to the discovery of new targets for these *Mtb* strains that are hard to eliminate and often result in poor clinical outcomes for those infected.

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Chapter 8

Web Resources on Tuberculosis: Information, Research, and Data Analysis

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Additional information is available at the end of the chapter

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Abstract

Since the first edition of this book in 2013, many new tools and databases have become publicly available, as well as several have been discontinued. Here, we present an updated version of web resources on tuberculosis, providing more detailed information on some key concepts. However, the purpose of this chapter is by no means to offer an exhaustive list of all the resources available on the Internet about TB, the topic of this book. This would be a massive and perhaps futile work since the evolution of the Internet occurs at a very fast pace. Rather, this chapter concentrates on a selection of the most important, relevant and stable websites with relevance to several aspects of TB, such as research, treatment, main institutions, funding, and specialized platforms. We think this should complement all the other information already presented in this book, offering the reader a more integrated view of the disease, as well as access to new platforms and systems specialized in the analysis of data generated by a series of new technologies such as DNA sequencing.

Keywords: tuberculosis, omics, bioinformatics

1. Introduction

The Internet has quickly become the universal exponent of the digital world as we know it. Today, billions of people around the world use the Internet as their primary source of information and benefit from electronic mail systems, information distribution, file sharing, multimedia streaming services, and online social networking, to cite just a few examples. With the popularization of smartphones, access to the Internet has become even more frequent, and many people remain connected all the time. This medium is an unprecedented form of

communication in the history of humanity, capable of bringing information from virtually anywhere almost instantaneously.

Scientists have been pioneers in benefiting from global interaction and globalized information. Nowadays, we deal with algorithms, data mining, terabytes and petabytes of data, teraFLOPS and petaFLOPS to measure computer performance, distributed computing, cloud computing, and terms and aspects of what we call big data, referring to data sets with huge sizes, exceeding the capability of commonly used software tools to manage and process these data within a feasible time frame. This kind of data stems from a variety of new (as well as old) high-throughput technologies, employed by researchers in different fields, such as astronomy and (recently) biology, as well as from information technology companies, such as Amazon, Facebook, and Google, among many other examples. For instance, the Large Hadron Collider, a particle accelerator at CERN, Switzerland, generates data on the order of terabytes per second.

However, the global connection is not restricted to the virtual world: with the ease of intercontinental travels, infectious agents can traverse the whole world in one single day. Tuberculosis (TB) is a disease considered to be at high risk for people who maintain prolonged and repeated contact with the host. Therefore, the risk of infection when traveling is reduced, but some conditions might present as a risk factor. Immunocompromised travelers (whether by the disease itself or by medication), smokers, children under 5 years old, and healthcare workers are particularly vulnerable to TB. Before traveling, the individual should consult a specialist to assess the risks of contracting TB in the area of stay, checking the incidence of TB and, especially, of resistant strains. Prophylactic measures should be taken when necessary, and medical follow-up should be provided when returning from the trip. These procedures are critical to containing the global circulation of TB strains [1].

TB is a pandemic disease, with an estimated one-third of the world's population contaminated by the bacillus *Mycobacterium tuberculosis* (Mtb). Although treatable, patients without proper follow-up usually abandon the therapy as soon as they feel better. This and the indiscriminate use of antibiotics are causing the emergence of new drug-resistant strains, with patterns of epidemic or outbreak resembling the dissemination of a new piece of information throughout the Internet.

On the other hand, there has also been a revolution in other areas. High-performance technologies, such as genomics, transcriptomics, proteomics, and metabolomics, for instance, offer a new and more integrated view of the genetics and metabolism of targeted organisms. Currently, thousands of mycobacterial genomes are already sequenced or are in progress. Hence, for example, comparing genomes of virulent and non-virulent strains of Mtb, scientists can pinpoint genes and/or polymorphisms possibly involved in pathogenesis; similarly, analyzing transcriptome data, researchers might gain an idea of the effects caused by a given drug in the bacillus' metabolism.

Since the Internet content is continually updated and changed, providing an exhaustive list of all the resources available online concerning TB would be a cumbersome and perhaps futile work. Hence, in this chapter, we present a selection of the most relevant and stable websites

exploring several aspects of TB, such as treatment, research, and funding, or providing access to analytical tools, databases, or specialized platforms for mycobacterial research. We hope this chapter can offer the reader an overview of online, publicly available, computational resources that could help us to fight TB.

2. Bioinformatics and omics

2.1. Bioinformatics

Bioinformatics is an interdisciplinary field that involves distinct areas, such as biology, computer science, mathematics, and statistics, comprising an extensive list of activities, such as research, development, or application of computational techniques or tools to acquire, store, organize, analyze, visualize, and integrate biological, medical, behavioral, or health data and information. Bioinformatics addresses scientific questions imposed by biological phenomena applying several analytical approaches such as image processing, computational simulations, network analysis, and data mining, among others, to perform comparative genomics studies, gene expression analysis, structural protein analysis, phylogenetics, and metabolic networks, just to cite a few examples [2].

However, bioinformatics preceded the term, as its origins can be traced back to the early 1960s when computers became essential tools in the field of molecular biology, as well as in all other research areas [3]. At that time, Margaret Oakley Dayhoff, a biochemist with a PhD degree in quantum chemistry, pioneered the development of computer methods for comparing protein sequences and for inferring evolutionary histories based on protein sequence alignments. Among many other significant scientific contributions made during her career, she cataloged all known protein sequences and made them available to the scientific community, publishing in 1965 the *Atlas of Protein Sequence and Structure*, containing sequence information on 65 proteins, considered the first molecular biology database [4].

Indeed, toward the end of the 1960s, several algorithms and computer programs were already available for analyzing structure, function, and evolution of nucleotide and protein sequences, as well as rudimentary protein databases [3, 5]. In the following decades, new computational methods and approaches were introduced such as algorithms for sequence alignments, public domain databases, efficient data search and retrieval systems, sophisticated methods for protein structure prediction, tools to automatically annotate genes and genomes, and systems for functional genome analysis, among others [6].

2.2. Omics

The cost of DNA and RNA sequencing has been gradually decreasing over the years, and the improvement achieved with new sequencing technologies allows sequencing the whole genome or transcriptome of an organism in few hours, creating new avenues for biological research. However, genome sequencing involves the generation of a massive amount of data,

in the order of hundreds or even thousands of gigabytes. In the same way, but with higher cost and some technological limitations, mass spectrometry and magnetic resonance imaging are also capable of identifying thousands of proteins and metabolites of an organism, respectively. In this context, new "omics" (an informal term that collectively refers to research fields of biology ending in -omics, such as genomics, proteomics, or metabolomics) approaches have arisen, involving not only the production of sequence data but also the analysis, treatment, and interpretation of biological data on a large scale [7]. The following sections briefly present some of the main "omics" approaches, exemplifying their application in TB research.

2.2.1. Genomics

Genomics is a field of biology that seeks to understand the structure, functions, and evolution of genes and genomes analyzing large-scale genomic data obtained with high-throughput sequencing technologies. For instance, whole genome sequencing (WGS) and comparative analyses of genomic segments (chromosomes or syntenic regions) or the entire genome content (protein-coding genes, RNA-coding genes, pseudogenes, non-translated regions, etc.) allow the investigation of complex biological phenomena, giving insights into genome evolution and adaptation over time or even helping to improve clinical diagnoses. In the last two decades, WGS has become a routine among researchers, and currently there are thousands of completely sequenced genomes available in public databases. In fact, the availability and accessibility of genomic sequences are increasing rapidly, with the results generated by teams of researchers in collaborative networks or even in independent laboratories, supporting many essential analyses in the field, such as comparison of biological sequences and searching for similarities between them, enabling the inference of functional and evolutionary relationships between genes, gene families, and genomes [8, 9].

For instance, comparative genomics, together with the combination of archeological data and DNA sequencing, has already established a plausible evolutionary scenario for the origin of the principal etiological agent of TB, the pathogen Mtb. Although it is a millennial disease, drugs for the treatment of TB have appeared less than 100 years ago. Since then, multidrug-resistant (MDR) strains have emerged, as well as extensively drug-resistant (XDR) strains, and more recently, several countries have reported cases of total drug-resistant (TDR) strains, frustrating the efforts to fight TB. A typical feature of bacterial agents that develop drug resistance is the occurrence of lateral gene transfer (LTG), but comparative genomic studies did not find evidence of LTG in *Mycobacterium tuberculosis* complex (MTBC) organisms. Thus, there is a pathogen adapted for living in human host cells, able to remain for a long time as a latent disease, surviving in the host and adapting to yield a persistent infection, often immune to treatments. The primary challenge is then to develop an effective vaccine against most Mtb strains, which would be possible by targeting conserved elements [10].

2.2.2. Transcriptomics

Transcriptomics is the analysis of gene expression through the sequencing of RNA in large scale (RNA-Seq). Transcriptome, the whole set of RNA transcripts of a given organism, organ, tissue, or cell lineage, contains different types of RNAs. Thus, transcriptomics provides

essential information of the biological sample under analysis, allowing both quantitative and qualitative approaches to gene expression, providing a profile of all coding and noncoding transcripts, in specific conditions. RNA-Seq technologies have been improved gradually, including novel techniques such as single-cell RNA-Seq, in which individual cells of interest obtained from culture, tissue, or dissociated cell suspensions are isolated, converting RNA into cDNA and sequencing of cDNA libraries. However, transcriptomics still faces too many challenges. RNA-Seq produces very short reads and presents a high error rate, yielding a tremendous amount of data from massively parallel sequencing, requiring significant computational resources, as well as specific algorithms and software, to analyze it [11–13].

Mtb has an extensively resistant cell wall, can adopt an opportunistic switching over to latency, and has many strategies that fool the host's immune system, compromising the effectiveness of therapeutic approaches available. The analysis of Mtb transcriptome signatures during infection, for instance, provided by the genome-wide expression profile, showed the expression of numerous genes used to evade the host immune responses, suitable to the intracellular lifestyle, and to respond to various antibiotic drugs [14].

2.2.3. Proteomics

Proteomics establishes a global analysis of a cell's proteins. Gathering information about the proteome and comparing it with genome and transcriptome data is the way to understand the functioning of the cell. The physicochemical properties of highly diversified amino acids, protein modifications, and degradations and the interconnectivity of proteins in complexes are examples of difficulties encountered in collecting data compared to the other "omics" sciences. Nowadays, mass spectrometry is known as the state-of-the-art proteomics and has been improved with the development of instruments, sample preparation, and new analytical software. Mass spectrometry is capable of characterizing almost a complete proteome, revealing the profiling of the expressed proteins at the cellular and subcellular levels, providing knowledge of the functional status of a cell in response to environmental stimuli [15, 16].

Among all sequenced Mtb strains so far, with different genotypes and phenotypes, only a few have a complete identification of protein-coding regions, lacking the knowledge of protein functions that play roles in the physiology of mycobacteria. This information would be essential to allow, for example, understanding the causes that lead to mechanisms of mycobacterial pathogenicity and drug resistance. Even then, proteomics has already enabled us to know some aspects of virulence and its mechanisms of action in Mtb strains. The next step for proteomics contribution in mycobacteria research, the analysis of the immune response of the host, is one of the ways to establish new treatment programs, especially in the current scenario of drug resistance [17].

2.2.4. Metabolomics

Metabolomics encompasses the study of metabolism within a living cell, that is, it deals with the identification and analysis of biochemical reactions products and their processes within the cell. The metabolome is the set of these products (metabolites), and the metabolism analysis can reveal the cell's organic response. Metabolic analysis is a valuable resource for identification of

specific metabolite biomarkers, which would help, for example, evaluating response to drugs or stress agents. Magnetic resonance spectrometry (NMR) and mass spectrometry (MS) are the appropriate technologies for accurate measurements of metabolites, providing a good phenotype representation of any cell [18, 19].

Being complementary to other "omics," the metabolomics fills some gaps to a better understanding of diseases caused by mycobacteria. It is known that mycobacterial exosomes can be used as biomarkers, since they come from infected cells, containing mycobacterial proteins, lipoarabinomannan, and metabolites. They have been used for TB diagnosis and in the research of new vaccines. It is important to emphasize that the signatures of TB biomarkers must be validated in geographical and ethnical context, given the worldwide and diversified nature of the strains, besides concomitant infections such as malaria and HIV, as they affect metabolic biosignatures [19, 20].

2.3. One example of integration of omics

An effective vaccine for all strains of TB and the development of therapeutic approaches appropriate to the variations and stages of the disease are the main goals of current research in TB. The achievement of the genomic expression catalog of a global collection of BCG vaccine strains, comparing genomes and transcriptomes of 14 of the most widely used BCG strains, is one example of interaction between "omics" sciences in TB research, contributing to display evidences for highly diverged metabolic and cell-wall adaptations. Moreover, quantitative proteomics has identified the major differences in protein expression, when changes observed in the proteome confirmed the changes observed in the transcriptome, showing how the adaptation to the environment causes phenotypic differences between BCGs [21].

3. Tuberculosis facts, information, and treatment research

This section presents websites covering diverse information about TB, including history, pathogenesis, transmission, epidemiology, diagnosis, treatment, and infection control, listed in alphabetical order (**Table 1**). They also provide information about courses, tokens, and links

	Agency	**URL**
Americas	American Lung Association (ALA) Lung Disease Programs	http://www.lung.org/
	American Public Health Association	https://www.apha.org/
	Bill & Melinda Gates Foundation	https://www.gatesfoundation.org/
	Centers for Disease Control and Prevention, Division of Tuberculosis Elimination (CDC-DTBE)	https://www.cdc.gov/tb/
	Food and Drug Administration (FDA)	https://www.fda.gov/default.htm
	Global Tuberculosis Institute	http://globaltb.njms.rutgers.edu/
	Institute for Tuberculosis Research	http://www.tuberculosisdrugresearch.org/

	Agency	URL
	National Institute of Allergy and Infectious Diseases (NIAID)	https://www.niaid.nih.gov/
	National Library of Medicine, PubMed	https://www.ncbi.nlm.nih.gov/pubmed/
	Pan American Health Organization (PAHO)	http://www.paho.org/
Africa	AllAfrica.com: TB News from Africa	http://allafrica.com/tuberculosis/
	Desmond Tutu TB Centre	http://www0.sun.ac.za/dttc/
	South African Tuberculosis Vaccine Initiative	http://www.satvi.uct.ac.za/
	Tb Alliance	https://www.tballiance.org/
Asia and Oceania	JATA, Research Institute of Tuberculosis	http://www.jata.or.jp/eindex/home.html
	National Institute for Research in Tuberculosis	http://www.trc-chennai.org/
	Pakistan Anti-TB Association	http://www.patba.org/
	TBC India	http://www.tbcindia.nic.in/
Europe	European Tuberculosis Surveillance Network	https://ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/european-tuberculosis/
	International Union Against Tuberculosis and Lung Disease (UNION)	https://www.theunion.org/
	Max Planck Institute for Infection Biology	http://www.mpiib-berlin.mpg.de/
	National Health Service in England	http://www.nhs.uk/conditions/Tuberculosis/Pages/Introduction.aspx
	Pasteur Institute	https://www.pasteur.fr/
	StopTB Partnership	http://www.stoptb.org/
	TBnet	http://www.tb-net.org/
Global	World Health Organization (WHO)	http://who.int/tb/en/

Table 1. Websites covering TB facts, information, and treatment research.

to other websites and general guidelines. We do not prioritize any of them since every effort made to combat this disease has become important because of its comprehensiveness.

4. Tuberculosis databases and computational tools

Bioinformatics had its origins in the 1960s when computers became essential tools in research. Since then, numerous computational resources have been created, providing the scientific community different analytical tools to interpret a range of biological data. Collectively, these online resources are publicly available and are dedicated to acquire, store, organize, analyze, visualize, and/or integrate the ever-increasing amount of biological data originated from scientific experiments, scientific literature, high-throughput technologies, and computational analyses.

In this section, we provide a selection of online publicly available resources (entirely or partially) dedicated to mycobacteria causing tuberculosis, categorized according to its purpose and functionality (**Table 2**). Each category is quickly reviewed, presenting the reference to the original paper describing each computational tool, as well as its electronic address.

Category		Resource	URL	Ref.
Generic and multifunctional resources	Resources for functional and evolutionary genomic study of the genus *Mycobacterium*, comprising extensive literature review and data annotation on mycobacterial genome polymorphism, virulence factors, *in silico* generated and manually reviewed information on the complete genome sequence of these organisms, and essential genes	MyBASE	http://mybase.psych.ac.cn/	[22]
		The MycoBrowser portal	http://mycobrowser.epfl.ch/	[23]
Genomic mapping and functional annotation	Systems supporting functional annotation, including protein analysis, subcellular localization prediction, and mycobacterial membrane protein identification and characterization	MycoMemSVM	http://lin.uestc.edu.cn/server/MycoMemSVM	[24]
		MycoSub	http://lin.uestc.edu.cn/server/Mycosub/	[25]
		Genolist (TubercuList, BoviList, BCGList)	http://genolist.pasteur.fr/	[26, 27]
Comparative genomics	Collection of databases dedicated to mycobacterial comparative genomics, providing precomputed data of comparative genome analyses among selected mycobacterial genera, as well as inferred orthologous groups, functional annotations, and protein features	GenoMycDB	http://genomycdb.fiocruz.br/	[28]
		MycoDB	http://xbase.warwick.ac.uk/mycodb/	[29]
		Mycobacterium tuberculosis Comparative Database	https://olive.broadinstitute.org/projects/Mycobacterium tuberculosis Comparative	[30]
Genetic diversity and epidemiology	Focused on genetic diversity and epidemiology of MTBC, providing information of epidemiological data, strain lineage, genotyping, and phylogeny. They offer analyses of mycobacterial interspersed repetitive units (MIRU), single nucleotide polymorphism (SNP), long sequence polymorphism (LSP), spoligotyping patterns, IS6110-based restriction fragment length polymorphism (RFLP), and regions of difference (RD) profiles	CASTB	http://castb.ri.ncgm.go.jp/CASTB/	[31]
		MIRU-VNTRplus	http://www.miru-vntrplus.org/	[32, 33]
		PhyTB	http://pathogenseq.lshtm.ac.uk/phytblive/index.php	[34]
		PolyTB	http://pathogenseq.lshtm.ac.uk/polytblive/browser.php http://pathogenseq.lshtm.ac.uk/polytblive/map.php http://pathogenseq.lshtm.ac.uk/polytblive/phy.php	[35]
		SITVITWEB	http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/	[36]

Category		Resource	URL	Ref.
		SpolSimilaritySearch	http://www.pasteur-guadeloupe.fr:8081/SpolSimilaritySearch/	[37]
		spolTools	http://spoltools.emi.unsw.edu.au/	[38]
		TB-Insight	http://tbinsight.cs.rpi.edu/	[39]
Gene expression and regulation	Provide data on mycobacterial transcription factors, predicted operons, predicted transcriptional units, gene expression, and regulatory networks	CMRegNet	http://lgcm.icb.ufmg.br/cmregnet/	[40]
		GeNET	http://bengi.cs.mun.ca/genet/	[41]
		MTBRegList	http://www.usherbrooke.ca/vers/MtbRegList/	[42]
		MycoperonDB	http://cdfd.org.in/mycoperondb/home.html	[43]
Structural biology	These tools use three-dimensional models of mycobacterial proteins to provide information of domain assignments, functional annotation protein-protein or protein-small molecule interactions, and structural analyses of mutations potentially associated with drug resistance	CHOPIN	http://mordred.bioc.cam.ac.uk/chopin/	[44]
		SInCRe	http://proline.biochem.iisc.ernet.in/sincre/	[45]
Drug targets and resistance	Tools for diagnosis of drug resistance in tuberculosis, new vaccines, and drug targets	AuTuMN TB-modeling	http://www.tb-modelling.com/mdr_tb_at_retreatment/	[46]
		MtbVeb	http://crdd.osdd.net/raghava/mtbveb/	[47]
		MUBII-TB-DB	http://umr5558-bibiserv.univ-lyon1.fr/mubii/mubii-select.cgi/	[48]
		MycobacRV	http://mycobacteriarv.igib.res.in/	[49]
		PhyResSE	http://phyresse.org/	[50]
		TB Drug Resistance Mutation Database	https://tbdreamdb.ki.se/Info/	[51]
		TB-Profiler	http://tbdr.lshtm.ac.uk/	[52]
		TDR Targets database	http://tdrtargets.org/	[53]
		TIBLE	http://mordred.bioc.cam.ac.uk/tible/	[54]
		TuberQ	http://tuberq.proteinq.com.ar/	[55]

Table 2. Online publicly available resources dedicated to mycobacteria causing tuberculosis.

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Chapter 9

Patients and Health System-Related Factors Impacting on Tuberculosis Program Implementation in Resource-Constrained Settings: Experience from Multi-TB Facilities in Oyo State, South-West of Nigeria

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Additional information is available at the end of the chapter

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Abstract

Background: Tuberculosis (TB) is one of the most prevalent human infections and is the second leading cause of deaths from infectious diseases worldwide, and Nigeria is the fourth among the 22 high-burden countries in the world for tuberculosis even though the exact burden of TB in Nigeria is not known.

Methods: The study used exploratory cross-sectional design. A multistage stratified random sampling technique was used to select 680 participants from 16 DOTS facilities in one state in Nigeria.

Results: The results show that 59.25% (410) of individuals believed that the quality of access to care was excellent, 78.44% (542) of individuals believed that the appearance of the healthcare facility they attended was excellent, 75.40% (518) of individuals believed that there were many people accessing healthcare facilities and 82.33% (559) reported that they waited less than 30 minutes at a healthcare facility.

Conclusions: Providing good quality care to patients is an ongoing practice that requires continued consultation with everybody involved including patients who are at the receiving end of the service in order to evaluate and improve on the services rendered. Such practices will motivate compliance to treatment and a collaborative relationship between patients and healthcare providers in TB management. Despite several challenges affecting treatment and patient care, this study reports that healthcare provision

was generally satisfactory. Findings from this study are relevant for policy formation and strategic implementation for TB control program in resource-limited settings.

Keywords: tuberculosis, barriers, Nigeria, individual, provider

1. Introduction

Tuberculosis (TB) is a chronic infectious disease affecting any part of the body but more commonly the lungs [1]. It is one of the most prevalent human infections and is the second leading cause of deaths from infectious diseases worldwide [2]. In 2013, 80% of TB cases occurred in 22 high-burden countries leading to 1.5 million deaths. Nigeria is the fourth among these 22 countries, wherein the World Health Organization (WHO) estimates an incidence rate for all forms of tuberculosis to be '311 per 100,000 populations, incidence of smear positive annually 131 per 100,000 population and prevalence of 546 per 100,000 populations [3, 4]'. Also, TB services are provided mostly as part of the primary health services followed by secondary and tertiary healthcare provided by public and private institutions. Within the public sector, TB consultations, diagnostic, drugs and hospitalization services are provided free of charge [4]. At the private facilities, TB diagnostic and treatment services are provided free of charge; however, all patients irrespective of their health problem visiting the facility pay administrative fees. Following diagnosis, TB patients admitted at the private hospitals are required to pay additional fees for accommodation and feeding. If in any way the care provided in these facilities is found to be substandard, then this will result in poor treatment outcomes, persistent infectiousness as well as possible emergence and spread of drug-resistant strains [2].

The facilities at which TB care is provided are called directly observed therapy (DOTS); their scope of service includes diagnosis of TB (where microscopy services are available), supervised TB treatment, health education and adherence counseling, as well as HIV counseling and testing [4]. While the DOTS approach has been in place and seems to have lessened the burden of care on patients, access and adherence to TB treatment still face multiple challenges at different levels including individual and those that are a result of the system [5–7].

Individual-level barriers involve physical (distance to TB services and access to transport), financial (the direct and indirect costs of seeking TB services), stigma (stigma surrounding TB and its association with HIV), health literacy (TB-related knowledge and education) and sociocultural (gender roles and status in the family) factors, whereas provider-/system-level barriers include provider's degree of suspicion for TB, the number and types of providers seen before TB diagnosis, provider adherence to national TB program guidelines and patient satisfaction with TB services [2, 6, 7]. Due to these challenges, a comprehensive understanding of barriers is needed in order to provide insight into TB service programs, research and policy. It is against this background that this study was designed to determine individual and provider's barriers and delays that limit access and adherence to TB services.

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2. Methods

2.1. Study design

This study was an exploratory cross-sectional design. The study was conducted from June 2016 until November 2016 in 16 randomly selected DOTS facilities in Ibadan, Oyo State, Nigeria.

2.2. Study location

Nigeria lies on the west coast of Africa between latitudes 4°16′ and 13°53′ north and longitudes 2°40′ and 14°41′ east. It occupies approximately 923,768 square kilometers of land stretching from the Gulf of Guinea on the Atlantic coast in the south to the fringes of the Sahara Desert in the north. The territorial boundaries are defined by the Republics of Niger and Chad in the north, the Republic of Cameroon on the east and the Republic of Benin on the west. Nigeria is the most populous country in Africa and the 14th largest in land mass. The country's last census conducted in 2006 placed the country's population at 140,431,790 with a national growth rate estimated at 3.2% per annum [8].

Ibadan is the largest indigenous city south of the Sahara and is located at an altitude generally ranging from 152 m to 213 m with isolated ridges and peaks rising to 274 m. It is the state capital of Oyo State (see **Figure 1** above) which is near the forest grassland boundary of south-west of Nigeria on longitude 3° east of the Greenwich meridian and latitude 7° north of the equator. It

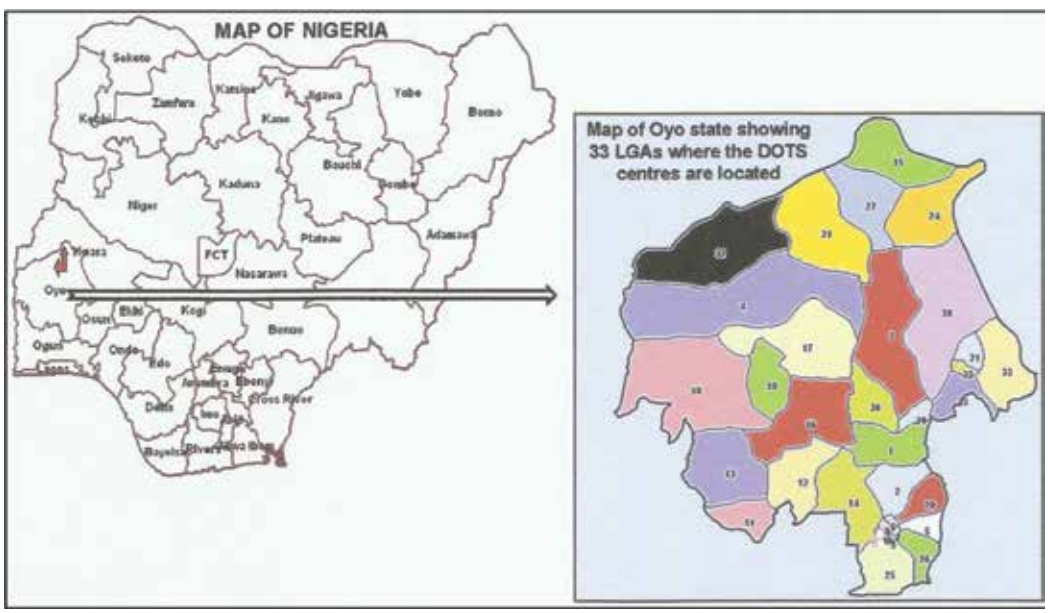

Figure 1. Nigeria (Ibadan, south-west of Nigeria). (Source: Nigeria Demographic and Health Survey, 2013).

is at a distance of about 145 km north-east of Lagos. Oyo State is divided into 33 local government area. It comprises largely the Yoruba-speaking tribe and other ethnic groups. Ibadan is dominantly a civil service city with some level of industrial activity, private businesses and other forms of trade and peasant jobs. The estimated population is 2.6 million people. Religious groups in the city are the Christians, Muslims and traditionalists. The study sites include those that are randomly selected from the under listed DOTS centers in Ibadan within the LGAs (strata): (i) Moniya Primary Health Care, (ii) Ojoo Primary Health Care, (iii) Odogbo Military Hospital, (iv) SDP Primary Health Care, (v) Iwo Road Primary Health Care, (vi) Alafia Hospital, (vii) Medical Outpatients-University College Hospital, (viii) Adeoyo Maternity Hospital, (ix) Jaja Health Services-University of Ibadan, (x) Alafara Primary Health Care, (xi) Agodi Prisons, (xii) OLA Catholic Hospital, (xiii) Sabo Primary Health Care, (xiv) Oniyanrin Primary Health Care, (xv) Atolu Primary Health Care, (xvi) Iyana Church, (xvii) Ejiku Primary Health Care, (xviii) Agbongbon Primary Health Care, (xix) SMG Catholic Hospital, (xx) Molete Primary Health Care, (xxi) Adifase Primary Health Care, (xxii) Chest Hospital Jericho, (xxiii) Olomi Primary Health Care, (xxiv) Ring Road State Hospital and (xxv) Apete Primary Health Care.

2.3. Sampling

The sampling technique was a multistage stratified random sampling technique. The first stage was to identify all the LGAs in Ibadan, classify the LGAs into strata and make a random selection of LGAs. The second stage was a random selection of the DOTS facility within the selected LGAs from which simple random selection of consenting TB patients attending DOTS facility at the hospitals/health facilities will be was attained. This multistage stratified random sampling technique was employed with the aim of precluding investigator bias and ensuring that the study population selected for the study is representative of TB patients in the study location.

2.3.1. Sample size estimation

Using the logic for calculating the analysis of variance (ANOVA) that is a collection of statistical models for the analysis of differences among group (DOTS centers) means (includes variations within and without/between groups). The assumption is that the groups are independent (unrelated). ANOVA has the advantage of assessing the importance of one or more factors by comparing the response variable means at the different factor levels:

- Effect size: 0.5
- Type 1 error: 0.05
- Type 2 error: 0.2
- Power: 0.80
- Number of groups: 2 (representing DOTS centers within each LGA)
- Critical F value: 4.15 (value which F should be over to get a significant result)

Therefore, the total sample size (participant per DOTS center) is 34, given that the study was conducted in 16 randomly selected DOTS facilities within the selected LGAs.

34 Participants × 16 DOTS facilities = 544 participants.

Assuming nonresponding rate of 20%.

Adjusted sample size (N^1) = N/1 − q.

where q = 0.2; N^1 = 544/1–0.2 = 680 participants (a *minimum of 680 TB patients were recruited into the study).*

2.4. Ethics

Ethical approval for the study was obtained from the University of KwaZulu-Natal (South Africa), Biomedical Research Ethics Committee's approval number (BE233/16). Additional approval was given by the Oyo State's Ministry of Health Ethics Committee (AD 13/479/1045). A full consenting process was applied in respect of all participants.

3. Results

A descriptive analysis assessing the association between individuals' sociodemographic and clinical characteristics (independent variable) and system-related barriers (dependent variables) was conducted. The individuals' sociodemographic characteristics were age, distance from facility, marital status, family type, education, religion, ethnic group and wealth index. The individuals' clinical characteristics were treatment status, where individuals access healthcare, how often individual access healthcare and HIV status. The system-related barriers were the quality of access to care, the healthcare worker attitude, the healthcare center's appearance, the number of people seeking treatment and the waiting time at the healthcare center. Chi-square tests were used to determine the associations between sociodemographic and clinical characteristic associations with the individual and system-related barriers. Logistic regression models reporting odds ratios (OR) and 95% confidence intervals were used to determine the relationship between sociodemographic and clinical characteristics with the individual and system-related barriers.

The results show that 59.25% (410) of individuals believed that the quality of access to care was excellent, 89.33% (611) of individuals believed that the attitude of healthcare workers was positive, 78.44% (542) of individuals believed that the appearance of the healthcare facility they attended was excellent, 75.40% (518) of individuals believed that there were many people accessing healthcare facilities and 82.33% (559) reported that they waited less than 30 minutes at a healthcare facility (see **Table 1**).

The sociodemographic descriptive statistics show that the distance from facility, family type and wealth index were significantly associated with the quality of access to care. Education was partially associated. Education was significantly associated with healthcare worker attitude. Family type was partially significant. The distance from the healthcare facility was associated with the appearance of the facility. Education, religion, ethnic group and wealth index were significantly associated with education which was significantly associated with the waiting time at the healthcare center. Family type was partially associated (see **Table 2**).

Access to care	Frequency (%)
Excellent	410 (59.25)
Not excellent	282 (40.75)
Healthcare worker attitude	
Positive	611 (89.33)
Not positive	73 (10.67)
Healthcare center's appearance	
Excellent	542 (78.44)
Not excellent	149 (21.56)
Number of people seeking treatment	
Many	518 (75.40)
Few	169 (24.60)
Waiting time	
0–30 minutes	559 (82.33)
More than 30 minutes	120 (17.67)

Table 1. Proportion of health system-related factors.

The clinical descriptive statistics show that where individuals access healthcare and how often individual access healthcare and HIV status were significantly associated with access to care. HIV status was significantly associated with healthcare worker attitude. Where individuals access healthcare and how often individuals access healthcare were significantly associated with perceptions about healthcare center's appearance. Where individuals access healthcare was significantly associated with the number of people seeking care. HIV status was partially associated. Where individuals access healthcare and HIV status was significantly associated with waiting time at the healthcare center (see **Table 3**).

3.1. Quality of access to care

The regression models show that those who lived 5 km–10 km from the healthcare facility were significantly more likely to believe that the quality of access to care was not excellent compared to those who lived within 5 km (OR, 2.48; CI, 1.72–3.56; $p < 0.001$). Those from polygamous families were more likely to believe that the quality of access to care was not excellent compared to those from monogamous families (OR, 1.38; CI, 1.00–1.90; $p = 0.049$) (see **Table 4**). Those individuals who did not usually get care at private clinics were significantly less likely to believe that the quality of access to care was not excellent (OR, 0.43; CI, 0.31–0.61; $p < 0.001$). Those individuals who accessed care not more than once a year were significantly less likely to believe that the quality of access to care was not excellent compared to those who accessed care more than once a year (OR, 0.54; CI, 0.37–0.78; $p = 0.001$). Those who did not know their HIV status were significantly more likely to believe that the quality of access to care was not excellent compared to those who were reactive (OR, 2.69; CI, 1.14–6.33; $p = 0.023$) (see **Table 5**).

Access to care	Total	Excellent	Not excellent	p-Value
Age				0.847
Less than 20	55	56.3631	43.64 (24)	
21–30	183	56.28 (103)	43.72 (80)	
31–40	200	59.00 (118)	41.00 (82)	
41–50	112	62.50 (70)	37.50 (42)	
51–60	66	59.09 (39)	40.91 (27)	
60+	46	65.22 (30)	34.78 (16)	
Distance from facility				<0.001
< 5 km	252	69.44 (175)	30.56 (77)	
5–10 km	255	47.84 (122)	52.16 (133)	
> 10	185	61.08 (113)	38.92 (72)	
Marital status				0.972
Never married	212	59.43 (126)	40.57 (86)	
Married	479	59.29 (284)	40.71 (195)	
Family type				0.049
Monogamous	440	61.82 (272)	38.18 (168)	
Polygamous	237	54.01 (128)	45.99 (109)	
Education				0.088
Pre-high school	233	54.08 (126)	45.92 (107)	
High school	282	60.64 (171)	39.36 (111)	
College/higher education	175	64.57 (113)	35.43 (62)	
Religion				0.337
Christian	330	59.70 (197)	40.30 (133)	
Islam	359	58.50 (210)	41.50 (149)	
Traditional	3	100.00 (3)	0.00 (0)	
Ethnic group				0.622
Yoruba	652	58.90 (384)	41.10 (268)	
Igbo	26	61.54 (16)	38.46 (10)	
Hausa	14	71.43 (10)	28.57 (4)	
Wealth index				0.003
Lower class	226	65.04 (147)	34.96 (79)	
Lower middle class	146	65.07 (95)	34.93 (51)	
Upper middle class	262	50.38 (132)	49.62 (130)	
Upper class	58	62.07 (36)	37.93 (22)	

Table 2. Sociodemographic characteristics stratified by access to care.

Access to care	Total	Excellent	Not excellent	p-Value
Treatment status				0.781
Retreatment	47	61.70 (29)	38.30 (18)	
Relapse	44	54.55 (24)	45.45 (20)	
New treatment	552	58.88 (325)	41.12 (227)	
Places where individuals access to healthcare				<0.001
Private clinic	179	44.13 (79)	55.87 (100)	
Non-private clinic	508	64.57 (328)	35.43 (180)	
How often do individuals access healthcare				0.001
More than once a year	443	52.60 (233)	47.40 (210)	
Not more than once a year	169	67.46 (114)	32.54 (55)	
HIV status				0.029
Reactive	49	63.27 (31)	36.73 (18)	
Non-reactive	567	59.44 (337)	40.56 (230)	
Do not know	41	39.02 (16)	60.98 (25)	
Healthcare worker attitude				<0.001
Positive	611	62 (279)	38 (232)	
Not positive	73	34.2 (25)	65.8 (48)	
Appearance of healthcare facility				<0.001
Excellent	611	62 (279)	38 (232)	
Not excellent	73	34.2 (25)	65.8 (48)	
Number of people seeking treatment				0.270
Many	518	60.4 (313)	39.6 (205)	
Few	169	55.6 (94)	45.4 (75)	
Waiting time				0.003
0–30 minutes	558	60.4 (342)	39.6 (216)	
More than 30 minutes	120	55.6 (56)	44.4 (64)	

Table 3. Participants' clinical and care-related characteristics.

3.2. Healthcare worker attitude

Those individuals who had a high school education were significantly less likely to believe that the attitude of the healthcare workers was not positive compared to those who only had a pre-high school education (OR, 0.44; CI, 0.24–0.81; p = 0.009) (see **Table 4**). Those individuals who did not know their HIV status were significantly more likely to believe that the attitude of the healthcare workers was not positive compared to those who were reactive (OR, 6.61; 1.34–32.63; p = 0.020) (see **Table 5**).

Access to care	**OR**	**95% Conf. interval**	**p-Value**
Excellent			
Not excellent (distance from healthcare center)			
<5 km (ref)			
5–10 km	2.48	1.72–3.56	<0.001
>10 km	1.45	0.97–2.16	0.069
Not excellent (family type)			
Monogamous (ref)			
Polygamous	1.38	1.00–1.90	0.049
Attitude of healthcare workers	OR	95% Conf. interval	p-Value
Positive			
Not positive (family type)			
Monogamous (ref)			
Polygamous	1.58	0.96–2.61	0.069
Not positive (education)			
Pre-high school (ref)			
High school	0.44	0.24–0.81	0.009
College/higher education	1.02	0.57–1.80	0.959
Appearance of healthcare facility	OR	95% Conf. interval	p-Value
Excellent (ref)			
Not excellent (distance from healthcare center)			
<5 km (ref)			
5–10 km	0.41	1.25–2.91	0.003
>10 km	0.99	0.60–1.63	0.965
Number of people at healthcare facility	OR	95% Conf. interval	p-Value
Many			
Few (education)			
Pre-high school (ref)			
High school	2.54	1.66–3.88	<0.001
College/higher education	1.26	0.76–2.08	0.379
Few (religion)			
Christian (ref)			
Islam	1.18	0.83–1.67	0.361
Traditional	Null (too few observations in sample)		

Access to care	OR	95% Conf. interval	p-Value
Few (ethnic group)			
Yoruba (ref)			
Igbo	1.74	0.76–3.98	0.190
Hausa	5.91	1.95–17.91	0.002
Few (wealth index)			
Lower class (ref)			
Lower middle class	0.91	0.55–1.50	0.720
Upper middle class	1.30	0.87–1.95	0.204
Upper class	0.43	0.19–1.01	0.054
Waiting time at healthcare facility	OR	95% Conf. interval	p-Value
0–30 minutes			
More than 30 minutes (education)			
Pre-high school (ref)			
High school	0.48	0.30–0.78	0.003
College/higher education	0.98	0.6–1.59	0.944

Table 4. Sociodemographic characteristic regression models.

Access to care	OR	95% Conf. interval	p-Value
Excellent			
Not excellent (places where individuals access care)			
Private clinic (ref)			
Non-private clinic	0.43	0.31–0.61	< 0.001
Not excellent (number of times accessed care)			
More than once a year (ref)			
Not more than once a year	0.54	0.37–0.78	0.001
Not excellent (HIV status)			
Reactive (ref)			
Non-reactive	1.18	0.64–2.15	0.600
Do not know	2.69	1.14–6.33	0.023
Attitude of healthcare workers	OR	95% Conf. interval	p-Value
Positive			
Not positive (HIV status)			
Reactive (ref)			

Access to care	**OR**	**95% Conf. interval**	**p-Value**
Non-reactive	2.81	0.6–11.86	0.160
Do not know	6.61	1.34–32.63	0.020
Appearance of healthcare center	OR	95% Conf. interval	p-Value
Excellent			
Not excellent (places where individuals access care)			
Private clinic (ref)			
Non-private clinic	0.45	0.31–0.65	< 0.001
Not excellent (number of times accessed care)			
More than once a year (ref)			
Not more than once a year	0.57	0.40–0.81	0.002
Number of people seeking care	OR	95% Conf. interval	p-Value
Many			
Few (places where individuals access care)			
Private clinic (ref)			
Non-private clinic	0.48	0.33–0.70	< 0.001
Few (HIV status)			
Reactive (ref)			
Non-reactive	0.57	0.31–1.04	0.068
Do not know	0.35	0.13–0.96	0.042
Waiting time at healthcare center	OR	95% Conf. interval	p-Value
0 to 30 minutes			
More than 30 minutes (places where individuals access care)			
Private clinic (ref)			
Non-private clinic	1.98	1.19–3.32	0.009

Table 5. Clinical characteristic regression models.

3.3. Healthcare facility appearance

Those individuals who lived 5 km to 10 km were significantly less likely to believe that the appearance of the healthcare facility was not excellent compared to those who lived within 5 km of the healthcare facility (OR, 0.41; CI, 1.25–2.91; p = 0.003) (see **Table 4**). Those individuals who did not access care at private clinics were significantly less likely to believe that the appearance of the healthcare facility was not excellent (OR, 0.45; CI, 0.31–0.65; p < 0.001). Those individuals who did not access healthcare more than once a year were significantly less likely to believe that the appearance of the healthcare facility was not excellent compared to those who accessed healthcare more than once a year (OR, 0.57; CI, 0.40–0.81; p = 0.002) (see **Table 5**).

3.4. Number of people accessing healthcare

Those with a high school education were significantly more likely to believe that there were few people accessing healthcare facilities compared to those with pre-high school education (OR, 2.54; CI, 1.66–3.88; $p < 0.001$). Those of the Hausa ethnic group were significantly more likely to believe that there were few people accessing healthcare facilities (OR, 5.91; CI, 1.95–17.91; $p = 0.002$). Those who did not access care at private clinics were significantly less likely to believe that there were few people accessing healthcare (OR, 0.48; CI, 0.33–0.70; $p < 0.001$). Those individuals who did not know their HIV status were significantly less likely to believe that there were few people accessing care compared to those who were reactive (OR, 0.35; CI, 0.13–0.96; $p = 0.042$) (**Table 5**).

3.5. Waiting time at healthcare facility

Those individuals who had a high school education were significantly less likely to report waiting more than 30 minutes at the healthcare facility compared to those who had a pre-high school education (OR, 0.48; CI, 0.30–0.78; $p = 0.003$) (see **Table 4**). Those individuals who did not access healthcare at private hospital were significantly more likely to report waiting more than 30 minutes at a healthcare facility compared to those who accessed healthcare at private clinics (OR, 1.98; CI, 1.19–3.32; $p = 0.009$) (see **Table 5**).

4. Discussion

In this study, we determined individual and provider's barriers and delays that limited access and adherence to TB services in 16 hospitals based in one state of Nigeria. We determined this through assessing the association between sociodemographic and quality of access to care, healthcare worker attitude, healthcare facility appearance, number of people accessing healthcare, as well as waiting time at healthcare facility. Our findings supported those reported in previous studies; for example, we report that living outside 5 km from the health facility was associated with poor perception of access to quality care [9–11]. This finding could be linked to the cost of time and transport incurred in traveling to the healthcare facility and the time taken to receive service upon arrival to the facility especially with treatment such as TB which requires continued contact with healthcare providers [12, 13]. We found that coming from a polygamous marriage or family was linked to significantly associating with not linking healthcare with good quality.

Also, individuals who were never exposed to private healthcare were likely to view public healthcare as providing excellent service. This finding might be due to their inability to compare the services they receive with those provided in private healthcare services. Private healthcare systems are associated with advanced resources, less waiting time and better treatment outcomes; it is therefore not surprising that in our study those who had a pre-exposure to private healthcare were likely to view the current healthcare service as not excellent [13, 14].

Those who knew their HIV status were likely to believe that the quality of care was excellent. This finding is significant because previous findings have shown that co-infection of HIV/TB

can lead to negative side effects, high drug burden and poor treatment outcome [14–16]. The perception by TB-/HIV-co-infected patients that healthcare service was excellent might mean that despite experiencing a double burden of the diseases, access to treatment may be less strenuous as they access treatment at the same facility and are more familiar with the operation of the facility as well as healthcare providers.

Our second aim was to understand the attitudes of participants toward healthcare workers; we found that those who were HIV positive and with high school education, respectively, were likely to perceive healthcare worker's attitude positively. The finding that having high school education was associated with positive attitude toward healthcare providers could be linked with patient's ability to understand the instructions with minimal dependence or assistance from healthcare workers. Also, the difference in satisfaction and sociodemographic factor such as education can be explained through the different expectations which patients may have toward how health providers should care for them. Although this may be the case, it is important that patients have a positive perception of healthcare workers in order to comply to treatment and hospital visits [6, 17]. A positive relationship between healthcare providers and patient was found to be linked to patients playing an active role in the management of their disease and adherence until the end of the treatment [18, 19].

5. Limitations

The following limitations in this study are acknowledged: the study was cross-sectional, collecting data at one point. The views of the participants may have changed after our first contact with them. Although our findings cannot be generalized because they were conducted in 16 health facilities in one country, the self-reported perception of participants was similar across the different facilities.

6. Conclusions

Providing good quality care to patients is an ongoing practice, which requires continued consultation with everybody involved including patients who are at the receiving end of the service in order to evaluate and improve on the services rendered. Such practices will motivate compliance to treatment and a collaborative relationship between patients and healthcare providers in TB management. Despite several challenges affecting treatment and patient care, this study reports that healthcare provision was generally satisfactory. Findings from this study are significant in guiding policy and interventions for resource-limited settings.

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Authors' contributions

OO and JMT conceptualized the idea and designed the study. Data collection, cleaning and processing were conducted by OO. Data analysis and interpretation were conducted by OO, LM and LM OA and supervised by JMT. All authors wrote the initial manuscript and read and approved the final manuscript.

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Competing interests

The authors declared no competing interests.

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Chapter 10

Clients' Perception of Quality of Multidrug-Resistant Tuberculosis Treatment and Care in Resource-Limited Setting: Experience from Nigeria

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Additional information is available at the end of the chapter

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Abstract

Background: Quality care is essential to the well-being and survival of people with multidrug-resistant tuberculosis (MDR-TB). The aim of this study is to explore how MDR- TB patients, who were voluntarily hospitalized, perceived care and treatment strategy and to assess the influence of psychosocial factors on their perception of care and treatment strategy in Nigeria. Methods: The study enrolled 98 MDR-TB patients on voluntary confinement in four MDR-TB hospitals in Nigeria. Patients' perceptions of quality of care and treatment strategy were evaluated with 28-item and 6-item instruments, respectively. Bivariate analysis was used to test for an association and multivariate analysis for factors that might contribute to the perceived quality of care. Results: Seventy-eight per cent (78%) of the participating patients perceived the quality of care to be good. Patients with better psychosocial well-being had five times higher odds to report good quality of care. Conclusion: The majority of MDR-TB patients perceived the quality of inpatient care to be good in Nigerian hospitals; however, their psychological

health influenced their perception significantly. Health care providers need to improve treatment strategies to encourage acceptance of care as poor perception to health care service delivery may deter treatment completion and also cause relapse among clients on treatment.

Keywords: satisfaction, quality of care, multidrug-resistant tuberculosis patients, Nigeria

1. Introduction

Tuberculosis (TB) is a major global public health crisis which was estimated to have led to 1.4 million deaths in 2015 [1]. Despite the availability of effective treatment for the disease, HIV co-infection has contributed to its re-emergence in more severe form with 5% of TB-infected patients reportedly experiencing multidrug resistant TB (MDR-TB) in 2013 [2, 3]. These incidents are growing particularly in developing countries such as Nigeria [4]. Nigeria is reported to have more than usual numbers of MDR-TB cases, which further poses threat to the control and eventual elimination of TB in the country [5, 6].

The acquisition of MDR-TB is a consequence of two main factors such as health service and patient-related. On the part of the health services, it is primarily a result of the compromised quality of care provided to TB patients. These include, inter alia, long waiting time to see the health workers, delayed retrieval of laboratory results, non-availability of anti-TB drugs and sub-optimal dosing of rifampicin and isoniazid among patients with TB/HIV co-infection [7]. While these established health service challenges impact acquisition of MDR-TB, there is the interplay of factors framed by patients' characteristics, disposition and behaviours to their TB treatment and care. Patients diagnosed with MDR-TB are also reported to experience psychosocial and economic challenges such as stigma, fear of being discriminated, lack of adequate funds, depression and psychological distress as a result of side effects from the treatment [8]. All these factors need to be addressed comprehensively particularly because of the significant role they play in determining treatment completion or adherence.

To cultivate consistent adherence, retention of patients and reduction of the prevalence of MDR-TB cases, a new treatment approach which is considerate of patients' needs should be adopted. This can be achieved through prioritizing patients as essential collaborating partners to the treatment team. It is important to listen to patients' voice because they have a potential to contribute to treatment innovations. They are also recognized as crucial in the caring process of culminating the widely encouraged patient-centred approach to care [9, 10].

In both policy and practice, it is becoming a standard requirement in the field of healthcare to give serious importance to patients' views [11]. Understanding the attitudes and perceptions of patients towards the care they receive is crucial in tailoring services which meet their needs. Such efforts will improve treatment success. For instance, in Nigeria, the treatment success rate for drug-resistant TB is 60% compared to 85.5% for the drug susceptible TB [12]. Among many factors contributing to, this is the possibility that the patients are not fully involved in their care and convinced or satisfied of the quality of services they receive.

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Often, patients have a lot of expectations from healthcare providers and the care they provide [13]. Without a concomitant quality service delivery, patients may become cynical and lower their own desire to participate actively in their care. With an increasing number of MDR-TB patients in need of treatment, research on the patients' perception of the quality of health care services they receive remains crucial. Understanding the perceptions will strengthen the primary care practices and provide insight on how patient centred care can be improved. Such information generated from research findings will be helpful to improve the quality of care and health service delivery to the patients [13, 14]. With this in mind, this study explored how MDR-TB patients on confined hospitalization perceived the treatment strategies and quality of care of the DOT programme, and assessed the influence of psychosocial factors (namely psychosocial and mental well-being) on their perception of quality of care.

2. Methods

2.1. Study settings and target population

This was a facility-based, cross-sectional study of all the patients who were managed for MDR-TB between January 2012 and October 2012 in four out of the five centres for the treatment of MDR-TB in Nigeria. The centres and the corresponding number of patients enrolled in the study were University College Hospital Ibadan (24 patients), Government Chest Hospital (GCH) Jericho, Ibadan (22 patients), Dr. Lawrence Henshaw Memorial Hospital (DLHMH), Calabar (12 patients) and Mainland, Lagos (40 patients). Admission of MDR-TB patients into the treatment programme started in January 2012 while recruitment for the study was in October of the same year. These patients were admitted in the hospital on the basis of having developed resistance to TB, and being willing to remain in isolation for the duration of 8 months for intensive treatment, with visitations limited to once a week from family members.

2.2. Measures

We used two questionnaires to measure different aspects of the patients' perceptions of care and treatment. The psychosocial well-being and mental well-being of patients were evaluated to assess their influence on perception prese nted in **Table 1** below. Psychosocial well-being as the independent variable was characterized as poor and good if respondents had an aggregated score of ≤9 and ≥ 10 points, respectively. Mental health was characterized as poor and good if the patient had an aggregated score of ≤5 and ≥ 6, respectively.

The patients' perception of treatment strategy was evaluated with a six-item instrument that asked whether they preferred the current method of treatment that is applied for their treatment during hospitalization. The responses were either 'Yes' or 'No' and coded 1 and 0, respectively. Respondents who selected 'Yes' supported the current treatment recommendations.

Variables	Items	Measure
Psychosocial well-being		Dichotomous
	Concerned that people will say bad things of me	
	Fear of Social rejection/isolation due to stigma	
	Worried that people will know I am treating TB in the hospital	
	Loneliness	
	Worried about my family	
	Miss being with my partner	
	Long hospital stay denies me the support from my family	
	Being separated from my family	
	Worried about disengagement from community	
	Hospital feels like a prison	
	Concerns about side-effects of medicines	
	Concerned that I have taken too many drugs	
	Have ability to tolerate side-effects	
	Low expectations of cure	
	Miss doing all the usual things I use to do	
	Worried that I will lose my job	
	Frustrations about the temporary inability to work/study/perform social responsibilities	
	Worried about income	
Mental health		Dichotomous
	Feel that I let myself and my family down	
	Has no pleasure in doing things	
	Feeling down, depressed or hopeless	
	Trouble falling or staying asleep or sleeping too much	
	Trouble concentrating on things	
	Being restless because I have been idle	
	Feels that it is better to be dead	
	Has poor appetite	
	Have little energy	
	Feeling bad about disengagement from my partner(s)	
Perception of quality of care	**Cronbach alpha = 0.949**	
Interpersonal relations	**Cronbach alpha = 0.904**	Scales
	Manner in which health workers receive	
	Respect shown by the doctors	
	Respect shown by the nurses	
	Respect shown by the hospital/ward maids	
	Respect shown by the cleaners	

Variables	Items	Measure
	Reassuring attitude of the health workers	
	Respect for privacy during the physical examination.	
	Doctors showed me interest	
	Have special knowledge of TB	
	Maintain confidentiality about my TB status	
	Give information about possible side effects of drugs	
	Give information about the use of my TB medication	
Health workers' competencies	**Cronbach alpha = 0.908**	Scales
	The explanations about my health problem were clear and complete	
	The explanations about the tests to be taken were clear and complete	
	The explanations about the treatment chosen were clear and complete	
	My involvement in the decisions concerning the treatment	
	Time spent in consultation with my doctor.	
	History of my problem taken by the doctor	
	Doctor's skill in making the physical examination more comfortable.	
	Appropriateness of the tests and physical examination	
	Correct diagnosis made by the doctor.	
	Execution of the care and treatments	
	Possibility of seeing the same doctor every time.	
	Time spent waiting to obtain test results.	
Social visits	**Cronbach alpha = 0.682**	Scales
	Improvement in my state of health	
	Lessening of my fears and anxieties	
	Return to my routine activities	
	Ability to react (what to do, who to contact) if my state of health deteriorates.	

Table 1. Variables and number of items with Cronbach alpha scores.

All the six items on treatment strategy were aggregated such that a higher score represented greater support of treatment strategy.

Patients' perception on the quality of care was evaluated with a 28-item tool (**Table 1**) that measured perception with a 5-point Likert scale, a measurement going from negative to positive indicated better perception of quality of care. For the purpose of further description of data and logistic regression procedure, the perception of treatment strategy score was characterized as 'does not support treatment strategy' and 'supports treatment strategy' if respondents had an aggregated score ≤ 3 and ≥ 4, respectively. For perception of quality of care, respondents were characterized as poor, indifferent and good if respondents had an aggregated score as follows: 28–73, 74–95 and 96–140, respectively.

2.3. Statistical analysis

We used summary statistics to show the distribution of the main variables, and the values were expressed as an absolute number with percentages and mean with standard deviation for categorical and continuous variables, respectively. We performed both bivariate analysis and multivariable logistic regression models to examine the associations between participants' socio-demographic and other characteristics with the perceived treatment strategy and quality of care. Univariate analyses were used to determine the unadjusted (crude) odds ratios, and bivariate analysis of the result for the association between independent variable (IV) and treatment perception was done using Student t-test when IV had two levels and ANOVA when the levels were more than two.

Multivariate logistic regression was performed to determine the factors that predict perceptions of both treatment strategy and quality of care. We considered variables for inclusion in the multivariable model if they reached a moderate level of significance ($p < 0.25$). The model significantly predicted the outcomes better than a model without the predictors. The model chi-square test value provides a measure of improvement due to the introduction of the independent variables. Nagelkerke R^2 provides an overall model fit. The following regression diagnostics were used to assess the goodness-of-fit of the model and to choose the parsimonious model: the Hosmer-Lemeshow goodness-of-fit test, tolerance test for multicollinearity and link test to check for model specification error. Thereafter, we performed receiver operating curves (ROC) (c-statistics) analyses to determine the predictive power of the final multivariable model. The ROC curve plots the sensitivity of the model against 1 minus sensitivity for different cut-off points of the predicted probability of having hypertension. The greater the ROC curve (upper limit =1), the better the model is at discriminating between hypertension cases. Results were presented as odds ratios (ORs) with 95% confidence intervals (CIs). All statistical analyses were performed using IBM SPSS version 20. The significance tests were two-tailed, and statistical significance was defined at the alpha level of 0.05.

3. Results

3.1. Socio-demographic, medical history and well-being of patients

A total of 98 patients were recruited into the study between January 2012 and October 2012 from 4 MDR-TB centres in Nigeria. Most (63.3%) of the participants were males. The mean age of patients was 36.1 years (SD 11.97). More than half (55.1%) of the patients were married. Most (65.3%) patients were Christians, while Muslims made up the remaining 34.7%. Most of the respondents had some form of education, as only 14.5% had no formal education. About three quarters (74.5%) were unemployed (see **Table 2**).

Table 2 indicates that some of the participants (34.6%) in the selected centres have been on TB treatment between 5 and 10 years, without cure resulting in their resistant to treatment. About

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Characteristics	Frequency n	Percentage %
Sex:		
Male	62	63.3
Female	36	36.7
Age (Mean ± SD) 36.1 ± 11.97		
≤30 years	34	39.5
> 30 years	52	60.5
Marital status		
Currently single	44	44.9
Currently married	54	55.1
Religion		
Christianity	64	65.3
Islam	34	34.7
Education		
No formal	13	14.5
Primary	25	27.8
Secondary	39	43.3
Post-secondary	13	14.4
Current employment status		
Unemployed	70	74.5
Employed	24	25.5
Income (Naira)		
≤100,000	28	56.0
10,001–20,000	7	14.0
20,001–50,000	8	16.0
>50,000	7	14.0
Family members visit		
Yes	72	80.0
No	18	20.0
Number of TB treatment(s) in the past		
1	16	16.3
2	29	29.6
3	36	36.7
≥4	17	17.3

Characteristics	Frequency n	Percentage %
Number of years on TB treatment		
1–2	21	21.4
3–4	27	27.6
5–10	34	34.7
>10	16	16.3
Current length of admission (months)		
1	4	4.1
2	16	16.3
3	9	9.2
4	10	10.2
5	35	35.7
6	1	1.0
7	16	16.3
8	7	7.1
Treatment Centre		
UCH, Ibadan	24	22.5
GCH, Ibadan	22	22.4
DLHMH, Calabar	12	12.2
Mainland, Lagos	40	40.8
Psychosocial well-being		
Poor	28	28.6
Good	70	71.4
Mental health		
Poor	39	39.8
Good	59	60.2

Table 2. Description of MDR-TB patients in isolation, Nigeria.

7.1% of patients were almost at the end of the DOT strategy, which lasted for a period of 8 months. Another 16.3% of patients were also near the completion of their treatment course at 7 months, and only 4% of patients were just beginning the treatment at 1 month. Results showed that a higher proportion of the patients had good psychosocial and mental well-being, 71.4 and 60.2%, respectively.

3.2. Perception of treatment and quality of care of patients

Table 3 shows that 63.8% accepted that patients with MDR-TB could be admitted for many months in the hospital for isolation and treatment. However, 43.5% preferred out-patients' treatment option. Similarly, a slight majority of 51.7% indicated a preference for treatment at

Item	Yes (%)	No (%)
Do you think this type of treatment where patients are kept in hospital for a long time is good?	63.8	36.2
*Would you have preferred to be treated as an outpatient?	43.5	56.5
*Would you have preferred to have a community health staff come to give you your medicine/injection at home?	51.7	48.3
Can you recommend this method of treatment to anybody close to you if they have drug resistant TB?	85.4	14.6
Should the government use this method to treat people with drug resistant TB?	73.6	26.4
Do you know about the progress of your treatment?	67.7	32.3

*Reversely coded for scoring.

Table 3. Perception of treatment strategy.

home through visiting community health workers. A considerable proportion (85.4%) indicated that they would recommend the treatment method to another person close to them if they had MDR-TB. A large number of participants (73.6%) were of the opinion that the government should use the in-care hospitalized intensive phase method to treat people with MDR-TB. The proportion who reported that they were informed about the progress of their treatment was 67.7%. Overall, the treatment strategy during hospitalization was supported by 69.4% of the patients (**Figure 1**). With respect to quality of care, internal consistency was shown to be excellent for overall quality of care, with Cronbach alpha = 0.949, and perception of interpersonal relations, Cronbach alpha = 0.904; perception of health workers' competencies, Cronbach alpha = 0.908, but lower for perception about visits, Cronbach alpha = 0.682. Mean score for overall perception of the quality of care was 109.2 (SD 24.00), out of a maximum score of 140 points with a minimum score of 28 (as shown in **Table 4**). Few patients (7.8%) had a

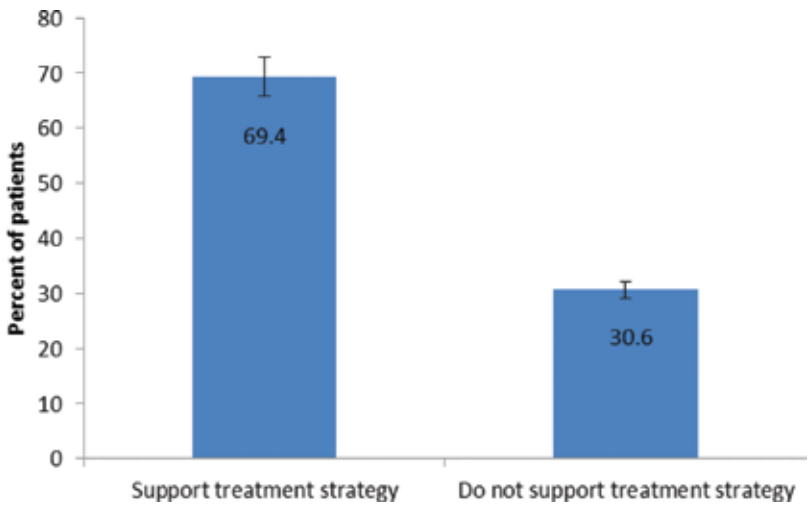

Figure 1. Patients' support for current TB treatment strategy.

	Number of items	N	Minimum	Maximum	Mean (SD)
Perception on treatment strategy	6	98	0	6	3.63 (1.69)
Overall Perception on quality of care	28	90	28	140	109.17 (24.00)
Interpersonal relations	12	91	12	60	46.61 (11.18)
Doctors work	12	93	12	60	46.04 (11.28)
Opinion on what I got from the visit	4	93	4	20	16.12 (3.36)

Table 4. Patients' scores on the perception of treatment strategy and quality of care items.

poor perception of the quality of care they received from the facilities; 78.9% had a good perception about the quality of care they received, while 13.3% were indifferent (**Figure 2**).

Correlation analysis was performed to test how the different domains of perception associated with overall quality of care (**Table 5**). Results showed a positive significant association between all the domains with overall perception. This implies that as interpersonal relations, perception about health workers' competencies, and perception about visit either increased or decreased, there was a corresponding increase or decrease in overall perception of quality of care. This association indicates the importance of the increased interpersonal relationship, professional competencies and social visits on improving the perception of quality of care. Only health workers' competence showed weak positive association with the perception of treatment strategy.

3.3. Perception of treatment and quality of care following socio-demographic, medical history and well-being status of patients

Results as shown in **Table 6** indicated that few variables were associated with the perception of treatment strategy. There was a significant difference in the means of perception to treatment

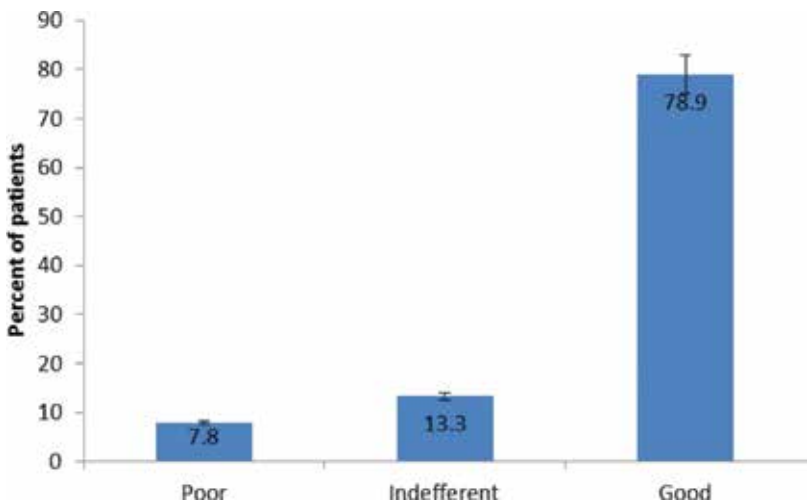

Figure 2. Patients' perception of quality of care.

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Domains	Perception on quality of care		Perception on treatment strategy	
	r	*p*	r	*p*
Interpersonal relations	0.952	<0.001	0.163	0.123
Health workers' competence	0.941	<0.001	0.200	0.054
Social visits	0.746	<0.001	0.045	0.668

Table 5. Association between domains of quality of care and overall quality of care.

	Perception on treatment strategy			Perception on quality of care		
Characteristics	**Mean (SD)**	**F**	***p***	**Mean (SD)**	**F**	***p***
Sex						
Male	3.74 (1.64)	0.699	30.405	108.83 (24.85)	0.037	0.849
Female	3.44 (1.77)			109.86 (22.59)		
Age						
≤30 years	3.67 (1.64)	0.010	0.920	113.00 (26.17)	1.406	0.239
> 30 years	3.71 (1.53)			106.39 (23.16)		
Marital status						
Currently single	3.79 (1.70)	0.734	0.394	114.81 (22.70)	3.740	0.056
Currently married	3.50 (1.69)			105.05 (24.30)		
Religion						
Christianity	3.78 (1.65)	1.423	0.236	109.79 (22.78)	0.106	0.745
Islam	3.35 (1.75)			108.06 (26.41)		
Education						
No formal	4.00 (0.816)	1.062	0.369	104.14 (25.50)	0.893	0.448
Primary	3.32 (1.43)			114.91 (17.518)		
Secondary	3.94 (1.83)			105.22 (28.54)		
Post-secondary	3.37 (1.84)			110.84 (21.77)		
Current employment status						
Unemployed	3.84 (1.72)	3.211	0.076	111.84 (24.78)	3.834	0.053
Employed	3.12 (1.59)			100.56 (20.41)		
Monthly income						
≤100,000	4.21 (2.02)	2.613	0.062	122.48 (15.11)	7.747	<0.001
10,001–20,000	2.71 (1.11)			114.71 (10.54)		
20,001–50,000	4.50 (1.85)			86.50 (31.08)		
>50,000	2.71 (0.95)			97.71 (25.54)		
Family member visits						
Yes	3.69 (1.61)	0.034	0.853	108.87 (22.08)	0.034	0.855
No	3.61 (2.03)			110.05 (30.50)		

	Perception on treatment strategy			Perception on quality of care		
Characteristics	**Mean (SD)**	**F**	***p***	**Mean (SD)**	**F**	***p***
Number of TB treatment in the past						
1	4.50 (1.75)	3.407	0.021	113.40 (16.75)	0.293	0.830
2	3.00 (1.60)			106.33 (27.60)		
3	3.88 (1.63)			108.90 (25.61)		
≥4	3.35 (1.57)			110.56 (21.10)		
Number of years on TB treatment						
1–2	3.80 (1.69)	0.250	0.861	107.10 (32.00)	0.205	0.893
3–4	3.40 (1.82)			107.95 (25.13)		
5–10	3.70 (1.71)			111.84 (18.88)		
>10	3.62 (1.54)			108.06 (22.19)		
Current duration on admission						
1–2	3.65 (1.92)	0.893	0.448	104.95 (26.05)	1.730	0.167
3–4	4.05 (1.80)			118.00 (24.10)		
5–6	3.30 (1.78)			112.20 (17.05)		
7–8	3.78 (1.16)			102.00 (29.77)		
Treatment centre						
UCH, Ibadan	3.12 (1.19)	4.366	0.006	108.16 (22.16)	7.150	<0.001
GCH, Ibadan	3.95 (1.13)			109.88 (28.35)		
DLHMH, Calabar	5.00 (1.59)			134.66 (5.26)		
Mainland, Lagos	3.35 (2.00)			101.00 (21.23)		
Psychosocial well-being						
Poor	3.79 (1.83)	0.591	0.444	107.40 (30.69)	0.188	0.666
Good	3.52 (1.60)			109.86 (21.12)		
Mental health						
Poor	3.85 (1.81)	0.685	0.410	110.48 (30.38)	0.153	0.697
Good	3.54 (1.64)			108.42 (19.66)		

Table 6. Perception of treatment strategy and quality of care distributed by socio-demographic, medical history and well-being of patients (higher mean values are associated with better perception).

strategy with the number of TB treatment in the past, as a higher mean score for the perception of treatment strategy was observed for patients who had only treated TB for the first time. Differences were observed in mean scores for treatment strategy by centre, with the DLHMH-Calabar registering a higher mean compared to the other centres. The bivariate analysis did not yield associations between psychosocial and mental well-being and perception of treatment strategy. Significant associations were, however, seen between income and treatment centre, and perception of quality of care such as patients in the low-income category had a

statistically higher mean for perception of quality of care. Furthermore, patients at the DLHMH-Calabar treatment centres had a significantly higher mean for the quality of care compared to those in other centres.

Independent variables that were significant in bivariate model and the two well-being variables—psychosocial and mental well-being—were added in a multivariate model with the two outcome variables using MANOVA, to examine the association between independent variables and the combined dependent variables. **Table 7** shows that all the variables remained significant in multivariate analysis, except psychosocial and mental well-being, indicating that there were significant differences in the means of the composite outcome variables with respect to the groups of each independent variable in the model. Partial eta-squared (η2) indicated some levels of contribution of some of the variables like employment status, treatment centre to the model, implying that these two variables did not exert a considerable effect on perceived treatment strategy and quality of care.

3.4. Predictors of perceived treatment strategy and quality of care

The variable psychosocial well-being became significant in the logit model predicting high perception of quality of care (**Table 8**). As patients with good psychosocial well-being were more likely to perceive the quality of care as good (OR: 5.1, CI: 1.46–23.94). Patients in the Mainland-Lagos and GCH-Ibadan were less likely to have a good perception with respect to quality of care compared with those in UCH-Ibadan, although the relationship was not significant. Patients who had spent ≥ 3 months in admission were less likely to perceive the quality of care in a positive light than those who had spent 1–2 month in admission. No difference was observed between patients treated for TB ≥ 4 times and those treated once previously (OR: 0.93, CI: 0.14–6.32) (**Table 9**). Employed participants were less likely to support treatment strategy (OR: 0.23, CI: 0.06–0.90) and less likely to perceive the quality of care as good (OR: 0.11, CI: 0.02–0.88), compared with the unemployed. Patients with secondary education and above were less likely to perceive the quality of care as good, while those who had primary education were twice more likely to report the quality of treatment as good than those who had no formal education.

Variables	Pillai's Trace F	*p*	η^2
Current employment status	5.563	0.031	0.582
Monthly Income	2.720	0.046	0.476
Number of TB treatment in the past:	2.755	0.044	0.479
Treatment centre	3.021	0.032	0.502
Psychosocial well-being	2.724	0.125	0.405
Mental health	1.330	0.317	0.250

Table 7. MANOVA for perceived treatment strategy and quality of care by selected variables.

	Perception of quality of care			
	Adjusted OR	**95% CI Lower bound**	**95% CI Upper bound**	***p***
Marital status				
Currently single	1			
Currently married	0.328	0.090	1.195	0.091
Employment status				
Unemployed	1			
Employed	0.272	0.071	1.044	0.058
Psychosocial well-being				
Poor	1			
Good	5.909	1.458	23.938	.013

Model Chi-square = 11.107, p = 0.011, Nagelkerke R^2 = 0.201. Selected variables entered on first step: Gender, age, marital status, religion, level of education, employment status, number of treatments in the past and psychosocial well-being.

Table 8. Adjusted logit model for support for good perception of quality of care.

	Perception of quality of care			
	Adjusted OR	**95% CI Lower bound**	**95% CI Upper bound**	***p***
Marital status				
Currently single	1			
Currently married	0.284	0.091	0.886	0.030
Religion				
Christianity	1			
Islam	0.224	0.065	0.774	0.018
Employment status				
Unemployed	1			
Employed	0.341	0.103	1.131	0.079
Number of treatments in the past				
1	1			
2	0.061	0.010	0.372	0.002
3	0.445	0.084	2.353	0.341
≥4	0.927	0.136	6.316	0.939

Chi-square = 28.891, p<0.001, Nagelkerke R^2 = 0.397. Selected variables entered on first step: Gender, age, marital status, religion, level of education, employment status, number treatment in the past and psychosocial well being.
[a]Model X^2 test = 26.39, p<0.001, R^2 = 0.412.

[b]Model X^2 test = 30.067, p<0.001, R^2 = 0.506.
All independent variables were added in the logistic regression model, but only variables that are significant in stepwise process are presented.

Table 9. Adjusted logit model for support for current treatment strategy.

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4. Discussion

This study sought to describe perceptions of TB infected patients on the method of treatment they were undergoing, the quality of care they were receiving while in hospitals, and the relationship between their perceptions as well as their psychosocial and mental well-being. To address these objectives, the current study used self-developed assessments. Our study found that the sample perceived the treatment strategy and quality of care received during the MDR-TB to be acceptable. For instance, 69.4% of the sample undergoing TB treatment in the four centres included in the study supported the treatment strategy offered, while 78.9% of the sample perceived the quality of the service to be satisfying. Similar findings were also shown in other studies conducted in Nigeria, Ethiopia and South Africa, respectively [15]. The study further suggests that patients' satisfaction was influenced by a positive interpersonal relationship between health workers, health workers' competence and the social visits patients got. These factors were also found to be associated with adherence to the treatment and continued use of the services. Other studies have reported similar findings suggesting that the patient-service provider relationship is an important reason for satisfaction with TB treatment service [15–19].

The study further reveals that treatment perception was influenced by employment status, the number of TB treatment in the past and the treatment centres. On the other hand, satisfaction with the quality of TB care was majorly influenced by marital status, employment status, monthly income and treatment centre at the bivariate level. A higher mean score for the perception of treatment strategy and quality of care is observed for unemployed patients than employed patients, and the result was confirmed by multivariate analysis. Similarly, patients who earn lower monthly income perceived quality of care to be better than patients who earned a higher income as the result showed. Our finding is similar previous findings which reported that TB patients who had no income were more satisfied with the TB care service than those who had savings [20]. Unlike our findings and those from other studies [21, 22], the authors reported a positive relationship between service satisfaction and educational level. This scenario may be due to people from lower social classes having modest expectations, partly due to limited awareness or limited ability to choose and hence vary expectation. They may also be reluctant to voice dissatisfaction for fear of losing the service. It is necessary to use other flexible and anonymised methods, for instance, qualitative research techniques, to determine client satisfaction. It is also asserted that patients from developing societies are often less likely to report dissatisfaction with the quality of care despite inadequate health resources and lack of access to health facilities in these places, compared with patients in developed societies where services are much better [22].

In comparison with patients who had previously received TB treatment, the study highlights that patients who were only treating TB for the first time showed a statistically higher mean for acceptance of treatment strategy, and this better perception dampened as numbers of time on treatment increased, but increased again slightly as patients experienced more TB treatment. The lower acceptance among patients who have treated TB more than once is because these patients have more experience of the treatment programme, and possibly unsuccessful in

repeated treatments; therefore, seeming to resign their trust in the effectiveness of the existing programme. In such circumstance where patients have undergone treatment for a repeated number of times, MDR-TB patients are to be encouraged to adhere, and not reject treatment programmes. The DOT centre where treatment strategy was more acceptable, with an equally better perception of quality of care was the DLHMH centre in Calabar. No notable associations were observed. All the well-being variables were not significantly associated with any perception outcome in bivariate analysis; however, after controlling for other independent factors in logistic regression analysis, the result showed a significant effect, as a higher likelihood of a good perception of quality of care among patients who had good psychosocial well-being was exhibited. Some studies have shown that patient's satisfaction with treatment during illness can be enhanced when the psychosocial needs of the patients are met [23, 24]. Patients who had spent 5–6 months on admission had higher odds of perceiving care positively as compared to those with either less or more time on admission. There was also an association between marital status and perception in logistic regression. Married patients were less likely to report acceptability of treatment method and a good quality of care. This could be explained as an issue of difference in expectations of this group of individuals. It is possible that married people have more expectation from the services than those who are not married because they may feel the weight of family responsibilities more than their unmarried counterparts, as such expect an even greater strategy that would improve their conditions so they can return to cater for those responsibilities.

5. Limitations

One of the major limitations of this study was the low sample size which may affect a more robust description of the perception of treatment strategy, quality of care and other likely associations between independent factors and outcome variables that is not detectable as a result of low sample size. In addition, this study did not measure the characteristics that are related to health service delivery which can be determinants of perception than merely individual's characteristics. Although this is the case, findings from this study provide insight into the perceptions of MDR-TB patients on quality care.

6. Conclusions

This study shows a considerably high acceptability of the DOT strategy for treatment and satisfaction with the quality of care that patients are receiving while hospitalized. Acceptability of the treatment method was influenced by marital status, employment status and the number of TB treatments in the past. The psychosocial well-being influences the patient's perceived quality of care.

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Competing interests

The authors declared no competing interests.

Authors' contributions

OO, BAU and KEO developed the concept of the study. Data collection, cleaning and processing was conducted by OO, EEU and KEO. Data analysis and interpretation was conducted by BAU, OO, EEU, KEO, KHM, DAA and LM. All authors wrote the initial manuscript, read and approved the final manuscript.

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Section 3

BCG and Non Tuberculous Mycobacterial

Chapter 11

Mosaic Structure as the Main Feature of *Mycobacterium bovis* BCG Genomes

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Abstract

Background: The genome stability of attenuated live BCG vaccine preventing the acute forms of childhood tuberculosis is an important aspect of vaccine production. The purpose of our study was a whole genome comparative analysis of BCG sub-strains and identification of potential triggers of sub-strains' transition.

Results: Genomes of three BCG Russia seed lots (1963, 1982, 2006 years) have been sequenced, and the stability of vaccine sub-strain genomes has been confirmed. A comparative genome analysis of nine *Mycobacterium bovis* BCG and three *M. bovis* strains revealed their specific genome features associated with prophage profiles. A number of prophage-coded homologs to Caudovirales ORFs were common to all BCG genomes. Prophage profiles of BCG Tice and BCG Montreal genomes were unique and coded homologs to herpes viruses ORFs. The data of phylogenetic analysis of BCG sub-strain groups based on whole genome sequences and genome restriction maps were in congruence with prophage profiles. The only fragmentary similarity of specific prophage sequences of BCG Tice, BCG Montreal, and BCG Russia 368 in pair-wise alignments was observed, suggesting the impact of prophages on mosaic structure of genomes.

Conclusions: The whole genome sequencing approach is essential for genomes with mosaic structure, harboring numerous prophage sequences. Tools for prophage search are effective instruments in this analysis.

Keywords: BCG sub-strains, *Mycobacterium bovis*, genome stability, genome rearrangements, prophages

1. Introduction

Tuberculosis (TB) is one of the top causes of death in the world. Currently, the only authorized vaccine for primary vaccination of children from TB remains BCG, first applied in 1921. It is broadly used in different countries as part of the national childhood immunization program. Despite the attempts of TB control through widespread introduction of vaccination it was estimated in 2014 worldwide that 9.6 million people have fallen ill with TB. Nevertheless, vaccination against TB reduced TB prevalence by 42% in 2015 compared to that in 1990 [1].

World Health Organization (WHO) controls BCG vaccine, and the WHO Expert Committee on Biological Standardization (ECBS) has developed the international requirements for the manufacture and control of BCG vaccine. In 2009, for BCG vaccines of three different sub-strains (Danish1331, Tokyo 172-1 and Russian BCG-I), WHO Reference Reagents were established by WHO ECBS. In addition, quality control requirements comprising molecular genetic characterization of final lots and working seeds of BCG vaccines were suggested [2]. Russian research laboratories performed whole genome sequencing (WGS) of BCG Russia sub-strain genome as WHO and good manufacturing practice (GMP) recommended [3–5]. Currently, ten whole genome sequences of BCG sub-strains including BCG Russia are available in GenBank. It should be noted that since the 1920s, cultivation of the original strain BCG resulted in the emergence of numerous sub-strains that have evolved from it. So, now we could investigate the evolution of BCG sub-strains and the endpoints of this evolution could be assessed likewise in the study of Darwinian biological species evolution [6]. The reason for BCG sub-strains' transition remains unclear because the progenitor of BCG strains was lost. The comparative analyses of genome features of different BCG sub-strains can help in solving this problem.

The attention was focused on mobile elements of BCG sub-strain genomes especially on prophage sequences because of their contribution to the bacterial genome patterning. Following Brüssow et al. [7], 12 years later, we can reaffirm that there is a renaissance of phage research because now we have a lot of information about bacterial and phage genomes in the international databases. It was noticed that reintroduction of the fitness factor by phages usually influences the pathogenic factors of bacteria cells [8]. Thus, phages are of great importance for bacterial short-term adaptation and our goal was to estimate a potential contribution of prophage sequences on the mosaic structure of vaccine BCG sub-strain formation.

2. BCG genome sequencing

M. bovis AF2122/97 (Accession Number NC_002945) was the first *M. bovis* strain, where complete genomic sequence was determined [9]. The first BCG genomic sequence was performed for BCG Pasteur 1173P2 (NC_008769.1) [10]. The sequences of these strains such as Tokyo 172 (NC_012207.1) and Moreau RDJ (NZ_AM412059.1) were generated from the small-*insert libraries (*1–4 kb) by using BigDye terminator chemistry on ABI377- or ABI3700-automated

DNA sequencers [9–12]. The large-*insert library (40 bp) preparation method was used for* Mexico BCG sub-strain (NC_016804.1) sequencing [13]. Orduña et al. [13] were first who used for BCG genome analysis – the next generation sequencing (NGS) based on 454 technology and Sanger method. Late shotgun DNA libraries for strain sequencing were performed by commercial NGS kits. The strains were sequenced by the combination of 454 and Illumina platforms and the Sanger method, for example, BCG 3281 (NZ_CP008744.1) isolated from the human patient with TB and Korea 1168P (NC_020245.2) [14]. Single-molecule real-time sequencing (SMRT) based on PacBio Systems was allowed to directly sequence DNA and achieve long sequencing reads (>10,000 bp) with uniform coverage [15]. SMRT in combination with Illumina was used for *M. bovis* 1595 (NZ_CP012095.1) complete genomic sequence determination [16]. The further development of SMRT technology was allowed to use this platform alone for complete genomic sequencing. Sequences of BCG sub-strain 26/ATCC 35735/Montreal (CP010331.1) and *M. bovis* 30 (CP010332.1) could be considered as the example of this approach [17]. Whole genome sequence of BCG Russia sub-strain was performed using 454 and Sanger technology for short-gun and paired-end libraries. Whole genome map (WGM) creation was useful for the control of the repeat regions.

3. Comparative genome analyses as proof of BCG Russia genome stability

In the vaccine manufacture, one of the important features of BCG sub-strain is the genome stability. So, BCG vaccines' quality control and production now include characterization of BCG sub-strain genome. The importance of molecular genetic characterization is confirmed by the WHO requirements. According to these requirements, WGS of the last seed lot of BCG Russia (BCG Russia 368, 2006 year) was performed. Besides, two BCG Russia sub-strains from seed lots of 1963 and 1982 years (BCG Russia 311 and BCG Russia 977) were analyzed on the basis of WGS.

Comparative analyses of three BCG Russia sub-strains from different seed lots revealed only two differences. The first difference was the *single-nucleotide polymorphism* (SNP) in the position 3,175,301 (numeration according to reference strain BCG Tokyo) in the sub-strain BCG Russia 368. This SNP leads to the synonymous mutation in the uridylyltransferase gene. In the generation of 1963 and 1982, this mutation was not registered.

The second change in genome-affected glycerol-3-phosphate acyltransferase gene is shown in **Figure 1**. The mutation that occurred in this gene in the position 2,744,580 (an insertion of TGT bases instead of C base) truncated the protein. Nevertheless, the mutation was not concerned with the conservative domain of glycerol-3-phosphate acyltransferase and the protein could be functional. It should be noted that not all reads had the insertion of TGT. The changes were registered only for 14% reads of BCG Russia 311 and 54% reads of BCG Russia 977. In the last BCG Russia 368 generation, this mutation wasn't found. So, the genome structures of three different BCG Russia seed lots remain stable. The last BCG Russia 368 generation that is discussed in the text later was deposited in GenBank with the Accession Number NZ_CP009243.1.

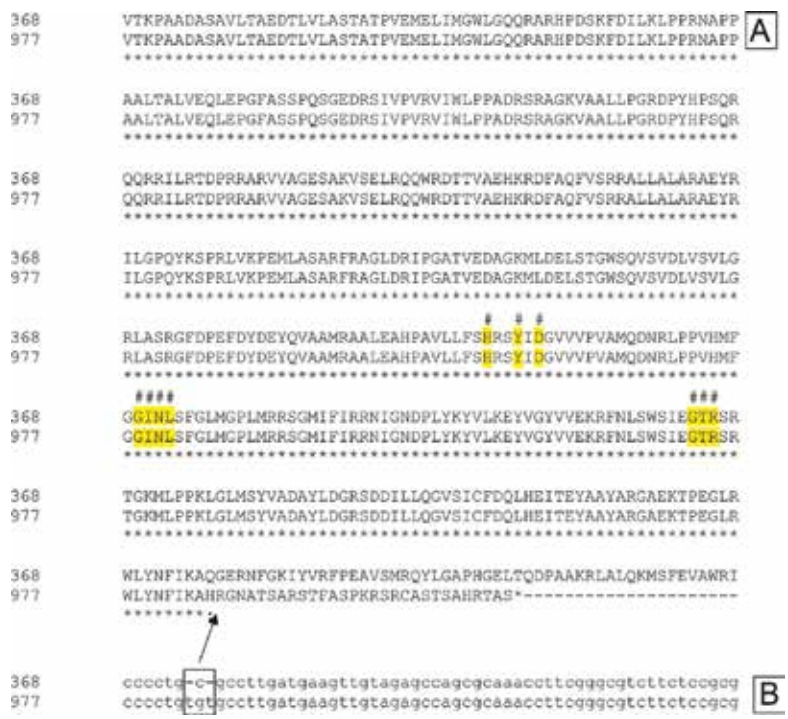

Figure 1. Comparison of different glycerol-3-phosphate acyltransferase variants in the three generations of BCG Russia. (A) Alignment of whole and truncated variants of glycerol-3-phosphate acyltransferase. (B) Fragment of glycerol-3-phosphate acyltransferase with mutation. Hash-amino acids residues in conservative domain of glycerol-3-phosphate acyltransferase important for the enzymatic activity.

4. *In silico* genotyping of BCG-Russia sub-strain

Genomic feature of BCG Russia, as well as all BCG sub-strains, is a large deletion of the 10-kb genomic region of difference 1 (RD1) [18].

Spoligotyping profile is the second known characteristic of BCG sub-strain. This method is based on detection of Direct Repeats (DR) on the right and left sides of IS6110. DR loci are members of a universal family of sequences, designated as clustered regularly interspaced short palindromic repeats (CRISPR) sequence family.

Spoligotype profile of BCG Russia was typical for *M. bovis* with the absence of spacers 3, 9, 16, and 39–43, the *in silico* pattern corresponded to spoligo-international-type number (SIT) 482 according to the SPOLDB4 Database [19, 20].

The Mycobacterial interspersed repetitive unit (MIRU) profile of BCG Russia sub-strain based on *12 MIRU loci was* 232,324,253,222 according to *in silico* genome analysis.

The whole data of BCG Russia MIRU-variable-number tandem repeats (VNTR) loci are summarized in **Table 1**. The repeat unit size (bp) and repeat number are indicated in the brackets,

Locus	Copy number of locus, bp*	Locus	Copy number of locus, bp
MIRU_2	(53 × 2) + 8	MIRU_23	(53 × 4) + 5
Mtub04	(51 × 0) + 30	MIRU_24	(54 × 2) + 30
ETR_C	(58 × 4) + 37	MIRU_26	(51 × 5) + 13
MIRU_4/ETR_D	(77 × 3) + 4	MIRU_27/QUB-5	(53 × 3) + 25
MIRU_40	(54 × 2) + 19	Mtub34	(54 × 2) + 51
MIRU_10	(53 × 1) + 51	MIRU_31/ETR_E	(53 × 2) + 3
MIRU_16	(53 × 3) + 18	Mtub39	(58 × 2)
Mtub21	(57 × 0) + 34	QUB-26	(111 × 4) + 24
MIRU_20	(77 × 2) + 11	QUB-4156	(32 + (59 × 0) + 19)
QUB-11b	(69 × 3) + 10	MIRU_39	(53 × 1) + 29
ETR_A	(75 × 5) + 20	QUB-3232	(56 × 5) + 48
Mtub29	(13 + (57 × 1) + 35)	VNTR-3820	(59 × 5) + 47
Mtub30	(58 × 1) + 53	VNTR-4120	(57 × 2) + 23
ETR_B	(57 × 5) + 8		

*(Repeat unit size (bp) × Repeat number) + Partial repeat size (bp).

Table 1. The copy number of MIRU-VNTR loci in BCG Russia genome.

partial repeat size (bp) is outside. One discrepancy in polymerase chain reaction (PCR) and *in silico* results was revealed. The number of repeat units in MIRU_4/ETR_D was three in accordance with MIRU-VNTR analysis of BCG Russia genome data obtained in three different laboratories (NZ_CP009243.1, CP011455.1, CP013741.1). The number of repeat units in MIRU_4/ETR_D of BCG Tokyo was identical. However, PCR analyzes of MIRU-VNTR performed by Supply et al. [21] for BCG Russia sub-strain and by Mokrousov et al. [22] for the strains isolated from BCGitis patients revealed only two repeat units in MIRU_4/ETR_D. The discrepancy in PCR and *in silico* analyses of MIRU-VNTR could be explained by difficulties in the amplification of high GC genomes. The similar discrepancy in PCR and *in silico* results was described by Iwamoto et al. [23] for *M. tuberculosis* H37Rv. The repeat number in Mtub39 locus was five in PCR analysis but two copies were revealed in *in silico* genome investigation.

5. Is the original BCG Russia sub-strain *recA* a mutant?

High degree of genomic stability of BCG Russia sub-strain is seen as an inexplicable fact by some scientists. One of the explanations of this fact proposed by Keller et al. is in the highly cited paper [24]. They postulated *recA gene inactivation* in BCG Russia sub-strain. RecA is a multifunctional and ubiquitous recombinase protein involved both in general recombination and in DNA repair. RecA-dependent recombination mediates genetic rearrangements

resulting in increased genetic instability, while RecA-mediated DNA repair mechanisms have been shown to be essential for intracellular survival and persistence [25].

Among the mechanisms of bacterial evolution, the leading role belongs to recombination events. The large-scale rearrangements, deletions and duplications were revealed during comparative genomics analyses in *M. leprae* [26], *M. tuberculosis* [27] and *M. bovis* BCG [10]. Gene duplication has led to the origin of the half tubercle bacillus proteins [28]. Tandem duplications and homologous recombination also make a significant contribution to the diversity of mycobacteria. As an example, recombination between adjacent repeats of IS6110 elements resulted in deletions of several genome regions in *M. tuberculosis* H37Rv [27], https//www.ncbi.nlm.nih.gov/pmc/articles/PMC2483709/ - B33 [29].

Keller et al. detected the single-nucleotide insertion of "C" at the 5′ end of the *recA* gene of BCG Russia sub-strain received from TD Allergen [24]. As a result, the stop codon was formed and recombinase A synthesis was absent. These data have not been confirmed by the genome analysis of the original BCG Russia sub-strain. Whole genome sequence (NZ_CP009243), obtained in our laboratory, and other sequences (CP011455.1, CP013741.1), did not have the single nucleotide insertion in the *recA* gene. So the complete reading frame for recombinase A was annotated. Thus, the original BCG Russia sub-strain is not a *recA* mutant and the stability of the BCG Russia genome cannot be associated with *recA inactivation.*

Keller et al. findings may indicate that the sub-strain used by authors was not original or has been changed during cultivation. The last one is possible. According to our data whole and truncated variants of glycerol-3-phosphate acyltransferase gene was identified in one of the BCG Russia generations (**Figure 1**).

6. The "early" sub-strain genomes comparison

Genome sequences were compared using BCG sub-strain Tokyo 172 genome, a member of the "early" sub-strains group as reference. First, among this group, BCG Tokyo sub-strain is closest to BCG Russia sub-strain as regards the time of its provision by the Pasteur Institute to Tokyo (in 1925). Second, it was lyophilized in the 1940s and used later as a freeze-dried vaccine, as BCG Russia sub-strain. Then, in 1960, the 172nd transfer on bile-potato medium was freeze dried and adopted as a primary seed lot [30]. Finally, one of the first BCG genomes that were accurately sequenced, assembled, and submitted to GenBank was the genome of this seed lot [11].

We observed no significant diversity in the sequences of the BCG Russia 368 and BCG Tokyo 172 genomes. The revealed genomic differences were summarized in **Table 2** and could be subdivided into three groups: region of differences (RDs), ins/del and SNP. Only two RDs were detected between the "early" sub-strains. First, a 22 bp insertion was found in the TetR family transcriptional regulator gene of BCG Russia 368 genome. One variant of Japan BCG vaccine (Type I), submitted in GenBank, included this deletion (RD16). The RD16 band identical to those of other BCG sub-strains was found in the Type II strain [31]. A 1602 bp deletion in BCG Russia

Type of differences	Number of differences	
	BCG Russia368/BCG Tokyo 172	**BCG Tokyo 172/BCGPasteur1173P2 [11]**
Region of Differences (more than 20 bp)	2	20
Insertions/deletions <20 bp (1–9 bp)	10	20
SNP in total	52	68
intergenic SNP	11	
synonymous SNP	8	
nonsynonymous SNP (without nonsense)	31	
Nonsense SNP as variant of nonsynonymous	2	

Table 2. Genomic differences of BCG Russia 368, Tokyo 172, and Pasteur 1173P2 sub-strains.

368 genome was the second RD, corresponding to the region from 4,110,452 to 4,112,053 bp in BCG Tokyo 172, beginning in JTY_RS19265 (ribonuclease gene), including JTY_RS19270 (antitoxin VapB48 gene) and finishing inside JTY_RS19275 (glutamate-cysteine ligase gene).

The sub-strains used for vaccine production in Bulgaria (BCG Sofia) and India were obtained from BCG Russia. Nowadays, UNICEF uses four variants of BCG vaccine on behalf of the Global Alliance for Vaccines and immunization. The Statens Serum Institute in Denmark produces BCG-Denmark; Bulbio (BBNCIPD) in Bulgaria; and the Serum Institute in India produces BCG-Russia (genetically identical to BCG-Bulgaria) and the Japan BCG laboratory produces BCG-Japan [32].

We could trace the genome characteristics of BCG Russia daughter sub-strains using published data. Stefanova et al. analyzed the BCG sub-strain used for production in Bulgaria (named Sofia SL222) with *M. tuberculosis* microarrays. They detected a 1.6-kb deletion that affects Rv3697c and Rv3698 homologs. The deletion of this region was also noted in BCG Russia but not in any other strains [33]. The authors concluded that RD 1602 bp is an old deletion, because BCG Pasteur was replaced with BCG Russia in Bulgaria BCG laboratory in the 1950s.

According to Seki M. et al. differences between the "early" sub-strain Tokyo and the "late" sub-strain Pasteur were more significant and the number of RD increased tenfold [11].

Less ins/del differences were found between BCG Russia and BCG Tokyo genomes, then between BCG Tokyo and BCG Pasteur genomes. The size of ins/del differences was small: only 1–9 bp.

However, the number of SNPs was nearly the same in the two pairs of the genomes. Nonsynonymous SNP in BCG Russia 368 amounted to 60%, but most of them were associated with conservative substitutions in the proteins. Only seven proteins had radical substitutions, though three of them were from the PE-PGRS/PPE family. This finding has emphasized the significance of these proteins for BCG sub-strain adaptation.

7. A whole genome restriction map analysis

The large array of published literature accentuated the important role of RD in BCG sub-strains differentiation. For checking these statements the methods by OpGen Incorporated Company was used. So, first of all, the assembly of DU2 region and the number of tandem duplications in this region in BCG Russia 368 genome were performed by the Argus™ Optical Mapping System. WGM of the sub-strain BCG Russia 368 was created by the laboratory of OpGen Incorporated Company (Maryland, the USA), according to the Argus™ Optical Mapping System user manual [34]. DNA was digested with *NheI*. Map Solver software version 3.2 was employed for creating the final circular WGM; the whole genome map of BCG Russia 368 is represented in **Figure 2**. The separate comparison of DU2 regions (**Figure 3**) has shown that genomes BCG Russia 368 and BCG Tokyo 172 are identical in this region, unlike from the BCG Pasteur optical map, which can be confirmed by presence of three copies (triple tandem duplications) in the DU2 region of BCG Russia 368.

The cluster construction based on map similarity of the six references of BCG sub-strains is shown in **Figure 4**. As you can see, the cluster was split into two groups: BCG Tice (ATCC 35743) was attributed to the group of the "early", while BCG Mexico to the "late" group of sub-strains in accordance with the *NheI* restriction fragments.

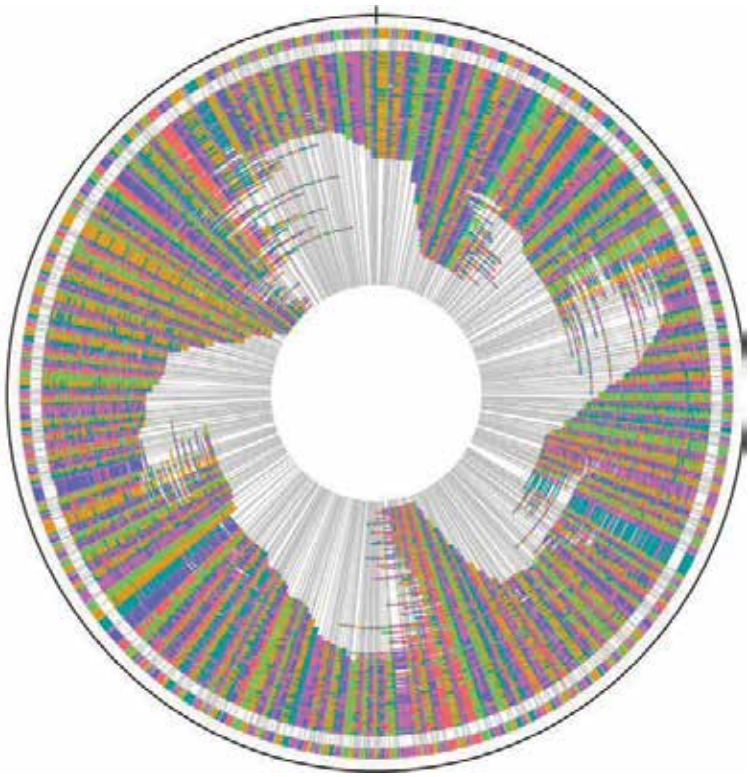

Figure 2. The circular restriction map of BCG Russia 368 whole genome. The restriction map was obtained by DNA digestion with *NheI*.

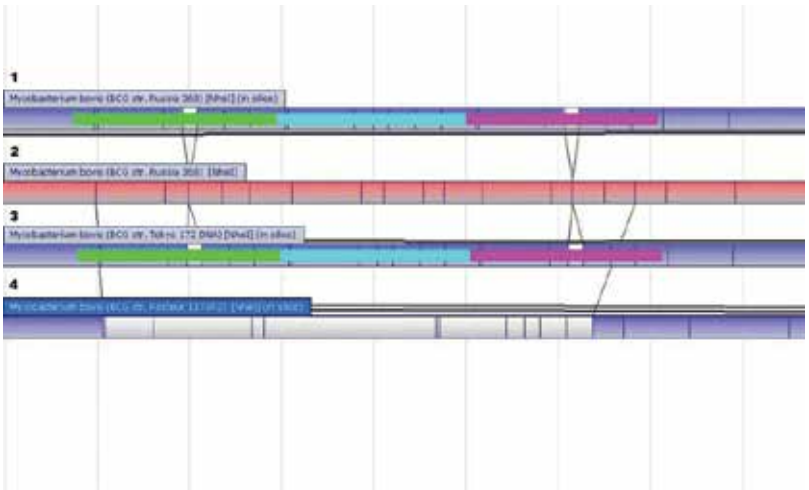

Figure 3. Aligned OpGen maps for BCG Russia 368 and reference BCG sub-strains created for DU2 region. (1) OpGen map created *in silico* for BCG Russia 368 genome fragment. (2) OpGen map of BCG Russia 368 whole genome digestion with *NheI in vitro*. (3 and 4) OpGen map created *in silico* for BCG Tokyo 172 and BCG Pasteur 1173P2 genome fragments. All OpGen maps were created by DNA digestion with *NheI*. Vertical lines are pointed out at restriction sites. Tree copies of the DU2 region in BCG Russia 368 and BCG Tokyo 172 genomes are marked as green, blue and purple bars. The genome region from the *astB* to the *sdhD* genes (DU2 region) is represented as the green bar. The *astB* gene in the second and the third copies of DU2 region (blue and purple bars) was truncated.

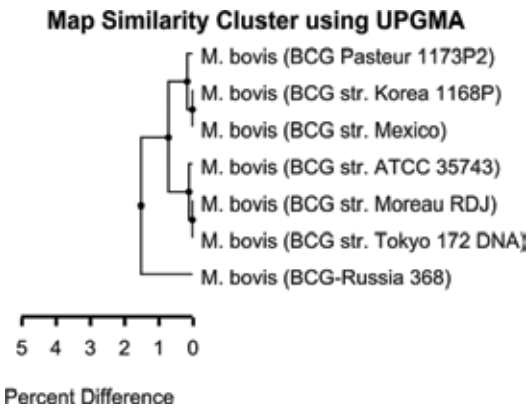

Figure 4. Map similarity cluster reconstruction for seven BCG sub-strain. The optical restriction maps of BCG Russia 368 and six reference BCG sub-strains obtained *in silico* were used for map similarity cluster reconstruction. The cluster construction was carried out using UPGMA (unweighted pair-group method using arithmetic averages) algorithm in OpGen MapSolver v.3.2.0. Program.

8. Genome map construction and the analyses of repetitive elements of BCG Russia 368

The whole genome gap-less BCG Russia 368 chromosome after the verification of the number of DU2 repeats was visualized in GeneWiz [35] (**Figure 5**). Genome atlas option of GeneWiz primarily GC Skew was selected as an appropriate instrument for verifying the accuracy of

genome assemblies and OriC detection. The place of the change in GC Skew agreed with the OriC and the first nucleotide position in the BCG Russia 368 genome. Other DNA properties, intrinsic curvature, stacking energy, position preference, global direct repeats, global inverted repeats, and AT-content, were essential for genome structure description.

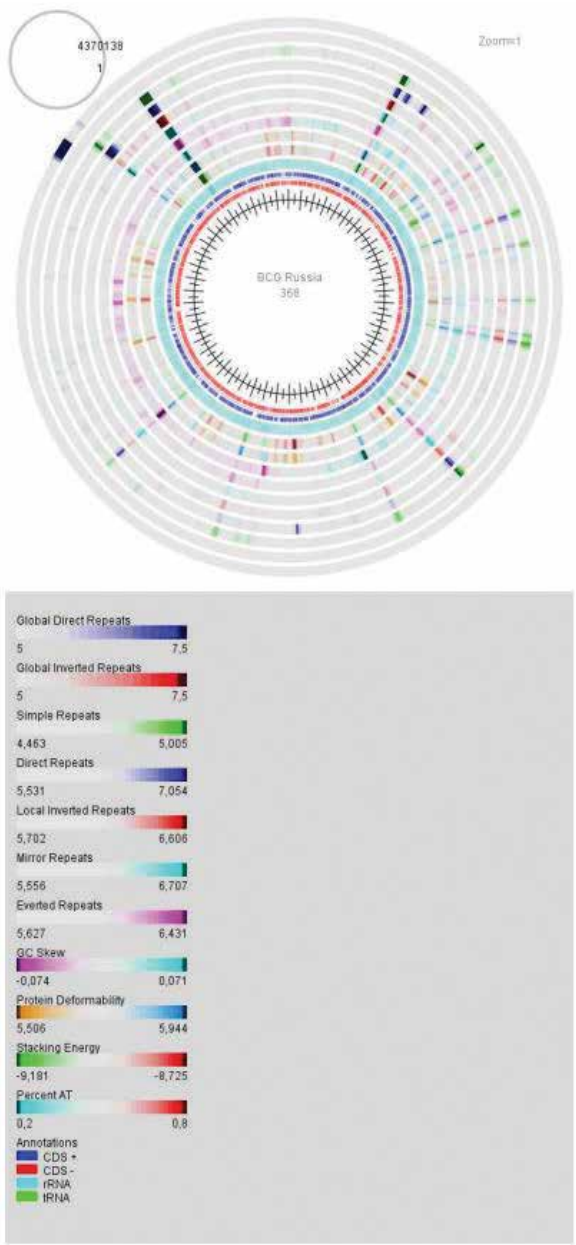

Figure 5. *M. bovis* BCG Russia 368 genome map.

Different types of repeats visualized by GeneWiz and shown in **Figure 5** correlated with the specific genome elements identified with the specific resources (**Table 3**).

Name	Number
REP	6
CRISPR	5
Prophage	2
PPE protein gene	66
PE protein gene	33
PE_PGRS protein gene	69
IS elements	41

Table 3. Specific genome elements in BCG Russia genome.

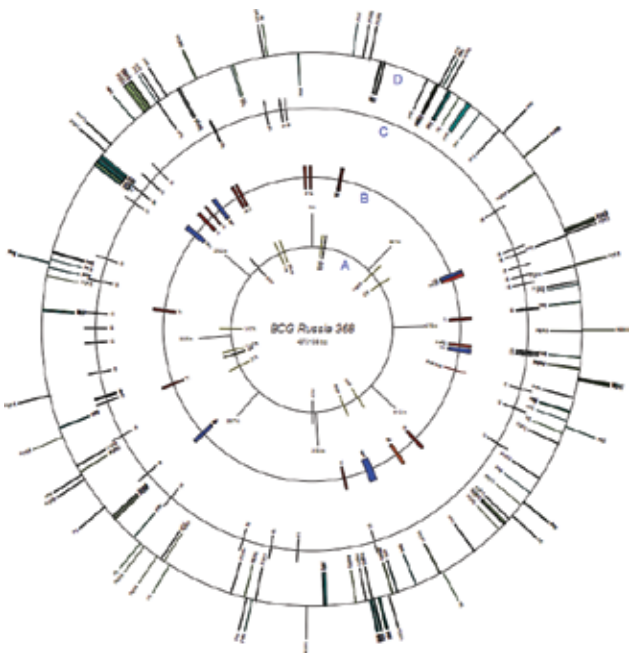

Figure 6. Localization of mobile elements (IS, repeats, prophage sequences), PE, PPE, and PE_PGRS genes in BCG Russia 368 genome. The circular map of BCG Russia 368 genome was visualized by the GenomeVx program. All of the prophage sequences were predicted by PHAST. Description of scheme: Repeats (REP, VNTR, and CRISPR elements) – circle A; phage sequences (according to PHAST) – B; IS elements – C; genes for PE, PPE, and PE_PGRS proteins – D. Accepted abbreviations: REP – Repetitive extragenic palindrome element; CR – CRISPR or possible CRISPR sequences predicted by CRISPRfinder; VNTR – Variable number tandem repeat; IS – Insertion sequence elements. Phages sequences: TI – BCG tice (CP003494.1); MN – BCG Montreal (CP010331.1); AF – *M. bovis* AF2122/97 (BX248333.1); PHR-2-rep – (922 bp repeat of 7.5 kb), PHR-1 – (11 kb), and PHR-2 – (7.5 kb) of BCG Russia. In the color code-ciphered phage sequences discovered in different *M. bovis* genomes: BCG tice (CP003494.1) – purple; BCG Montreal (CP010331.1) – blue; *M. bovis* AF2122/97 (BX248333.1) – orange; BCG Russia 368 (CP009243) – red.

The **insertion sequences (IS) elements and** affiliated resolvases, transposases, and integrases genes **were predicted by** ISfinder. Classification of IS elements and determination of inverted repeats flanking IS were made by the use of ISfinder database [36, 37]. Clustered regularly interspaced short palindromic repeats (CRISPR) and RISPR-associated Cas and Csm family proteins were predicted by CRISPRfinder [38, 39].

Locations of IS elements, repeats, prophage sequences, and PE, PPE, and PE_PGRS genes in BCG Russia 368 genome are visualized in **Figure 6**. Most of the repetitive elements of BCG Russia 368 genome, including some prophage sequences, are coinciding, overlapping, or interconnecting. So, it is hard to annotate some fragments of BCG Russia 368 genome. Special difficulties have arisen in the differentiation of bacterial and phage genes during PE/PGRS genes characterization.

9. The phages predicted in *M. bovis* genomes

Along with other mobile elements, the variability of predicted prophages may be the best indexes for characterization of mosaic structure genome. All of the prophages described in *M. bovis* genome were computed us by PHAge Search Tool (PHAST) [40, 41]. GenVision Plug-In of the DNASTAR Lasergene program package was selected for the visualization of prophage sequences. According to PHAST data, all of the predicted phages could be shared into three groups (see **Figure 7**). So, the first group composed of the common ones for *M. bovis* and *M. bovis* BCG prophages. A 7.5-kb prophage was revealed in most of the BCG genomes and in three *M. bovis*; exceptions include– BCG sub-strains Tice and Montreal. A 20.3-kb prophage in "early" sub-strain genomes (BCG Tokyo, Moreau, Russia) was replaced by an 11.2-kb one but was lost in "late" sub-strain genomes. The second group represented six BCG Montreal prophages, the third represented 15 BCG Tice sub-strain prophages. The prophages in the second and third groups were unique and did not coincide with the prophages of other sub-strains (see circle B in **Figure 6**). Like most of phage ORFs in the common ones, *M. bovis* prophages were annotated as genes belonging to the order Caudovirales (Myoviridae, Siphoviridae, and Podoviridae family), while most of phage ORF in BCG Tice or BCG Montreal prophages were similar to the genes of various Herpesviruses (Human, Bovine, Macaci, Alcela, Anguil).

The mosaic BCG genome structure has been verified by comparative prophage analyses. A partial similarity of BCG Tice/BCG Montreal prophage fragments has been identified after pair-wise alignment of BCG Montreal and BCG Tice phage sequences with BCG Russia 368 whole genome. The regions of similarity defined as the purple (BCG Tice) and blue (BCG Montreal) blocks on circle B are phage sequences discovered in different *M. bovis* genomes (**Figure 6**). In BCG Russia 368 genome, Tice-specific prophages (13.4 and 13.9 kb) were split into five and three parts, respectively. Fragments which are homologous to BCG Montreal-specific prophages represented in the genome of BCG Russia 368 as a sequence with multiple gaps ranging from 14 to 128 bp.

In turn, the 7.5-kb BCG Russia 368 prophage was split on 0.9- and 6.6-kb fragments located in different regions of BCG Tice/Montreal genomes. Moreover, these fragments lacked the transposase gene, which was specific to 7.5-kb BCG Russia 368 prophage.

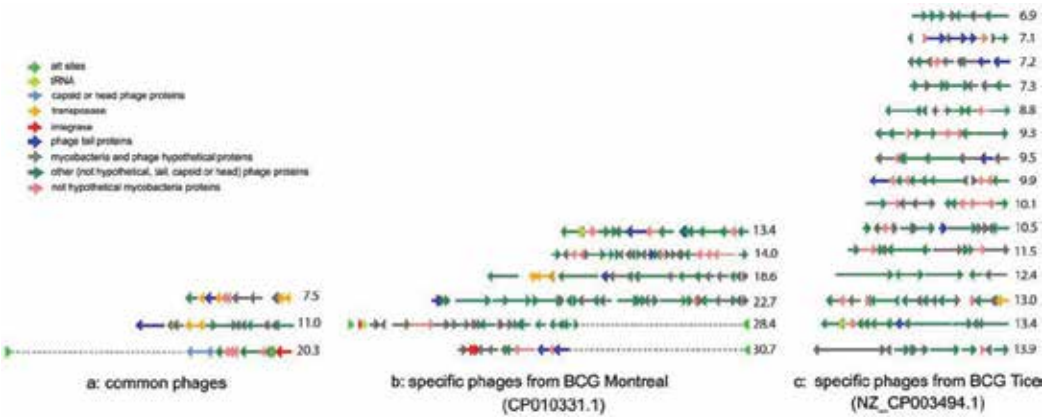

Figure 7. Comparative analyses of phage types from BCG Russia 368 and references *M. bovis* genomes. Color arrows indicate the location of phage genes. (a) Phages common for all analyzed genomes: 7.5, 11.0, and 20.3 kb – In all the analyzed strains (accession NZ_CP009243.1, NC_012207.1, NC_008769.1, NZ_CP003494.1, CP010331.1, NC_016804.1, NC_002945.3, NZ_AM412059.1, NZ_CP008744.1, NZ_CP012095.1, CP010332.1, NC_020245.2); (b) phages specific for BCG tice: 13.4, 14.0 18.6, 22.7, 28.4, and 30.7 kb; (c) phages specific for BCG Montreal: 6.9, 7.1, 7.2, 7.3, 8.8, 9.3, 9.5, 9.9, 10.1 10.5, 11.5, 12.4, 13.0, 13.4, and 13.9 kb.

Also, the 7.5-kb BCG Russia 368 prophages had 922-bp repeats in BCG Russia 368 genome (red bar in **Figure 6**, circle B) and was located near ISMt1 insertion element. Interestingly, besides prophage fragments, two intact prophages associated with the insertion elements have been predicted by PHAST in BCG Russia 368 genome. Thus, 11-kb and 7.5-kb prophage sequences were linked with IS6110 and IS1560 elements, respectively. So, the connection between prophage sequences and the IS elements has a considerable impact on the BCG genome evolution.

10. Phylogeny reconstruction

Phylogeny reconstruction was made using the genome sequences of analyzed *M. bovis* strains and BCG sub-strains. The full-genome comparison and phylogeny reconstruction were based on BLAST alignment and neighbor-joining algorithm [42] used in NCBI BLAST. The trees were represented by MEGA 6.0 [43]. Taking into account the prophage profile data, the congruence of obtained phylogenetic tree and vaccine sub-strains genealogy based on the DU2 region [10, 44] has been evaluated. *M. bovis* strains and BCG sub-strains formed different clusters on the tree (see **Figure 8**). As was expected, the "early" (Russia, Tokyo) and the "late" (Pasteur 1173P2, Korea 1168P, Mexico) BCG sub-strains formed separate but closely related groups. The basal branch in the BCG cluster was represented by BCG Moreau sub-strain. The unexpected position of the BCG Tice and the BCG Montreal has been revealed. The BCG Montreal showed some relationship with the "early" sub-strains. The BCG Tice has been placed in the most divergent basal position on the tree. In turn, BCG 3281, isolated from a pulmonary TB, patient had the relationship

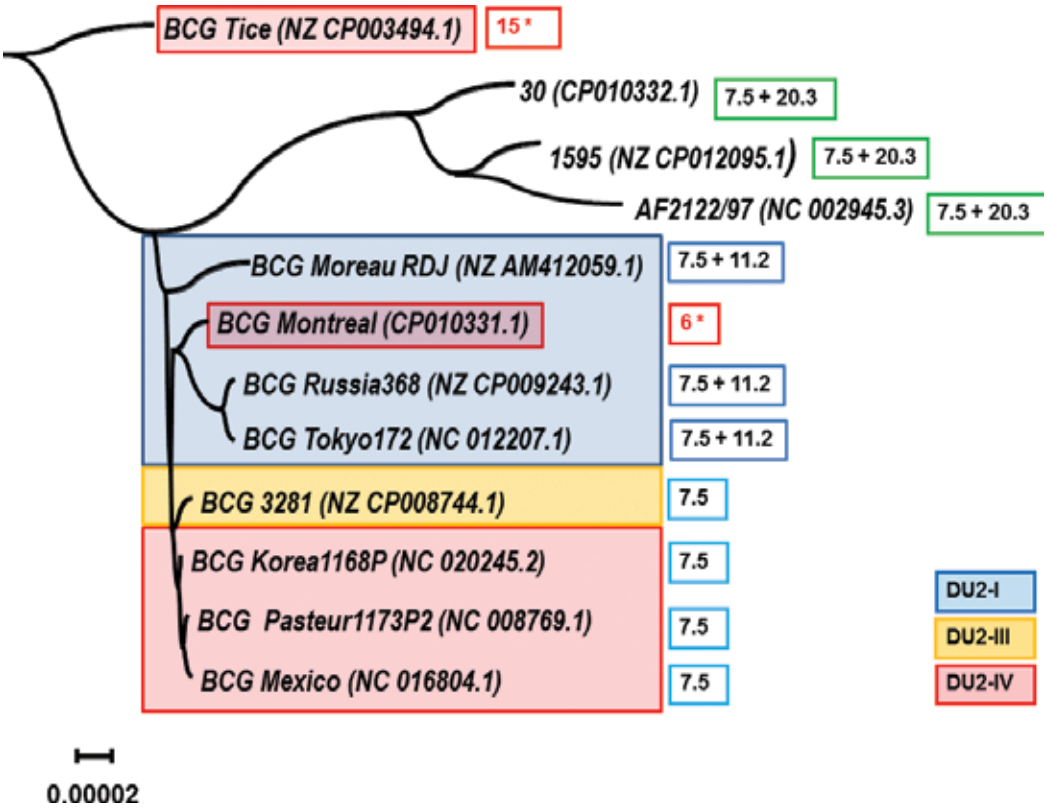

Figure 8. Whole genome phylogeny of *M. bovis* strains. Colored blocks describe the DU2 region type in BCG sub-strains: blue – DU2-I, yellow – DU2-III, red – DU2-IV. Unpainted blocks with colored borders and numbers inside define types of prophage profiles and prophage size in kb. Red borders with asterisks indicate the number of unique prophages identified in BCG tice and BCG Montreal genomes.

with the "late" sub-strains. Remarkably, all phylogenetic groups of the tree were characterized by specific sets of prophage sequences. The DU2 region genealogy only partially correlated with the whole genome phylogeny obtained in this study. If the DU2-I "early" sub-strain group showed common origin but the DU2-IV group split apart (see **Figure 8**). The BCG 3281 that represented DU2-III group [45] took an intermediate position. One of the possible explanations for this discrepancy may be numerous prophage-associated genome rearrangements.

11. Discussion

Numerous comparative genomics investigations of BCG sub-strains confirmed significant genomic polymorphism of BCG sub-strains which arose from one progenitor. RDs, indels and SNPs are real evidences still going on in the *in vitro* evolution of BCG sub-strains.

Here we supposed that genomic evolution and the BCG sub-strains diversity is a direct consequence of prophage-associated genome rearrangements. It is well known that 10–20% of bacterial genomes represented prophage sequences. Most of the prophages are damaged

and mutated. Nevertheless, recombinant events between homologous prophage sequences are possible. Moreover, some genes of defective prophages can be still working [46]. So, prophage genome content is an important biological driver/trigger of genomic rearrangements and evolution. Extensive contribution of prophages to bacterial fitness was supposed as a result of unexpected evolutionary prophage patterns [47]. Suggesting our assumption, outstanding differences between prophage profiles have been revealed in our comparative genome analysis of nine BCG sub-strains and three *M. bovis* strains. Big differences in the number and composition of prophages in the genomes of the late strains Tice and Montreal were discovered. According to the Brosch et al., both BCG Tice and BCG Montreal or Frappier were taken from the Pasteur Institute after 1934. They had close phylogenetic relations because they fall in one phylogenetic group, "DU2 IV, Δint" [10]. Dr. Rosenthal, who received the first Tice sub-strain from the Pasteur Institute, demonstrated heterogeneity of the "late" BCG sub-strain. It was a progenitor of at least six different daughter BCG sub-strains: H, K, E, L, LH, and BL. The sub-strain BL was strongly attenuated in laboratory studies. In 1952, BL was mixed with a new routine 'P' strain, received from the Pasteur Institute in 1951, in the ratio 3:1. This new sub-strain was called BLP. Since 1953 only freeze-dried BCG vaccine from this mixed strain has been produced [30]. The history of BCG Montreal sub-strain is also well known. Three times these BCG sub-strains were sent to Canada from the Pasteur Institute [30]. Significant changes of BCG genomes have been reflected in the appearance of new prophage profiles in BCG Tice and BCG Montreal sub-strains. It could also impact on vaccine properties of the sub-strains. According to Zhang et al. [48], BCG Tice, BCG Montreal/Frappier along with BCG Prague, and BCG Phipps sub-strains have lost the largest number of T-cell epitopes, defining its vaccine properties. In contrast, BCG Russia and BCG Tokyo sub-strains still have the largest number of T-cell epitopes among other BCGs. So an extended genomic sequencing is very important to identify prophages as potential markers of genomic rearrangement. Prophage studies could enhance our understanding of the genetic features of various BCG sub-strains and may also be useful for checking the genetic stability of the seed-lot sub-strain.

12. Conclusions

People migration from regions with a high incidence of TB and the growth of the number of HIV-infected individuals last decade resulted in the necessity of TB vaccination not only among children but also among adolescents and adults. In 2015, 15 vaccine candidates were considered in clinical trials. BCG vaccine replacement or vaccine boosting for the protection of adolescents and adults were considered. Recombinant BCGs, recombinant viral-vectored platforms, protein/adjuvant combinations, attenuated *M. tuberculosis* strains and mycobacterial extracts were included in the list [1]. A subunit vaccine developed in N.F. Gamaleya Research Center was based on the fusion of mycobacterial proteins with cellulose-binding domain [49]. On the other hand, new areas of BCG vaccine application have been proposed. As most humans are born in bacteriological environments characterized by a low microbial diversity, the effects of BCG vaccine administrated immediately after birth, as a modulator of Th-1/Th-2 responses, is very important and should be analyzed [50]. In this situation, the control of BCG genome stability is the important task, which will continue to be relevant.

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Chapter 12

Virulence Factors and Pathogenicity of Mycobacterium

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Additional information is available at the end of the chapter

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Abstract

Virulence, is referred as the ability of a pathogen to cause disease, and for mycobacteria it depends on their ability to reside within host cells and evade the microbicidal mechanisms of macrophages. The outcome of tuberculosis (TB) infection is highly variable and it seems that the closest relationship between the *Mycobacterium* genre and humans has shaped the mycobacterial genome to encode bacterial factors that reflects a highly evolved and coordinated program of immune evasion strategies that interfere with both innate and adaptive immunity causing disease even in fully immunocompetent host. Although *Mycobacterium tuberculosis* (MTB) does not have classical virulence factors, it has described many virulence-associated genes and virulence lifestyle genes from *Mycobacterium tuberculosis* complex (MTBC). In this chapter, we describe the most important gene/molecule involved in the host defense modulation response, also the plethora of strategies to evade immune mechanisms of macrophage. We review the main genes whose inactivation in the mycobacterial genome leads to a measurable loss in virulence in the different validated TB models.

Keywords: virulence factor, pathogenicity, mycobacterium, cell wall, immune evasion, tuberculosis

1. Introduction

Tuberculosis (TB) is mostly caused by *Mycobacterium tuberculosis* (MTB) and *Mycobacterium africanum*, both members of the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related species which are adapted to human and animals. The outcome of TB infection is highly variable and is determined by the response of the immune system and environmental variables, but a deeper knowledge of the global genomic diversity in the MTBC suggests that bacterial factors are also involved. To better understand the virulence mechanisms of the

MTBC, it is necessary to define what constitutes a virulence gene. There are a wide variety of conditions and parameters to define it, but undoubtedly a true virulence gene codifies for factors or enzymes producing factors that are involved in the interaction with the host, and are directly responsible for the pathological damage during infection and are absent in non-pathogenic bacteria. Perfect examples of virulence genes are the toxins produced by *Vibrio cholera* and *Escherichia coli* O157. MTB does not have classical virulence factors; however, many virulence-associated genes and virulence lifestyle genes from MTBC have been described [1]. Virulence-associated genes, are genes coding for factors that regulate expression of virulence genes and activate virulence factors by translational modifications, processing or secretion; whereas, virulence lifestyle genes codifies for factors that enable colonization of the host, enable evasion of the host immune system and enable intracellular survival. Besides, unlike other pathogens, MTB virulence is directly linked to its transmission. Thus its virulence can be measured through (1) the ability of the bacteria to survive the host immune response, (2) their capacity to cause lung damage and (3) to be successfully transmitted to infect a new host [2]. MTB pathogenicity, defined by its ability to cause disease in a host organism, has co-evolved with its physiology as specie. Initial infection is mainly through respiratory tract, here the alveolar macrophages are the most common cell type infected by MTB and the inflammatory signals arising from infection promote the influx of additional monocytes and macrophages, which become infected as well. Inflammation is required for initial control of infection but can also cause extensive tissue damage. Moreover, the bacteria exploit the host inflammatory signals to spread to other individuals. Because of that, it is believed that its pathogenicity is likely to have evolved from its specific adaptations to host immunity. In fact, Comas et al., showed that a large majority of the T cell epitopes, representatives of several linages of the MTBC, had high sequence conservation, indicating that there is a high strong selection pressure to keep those T cell epitopes unchanged, which suggest that MTBC might benefit from being recognized by T cells, because as referred above, MTB virulence depends on its transmission [3]. While a significant number of genes have been shown to be important for the progression of TB, we focus on only few examples. We place emphasis in those genes whose inactivation in the mycobacterial genome leads to a measurable loss in virulence in the different validated TB models. The mycobacterial cell wall and envelope are unique among bacteria and many of their components are known to play an important role in the TB pathogenesis. Taking these concepts into consideration, we analyzed separately their lipids, secreted proteins and systems that play a role in the synthesis of various cell surface molecules. Thereafter, we describe the main proteins that inhibit antimicrobial effectors of the macrophage.

2. Models for measuring MTB virulence

MTB virulence is studied in cell culture and animals models. Thus different parameters of pathogenicity according the TB models are used. A hallmark of MTB pathogenicity is the ability to infect and survive within macrophages; thus, primary macrophages and cell lines are used to analyze the virulence of MTB and mutants at the early stages of infection. Primary macrophages are more representative of the natural *in vivo* situation but they are difficult to propagate to

sufficient numbers for virulence experiments. Primary murine bone marrow-derived macrophages (BMDMs) and human macrophages from peripheral blood monocytes (HMDMs) are the most common primary macrophages used, whereas the immortalized cell lines THP-1, J774 and MH-S cells have been widely used to study MTB-macrophage interactions. Data from both cell type should be interpreted with care because the response of MTB to intracellular environment can vary greatly depending on the cell type used [4]. In addition to evaluate the survival, replication and the intracellular bacillary load of MTB in the macrophages models, it is possible to study some of its mechanisms to counteract the macrophage microbicide ability, such as (1) the resistance to reactive oxygen/nitrogen intermediates (ROI/RNI), (2) the phagosome arresting and (3) the inhibition of apoptosis [1]. On the other hand, in animal models it is possible to study all the stages of the TB infection, although their ability to replicate the different aspects of human TB pathology varies. The major models used are mice, guinea pigs and rabbits [5]. Mice is the most frequent *in vivo* model used because of their well-characterized genetics and the huge collection of immunological reagents as well as the existence of inbred strains, their susceptibility to MTB is low and their pathology is unlike that in humans [5]. Guinea pigs are very sensitive to MTB and develop a disease with multiple similarities to human disease, such as lung necrosis, lymphadenopathy and disease dissemination. And the rabbit model develops lung granulomas which closely resemble the histology of human TB when they are infected with *M. bovis*, due to their size, high cost and lack of immunological reagents that make the model less tractable than mice. The close similarity between MTB and *M. bovis* make the cattle model a very attractive way to study TB pathogenesis. The bovine TB disease pathology is very similar to human TB, with caseating granulomas in the lungs and a similar latent phase in the infection. The major advantage of the cattle model is the possibility to conduct field trials, which makes it very attractive for vaccine studies [6]. The non-human primate model are the only TB model that develops all the clinical disease states found in human TB, and although its use has been invaluable in TB research, it is limited by ethical concerns and high cost. The most important parameters associated with virulence in animal models, besides mortality and morbidity, are: the bacterial load, the numbers of bacteria found in the infected host after the initial infection, histopathological changes and inflammatory responses. Finally, it is important to mention that zebrafish model has also been shown to be very useful to elucidate the early events of the mycobacterial infection, particularly in the study of the mechanisms of granuloma formation and its role in controlling the infection [5]. Infection of zebrafish with *M. marinum*, a closest relative of MTBC, resembles many aspects of human tuberculosis; in fact, crucial virulence factors, host genes and immune cell types are conserved in the zebrafish-*M. marinum* model [7]. Using this model, the bacterial RD1 locus was found to be required for efficient granuloma formation and that the ESX-1 system was responsible for the cell death of infected macrophages [8].

3. Molecules involved in pathogenesis and virulence

3.1. Mycobacterial lipids

Mycobacterial cell wall is rich in lipids and has exceptional physico-chemical properties as a strong impermeability; and even it has peptidoglycan on it, mycobacteria are acid fastness

organisms due to the large amount of lipids. In this section, we describe mycobacterial cell wall components and their relation with pathogenicity and virulence, the second part of the chapter is dedicated to study the phenomenon produced by some of the mycobacterial molecules.

3.1.1. Lipoarabinomannan (LAM)

Lipoarabinomannan (LAM) is a glycolipoconjugate composed by an anchor mannosyl phosphate inositol (MPI), a polysaccharide backbone and diverse capping motifs species [9]. Correct translocation of LAM in to the cell wall constitutes an important feature for the mycobacterial stability, the lack of *O*-manosilation was associated with increased production of LAM and increased release of LAM/LprG protein and consequently with a reduction in virulence of MTB *O*-manosilated deficient strain [10]. Variation in the mannose-capped arabinan ManLAM motifs between LAM from different strains and clinical isolates, may be responsible of the production of interferon-γ (IFN-γ) in CD-1b-restricted cell lines; and also, in differences in adherence to macrophages [11]. LAM inhibits phagosome maturation characterized for the presence of immature phagosome marker rab5, and allows intracellular surviving. LAM is released in the macrophages and intercalates with endomembranes, phenomenon essential in order to block phagosome arresting and also, the delivery of lysosomal hydrolases via the molecule EEA1, which blocks impeding phagosomal acidification [12]. There are a large number of publications compiled in the review [13] that describe the cytokine expression activity trigger by LAM in macrophages and dendritic cells (DC). Although there are differences in methodology and results among the data; to compile, the 20 publications analyzed include: an increased expression of tumoral necrosis factor alpha (TNF-α) in most of the publications, increased of IL-10 in some of the data and results showed different IL-6 and IL-12 expression. ManLAM from MTB induce the expression of IL-12 and apoptosis in macrophages [14], whereas in T cells, lipid microdomains suffer insertion of PILAM with no apparent interaction with a specific receptor, this phenomenon trigger Th2 cytokine production and a decreased Th1 cytokine expression [15].

3.1.2. Lipomannan (LM)

Lipomannan (LM) is a multiglycosylated lipid or polymannosylated Phosphatidylinositol mannoside (PIM). LAM and LM coexist in the mycobacterial cell wall. LM has been considered an innate immunity antigen; tetra-acylated LM activates macrophages using TLR2/TLR4 in a dependent way of MyD88. Di-acylated molecules regulate and inhibit the production of NO secretion and cytokine in macrophages activated by lipopolysaccharide (LPS) [16]. LM purified from *M. kansasii* and *M. chelonae* in a CD14-TLR2-dependent mechanism, induce secretion and expression of mRNA of IL-8 and TNF-α in THP1 macrophages [17]. It had demonstrated that LM from *M. kansasii*, *M. bovis* bacillus Calmette-Guérin (BCG) and *M. chelonae* induced a dual function in macrophages: activation with surface expression of CD40, CD86; production of TNF and NO secretion in a TL2 MyD88 dependent way; and also, and inhibition of expression of TNF and IL-12p40 and NO in macrophages activated with lipopolysaccharide (LPS) in a TLR independent way [18]. LM from *M. kansasii*, *M. chelonae*

and MTB induced apoptosis and IL-12 in THP1 macrophages [14]. Tri-tetra-acylated forms of BCG LM were suggested as responsible of the pro-inflammatory response. Try-acyl LM response is dependent of TLR2/TLR1 and MyD88 TIRAP and produce IL12 and NO. In *M. smegmatis*, structural changes of LM and LAM unleash the loss of acid-fastness, faster killing by macrophages by THP1 macrophages and led to higher sensibility to antibiotics; whereas in MTB lead to an attenuated infectivity in mice and antibiotic sensibility [19].

3.1.3. Phosphatidylinositol mannosides (PIMs)

Phosphatidylinositol mannosides (PIMs) constitute a substantial component of the cell envelope, precursor of LAM and LM. PIM has a variable number of mannose units and acylation, virulent species have high order PIM (5 or 6 mannoses) that contribute to the uptake of macrophages by mannose receptor (MR); lower order PIM with few mannoses interact with DC-specific intercellular adhesion molecule-3-grabbing non-integrin DC-SIGN from DC [20]. The acylation state of PIM can induce granuloma formation and cell recruiting in BCG infection; specifically PIM_4, PIM_6 were used, and the acyl chain was responsible for NKT recruitment [21]. In contrast, glycolipids from MTB as PIM and ManLAM inhibited $CD4^+$ T cell activation by interfering in the phosphorylation and T cell receptor signaling [22]. Host inflammatory response such as TNF, IL-12p40 was inhibited by PIM in murine macrophages through CD14-dependent and CD14-independent mechanisms [23]. PIM induced an increased presence in culture supernatants of alveolar epithelial cells (AEC) of the anti-inflammatory cytokine transforming growth factor beta (TGF-β) and a significant production of reactive oxygen species (ROS) [24]. Diacyl-phosphatidylinositol dimannoside (Ac_2PIM_2), acylphosphatidylinositol hexamannoside ($AcPIM_6$) and diacylphosphatidylinositol hexamannoside (Ac_2PIM_6) from virulent MTB stimulate and drive proliferation in bovine PBMC from *M. bovis*-infected cattle; also the IFN-γ expression in PBMC was increased only during exposition to $AcPIM_6$ [25].

3.1.4. Trehalose-6,6´-dimycolate (TDM)

Trehalose-6,6´-dimycolate (TDM) also known as cord factor, is the most abundant and toxic lipid in the mycobacterial cell envelope. TDM is composed by two polar trehalose head group where two mycolic acids (MA) are esterified. MA variations constitute a strong determinant of the inflammatory response of TDM. TDM has biological functions, promoting angiogenesis [26], inhibits acidification of phagolysosome, prevents Ca^{2+} dependent phagosome-lysosome fusion and mycobacterial surface lipid removal, which increased trafficking of bacteria to the acidic compartments, causing 99% of killing in macrophages after 3 days of infection [27]. TDM coating beads produce a delayed maturation of phagosomes characterized by a non-acidified and hydrolytically restricted phagosome [28]. Cytokine production in macrophages exposed to TDM has been extensively described; there are a high diversity number of publications about it. The effect of these molecules has been correlated with the innate, early adaptive response (humoral and cellular immunity); to resume: cytokines as IFN-γ, TNF-α, IL-4, IL-6, IL-10; chemokines as MCP1, IL-8, are induced as response to exposition of TDM [29]. Reduction in the expression of MHCII, CD1d, CD80, CD40 and CD96 in the surface of

macrophages is induced by the exposure of the cells to TDM [30]. Microspheres coated with TDM showed an increased expression of enzymes and matrix metalloproteinases; molecules associated with tissue remodeling and tissue destruction during caseating granulomas [31]. The inflammatory profile induced by TDM has been related with granuloma development and maintenance, this phenomenon is related with TDM, and is dependent of TNF-α and IL6 expression; also C5a complement factor has been described as part of the granuloma maintenance microenvironment molecule [32].

3.1.5. Phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL)

Phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL) include a group of related cell wall lipids, non-covalently bounded to the mycobacterial surface. PDIM and PLG are major virulence factors of mycobacteria. PDIM and PGL are molecules required for bacterial duplication during the acute phase [33]. PDIM is involved in mycobacterial resistance to detergents, and also is linked with the permeability and envelope solidity [34]. PDIM is present in *M. marinum* and species from the MTBC, but is absent in *M. smegmatis*; it contributes to the intracellular bacterial surviving, protecting them against the action of reactive nitrogen intermediates species, and regulates TNF-α expression [35]. Phagosome acidification resistance is caused by exclusion of vacuolar proton-ATPase in the phagosomal membrane; PDIM deficient mutants can also provoke macrophages death [36]. PDIM is involved in the phagocytosis dependent of receptor through a macrophage plasma membrane reorganization mechanism [37]. Recent findings suggest that PDIM and ESAT-6 protein act together to induce phagosome membrane damage and apoptosis [38]. PGL is produced principally by fast growing mycobacteria; most of MTB isolates and H37Rv are unable to produce it, phenomenon caused by a mutation in the *phs15/1* gene. PLG1, an immunogenic glycolipid produced for all *M. leprae* isolates, contains a trisaccharide moiety, different to the MTB PLG; and could be obtained from *M. leprae* from infected tissues. PLG1 is responsible for the demyelination and damage in the axons, confers neurotoxic proprieties to the macrophages and increase the reactive nitrogen species (RNS) synthesis [39].

3.2. Secretion systems in mycobacteria

Molecular migration across the mycobacterial cell wall, constitute an important event related with the environment and host cells interaction. Mycobacterial waxy cell envelope controls the molecular movement and the secretion of substances across this structure is dependent of specialized proteins systems, some of these protein structures will be described below.

3.2.1. The twin-arginine transporter (TAT transporter)

The twin-arginine transporter (TAT transporter) is located in the cytoplasmic membrane and transport folded proteins. This system is composed by three membrane proteins named as TATA, TATB and TATC. *M. smegmatis* deletion mutants to *tatC* and *tatA* showed a growth defect on agar, defective exportation to active beta-lactamases and hypersensitivity to

sodium dodecyl sulfate (SDS), reason that suggests that TAT genes could be good candidates for vaccines and drug development [40]. Mycobacterial phospholipases, virulence-related molecules encoding by *plcA, plcB, plcC and plcD* genes, are secreted by the twin-arginine transporter [41]. TAT system is involved in secretion of relevant proteins, as example; the secreted protein encoded by the Rv2525c gene, which is involved in the cell wall biogenesis, this protein is conserved in *M. leprae* and present in MTB, it has been described as important for virulence and it is involved in the resistance to beta-lactam antibiotics [42]. The resuscitation-promoting factor, RpfB associated with the MTB reactivation stage, interacts with the virulence factor RipA, an endopeptidase protein secreted together with the chaperone MoxR1, which requires the TAT secretion system. Inhibition of this system increased the sensitivity to beta-lactam antibiotics and prevents the localization of the peptidoglycan hydrolase [43].

3.2.2. The ESX transporter

The ESX transporter has no counterpart in LPS bacteria; it is located in the cytoplasmic membrane and exports and secretes proteins across the mycobacterial cell envelope. ESX genes are encoded at the genome and plasmids [44] and codify to for ESX type proteins EspA, EspB, EspC, EspG, etc, the secreted proteins ESAT-6 y CFP-10; PE-PPE; and the conserved components EccB, EccC, EccD and MycP [45]. The ESX systems are named as ESX-1 to ESX-5, depending on the variation of the diverse systems and their components.

3.2.2.1. ESX-1

ESX-1 transporter system is important during mycobacterial infection in MTB and other pathogenic mycobacteria, in BCG the loss of the region of difference 1 (RD1) and the partial loss of the ESX-1 encoding region is related with the attenuation of the strain [46]. ESX-1 allows cytosolic contact and mediates vacuoles rupture [47]; the protein intervenes in host cell lysis in a contact dependent way, producing gross membrane disruptions [48]. DNA transfer through conjugation is also a function of ESX-1 system [49]; a phenomenon called "distributive conjugal transfer" that describes a genetic exchange between recipient and donor is dependent of ESX-1 in *M. smegmatis* [50]. *espB* gene located in the RD1 region has been related with cytotoxicity and their presence in the surface of apoptotic cells for clearance by macrophages through efferocytosis [51].

3.2.2.2. ESX-3

ESX-3 is involved in Zn and Fe uptake. ESX-3 proteins: EsxG and EsxH are associated with the (proline-glutamic acid, proline-proline-glutamic acid) PE and PPE secretion [52]. The EsxG and EsxH heterodimer, which harms macrophage phagosome maturation, is secreted by the ESX-3 system [53] and inhibits the endosomal-sorting complex required for transport (ESCRT) impairing MTB antigen-specific $CD4^+$ activation by macrophages and DC [54]. In *M. abscessus* ESX3 is composed by the genes *esxH, esxG, esx-3*, EsxG and EsxG proteins are related with enhancement of inflammatory cytokine generation in macrophages, *M. abcessus esx3* mutant resulted in less inflammatory response [55].

3.2.2.3. ESX-5

ESX-5 secretion system only present in slow-growing mycobacteria is linked to PPE and PE exportation and pathogenicity. The secretion mechanism of ESX-5 is activated in response to phosphate limitation through phosphate sensing of Pst/SenX3-RegX3 system [56]. In MTB, disruption of ESX-5 showed a strong attenuation, failure in the cell wall integrity and the loss of the secretion of the PPE protein [57]. ESX-5a region, from ESX5 is composed by duplicated genes and had been related with inflammasome activation [58]. Also, mutations in the ESX-5 system components as *esxC5* are related with ofloxacin resistance in MTB [59].

3.3. PE proteins: PE-PPE and PE-PGRS

The PE domain permits transportation of proteins, which share the domain. PE-PGRS and PE-PPE interacts with the TLR-2 on DC and macrophages, inducing: cytokine secretion, necrosis and apoptosis and enhance mycobacterial survival [60]. PE-PGRS33 interaction with TLR mediates macrophage entry [61]. PE-PGRS30 mutant showed an attenuated phenotype, specifically inhibits phagosome-lysosome fusion and showed decreased lung colonization and reduced tissue damage [62]. PE-PGRS32 gene is highly conserved in MTB strains, because it has been related with mycobacterial survival in macrophages, persistence and replication [63]. PPE10 has been described as an ESX-5 substrate in pathogenic mycobacteria; mutation of both of them reduced the envelope integrity and mycobacteria hydrophobicity [64]. Co-localization of PE-PGRS33 in the host cell mitochondria induces cell death: necrosis and apoptosis [65]. PE-PGRS47 disruption led to an *in vitro* and *in vivo* attenuated growth and autophagy inhibition in infected phagocytes [66].

3.4. Lipoproteins

MTB genome analysis showed around 90 putative lipoproteins, most of them are part of the mycobacterial cell envelope and the plasma membrane; their function is related with molecular exportation, cell wall homeostasis and nutrient uptake; their presence contribute to host-pathogen interaction.

3.4.1. LpqH (19 kDa protein)

This lipoglycoprotein *O*-glycosylated and acylated, is a major cell wall antigen. LpqH is recognized by the immune system and induces T cell proliferation *in vitro*, stimulates DC maturation and autophagy and activates TLR-2 [67]. The protein alters the expression and presentation of antigens by MHCII [68]. 19kDa protein, induces macrophage apoptosis by caspase-dependent and -independent mechanisms with activation of the initiator caspase 8 and executioner caspase 3 [69]. DC that phagocyte apoptotic macrophages induced by cell wall extract expressing LpqH, activates CD8 T cells through cross-presentation [70].

3.4.2. LppX

LppX is related with the release of complex lipids to the culture filtrate; LppX structure showed a large cavity that probably binds big motifs as the one present in PDIM. In a mice model, LppX-deficient mutant showed attenuation [71].

3.4.3. Mpt83

Mpt83 glycosylated lipoprotein related with host cell adhesion, is present in MTB and *M. bovis* and both proteins are identical, but with some glycosylation differences. Protein is recognized by the MR, and the native protein induces IL-6, IL-12 and TNF-α [72]. T cell proliferation and IFN-γ expression was detected in PBMCs from donors vaccinated with BCG or with latent tuberculosis after exposition to non-lypidated synthetic Mpt83 [73].

3.4.4. LprG

LprG also known as P27 lipoprotein, is a ligand of TLR2; inhibits antigen processing in macrophages MHC II [74]. It has a large cavity that binds triacylated agonist of TLR2: LM, LAM and PIM [75]; and determines LAM envelope localization and control phagosome-lysosome fusion [76]. Expressed in an operon with Rv1410c, binds triacylglyceride (TAG) in the cavity and regulates TAG levels, growth rate and virulence [77]. Involved in cell wall composition, MTB mutant deleted *lprG* showed a decreased amount of surface LAM, affecting interaction with the MR and host cells, phagosome-lysosome fusion disturbance, and in mice model showed a decreased number of bacteria in the lungs. [78]. *M. bovis* P27 is required for arresting phagosome maturation and replication in bovine macrophages [79].

3.4.5. RpfB

RpfB multidomain lipoprotein related with resuscitation after mycobacterial dormant state drives in DC Th1-type immunity through interaction with TLR-4 [80].

3.4.6. LpqS

LpqS MTB protein conserved in slow-growing pathogenic mycobacteria, is a protein related with survival during latency. *LpqS* mutant showed attenuated virulence in guinea pig models and provide better immunization against pulmonar tuberculosis in comparison with BCG [81]

3.4.7. LprN

LprN lipoprotein related with cellular entry and survival, is part of the *mce4* operon. Recombinant LprN expressed in *E. coli*, showed in *in vivo* assays on mice, an increased expression of IFN-γ and TNF-α, and also a higher T cell proliferation, but failed to protect mice against MTB challenge [82].

3.4.8. Lprl

Lprl lipoprotein present only in bacteria from MTBC, showed upregulation during mycobacterial macrophage infection. Lprl strongly attaches lysozyme, annulling completely their enzymatic activity. Lprl expression in *M. smegmatis* enhanced phagocytosis and survival in macrophages derivate from peritoneal monocytes; also protect the bacteria against lysozyme activity [83].

3.4.9. PstS

PstS phosphate transporter, glycolipoprotein, is recognized by MR, induces phagocytosis and reduces the production of ROS [84]. PstS-1, PstS-2, PstS-3 DNA vaccine was used in mice, only animals vaccinated with PstS-3 showed reduction of CFU on lungs and spleen [85].

4. Immune system evasion

Unlike other pathogens, MTB infects and resides within immune cells, this bacterium has the ability to live within the dynamic and heterogeneous environment of macrophage phagosome. Here, the bacilli use a plethora of strategies to evade the microbicidal mechanisms of macrophage, including: phagosome-lysosome fusion, recruitment of hydrolytic lysosomal enzymes, production of reactive oxygen/nitrogen species, antigen presentation and apoptosis. Disruption of those functions in turn disrupts the adaptive immune response. Phagocytosis is an active process that depends on the interaction with various surface receptors expressed on the macrophage such as complement receptor type 3 (CR3), FCγ receptors and lectin receptors and it can be opsonic or non-opsonic. However, non-opsonic phagocytosis of MTB results in higher intracellular survival, although it is difficult to assess if the engagement of specific receptor determines the course of infection [86]. MTB uses PDIM lipids to evade detection by TLRs, thereby preventing mycobacterial delivery into microbicide macrophages expressing iNOS [33]. Moreover, MTB actively blocks the phagosome maturation by their cell wall components or through the secretion of various macromolecules that interferes with this process, which enables bacterial survival in a non-acidified intracellular compartment [12].

4.1. Phagosome arresting

PtpA and SapM are two phosphatases that contribute with the phagosome arresting. PtpA binds to subunit H of the vacuolar V-ATPase in order to dephosphorylate its substrate, the vacuolar protein sorting 33B (VPS33B) resulting in the exclusion of V-ATPase from mycobacterial phagosome thus inhibiting phagosome acidification [87]. MTB mutant in PtpA was severely attenuated when infecting THP-1 cell line compared with wild type strain, these results show that PtpA is essential for mycobacteria survival within macrophage [88]. SapM is a secretory phosphatase that dephosphorylates phosphatidylinositol 3-phosphate (PI3P) on the phagosome membrane [87]. PI3P is essential for phagosomes to acquire lysosomal constituents; it is involved in the docking of rab effector proteins early endosomal autoantigen 1 (EEA1) and hepatocyte growth factor-regulated tyrosine kinase (HRS) substrate, which are important for phagosome maturation [89]. Disruption of *sapM* in MTB resulted in a highly attenuated strain with an impaired ability to grow in the THP-1 macrophages as well as in the guinea pig tissues [33].

Ndk is a nucleoside diphosphate kinase with ATP- and GTP-binding activity and it is widely conserved across all the three domains of life. This protein is autophosphorylated and secreted into the culture medium by MTB and possesses GAP activity towards Rho GTPases Rab5 and

Rab7, leading to reduced phagolysosome fusion [90]. Besides, Ndk also targets and inactivates the small GTPase Rac1, an essential component of the macrophage NADPH oxidase (NOX2) complex, inactivation of Rac1 was associated with reduced NOX2-mediated production of reactive oxygen species (ROS) and ROS-dependent apoptosis thus contributing significantly to mycobacterial virulence [91]. Another factor crucial for inhibition of phagolysosome fusion is the serine/threonine protein kinase G (PknG). In contrast to other mycobacterial kinases, autophosphorylation on Thr residues at the N terminus of PknG are not involved in the regulation of this kinase; however, it is essential for the capacity of PknG to block lysosomal delivery of mycobacteria and for the bacterial survival in murine BMDM [92].

The PE_PGRS protein family includes around 60 proteins but the role and function of these proteins remains elusive. Nonetheless, PE-PGRS30 was the first PE-PGRS protein with a certain role in the virulence of MTB. PE_PGRS30 mutant was impaired in its ability to colonize lung tissue and to cause tissue damage; and inactivation of PE_PGRS30 resulted in an attenuated phenotype in murine and human macrophages due to the inability of the MTB mutant to inhibit phagosome-lysosome fusion [62].

Several factors have been implicated in phagosome maturation arrest, such as LAM, TDM, LpdC, Zmpq and the Esx-1 secretion system [27, 93–95]. But more importantly it seems that the requirement for these processes in the different mycobacterial species may not necessarily be identical; for example, BCG is able to arrest phagosome maturation in spite of the absence of RD1 locus, thus phagosome arresting in the case of BCG can be without ESAT-6 and CFP-10 although these proteins are necessary for this arrest in *M. marinum* [94, 96]. Therefore it is noteworthy that phagosome maturation and its arrest are complex processes with multifactorial requirements.

4.2. Resistance to reactive oxygen and nitrogen species

Upon phagocytosis of mycobacteria, macrophages produce antimicrobial reactive oxygen and nitrogen species (ROS and RNS) via the enzymatic activity of NADPH oxidase (NOX2) and inducible nitric oxide synthase (iNOS), respectively. NOX2 is a multiprotein enzyme complex that assembles and activated in response to phagocytosis. This enzyme complex transfers electrons across the membrane from cytosolic NADPH to molecular oxygen, the reaction produce superoxide anions (O_2^-) which dismutates into hydrogen peroxide (H_2O_2) and generates toxic hydroxyl radicals [48]. iNOS is induced upon IFN-γ activation and produces nitrite and nitrate via nitric oxide (NO), this reacts with O_2^- and forms peroxynitrite ($OONO^-$) [97]. This reactive oxygen and nitrogen intermediates (ROI and RNI) react with a wide range of molecules, such as nucleic acids, proteins, lipids and carbohydrates, thus for intracellular pathogens like MTB survival upon exposure to oxidative stress is critical.

Among the factors that contribute to MTB success as a pathogen are: its ability to survive the redox stress manifested by the host and its capacity synchronize its metabolic pathways and expression of virulence factors. Two component proteins, namely DosS and DosT, are employed by MTB to sense changes in oxygen, nitric oxide and carbon monoxide levels, while WhiB3 and anti-sigma factor RsrA are used to monitor changes in intracellular redox

state [98, 99]. Using these and other unidentified redox sensors, mycobacterium orchestrates its metabolic pathways to survive in nutrient deficient, acidic, oxidative, nitrosative and hypoxic environments inside granulomas or infectious lesions [97]. MTB employs versatile machinery of the mycothiol and thioredoxin systems to ensure a reductive intracellular environment for optimal functioning of its proteins even upon exposure to oxidative stress [97]. Mycothiol is a low-molecular-weight thiol and functions like glutathione, the archetypal redox buffer, which is not produced by mycobacteria. Therefore it has antioxidant activity as well as the ability to detoxify a variety of toxic compounds. The thioredoxin (Trx) system is composed of NADPH, thioredoxin reductase (TrxR), and Trx is a small redox protein with two redox-active Cys residues in its active site [100]. Trx is responsible for maintaining a reducing intracellular environment, regenerating the reduced forms of methionine sulfoxide reductase and peroxiredoxins, as well as the redox regulation of enzymes and regulatory proteins by oxidoreduction and the detoxification of ROS [100]. MTB contains three types of Trx, although TrxB is the only one essential to fight against host defenses and for *in vitro* growth [101]. Additionally, mycobacterium employs a battery of protective enzymes, such as superoxide dismutase (SOD), catalase (KatG), alkyl hydroperoxidase (AhpC) and peroxiredoxins to detoxify and neutralize these redox stresses [97]. SOD is a metalloprotein produced by prokaryotes and eukaryotes to detoxify superoxide radicals. This enzyme dismutates O_2^- into H_2O_2 and molecular oxygen and has been shown to contribute to the virulence in a number of pathogens, including MTB, which have two genes, *sodA* and *sodC* [97]. Pathogenic mycobacterium species express and secrete higher levels of SodA compared to the non-pathogenic species. In fact, MTB mutants with reduced SodA expression displayed increased susceptibility to H_2O_2 and were markedly attenuated in mice [102]. Although SodA lacks a classical signal sequence for protein export, it is a protein dependent on SecA2 for secretion. MTB mutants in *secA2* are defective in the export of SodA and KatG, and are unable to grow in non-activated macrophages and showed reduced growth in mice [103]. SodC is a Cu/Zn superoxide dismutase anchored in the outer-membrane to protect MTB from reactive oxygen intermediates at the bacterial surface. Mutants in *sodC* show decreased survival in IFN-γ-activated murine peritoneal macrophages and have increased sensitivity to hydrogen peroxide [104].

Catalase peroxidases are enzymes that protect the bacterium from ROS damage and are used to detoxify H_2O_2, KatG from MTB degrades H_2O_2 and organic peroxides. Thus the major role of KatG in TB pathogenesis is to catabolize the peroxides generated by the phagocyte NADPH oxidase; although in the absence of this host antimicrobial mechanism, KatG is apparently dispensable [105]. Moreover, KatG also activates the anti-tuberculosis drug isoniazid (INH) converting it to several reactive species that inhibits a mycolic acid biosynthesis [105]. Although isoniazid resistance is multigenic, mutations in *katG* predominate among the INH-resistant strains, but the effect of these mutations on MTB virulence is variable. In general, *katG* mutations render MTB strains sensitive to endogenous or exogenous peroxides, generated during bacteria respiration or by phagocytes during infection [105], maybe because mutations also affect the peroxidase domain of KatG [106] though the activity of the alkyl hydroperoxidase C (AhpC) has been described as an important compensatory mechanism in INH-resistant strains [107].

AhpC is a member of the peroxiredoxin family that detoxifies organic peroxides into less reactive alcohol derivatives and confers protection against both oxidative and nitrosative stress. AhpC mutants show an essential role in the resistance to host oxidative agents in the early stages of infection [108]. Besides, phenotypic *M. bovis* mutants producing less AhpC were less virulent in guinea pig model than the wild type [109].

Peroxiredoxins (Prx) proteins are multifunctional antioxidant enzymes that reduce and thus detoxify hydroperoxides, organic hydroperoxides and peroxynitrite using electrons from Trx. Five Prx enzymes have been identified in MTB: AhpE, TPx, AhpC, Bcp and BcpB [97]. Tpx is the principal and most effective enzyme involved in the detoxification of H_2O_2 and peroxynitrite in mycobacteria. In macrophages, the *tpx* mutant shows impaired replication in activating and resting cells, and in mice the mutants were less lethal and persistent that the wild-type strain [110].

Deletion of genes that encode methionine sulfoxide reductase (msrA), Mtb proteasome (prcBA), nucleotide excision repair (uvrB) and F-420 biosynthesis (fbiC) are also hyper-susceptible to RNS [111]. Likewise, α-crystalline (HspX), bacterioferritin (bfrB) and the DosR regulon are upregulated by conditions that inhibit aerobic respiration; however, their role in MTB virulence is little understood [1].

4.3. Inhibition of apoptosis

During MTB infection, several forms of cellular fates have been observed such as necroptosis, apoptosis and autophagy, among those, apoptosis and autophagy have been recognized as innate macrophage defense mechanisms. Apoptosis is highly regulated process where the cytoplasm and other cellular organelles of dying cell are enclosed in membrane bound vesicles called apoptotic bodies. The apoptotic bodies are taken up by macrophages via receptor-mediated phagocytosis in a process defined as efferocytosis without elicit any inflammatory response [112].

Apoptosis reduces the viability of different mycobacterial species, including MTB; in fact many attenuated strains of mycobacteria induce more apoptosis than their wild type counterparts and exists a reciprocal relationship between virulence and apoptosis [113]. MTB infection mainly results in necrosis, while attenuated mutant strains including BCG and H37Ra primarily induce apoptosis [113]. Most of the factors that have been described as anti-apoptotic molecules play roles in the bacterial redox homeostasis (*katG, sodA, secA2* and *pknE*) because phagosome ROS promotes the apoptosis.

The *nuoG* gene of MTB encodes the NuoG subunit of the type I NADH dehydrogenase (NDH-1) that is important in the inhibition of host macrophage apoptosis, since MTB mutant-induced apoptosis in human THP-1 cells. Moreover, BALB/c and SCID mice infected with this mutant survived longer and the bacterial load in lungs were smaller than of the wild-type strain [114]. In addition, MTB can neutralize ROS in order to inhibit TNF-mediated host cell apoptosis via a NuoG-dependent mechanism [115]. Similar function can also be observed for SecA2, which is the ATPase of the canonical bacterial Sec secretion system, and as stated above SecA2 is required for intracellular growth of MTB in macrophages preventing phagosome maturation

[116]. Mutant of MTB in *secA2* induces more apoptosis than wild type in infected macrophages, but more importantly the author shows that SodA secretion was the major SecA2 process involved in the inhibition of apoptosis [117]. Therefore, it can assume that the SecA2 secretion system, most likely through SodA, inhibits apoptosis in a mechanism probable independent of oxidative burst [118].

The serine/threonine kinase E, pknE contributes to the survival response of MTB by regulating the bacilli machinery to resist apoptosis during nitrate stress. Deletion of *pknE* results in a mutant that was more susceptible to NO exposure, inducing higher level of apoptosis than wild-type strain and less production of TNF-α and IL-6. However, further *in vivo* studies are needed to establish the role of PknE in the virulence of MTB [119].

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Chapter 13

Overview of Non Tuberculosis Mycobacterial Lung Diseases

Chamila Priyangani Adikaram

Additional information is available at the end of the chapter

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Abstract

Nontuberculosis mycobacteria (NTM) are ubiquitous in nature and opportunistically infect different animals, including humans. Currently, NTM is emerging as an important cause of pulmonary infection among both immunocompromised and immunocompetent persons worldwide. The clinical relevance of pulmonary NTM varies among species while showing geographical heterogeneity in distribution as well as pathogenicity. The outcome of the respiratory NTM disease is a consequence of a complex interplay between microbial factors and host susceptibility. Furthermore, HIV infection, cystic fibrosis, cancer, underlying chronic lung disease and history of tuberculosis (TB) may be associated as risk factors for active nontuberculosis pulmonary diseases (NTMPD). The diagnosis of NTMPD requires the presence of symptoms, radiographic evidences, microscopic observations and definitive laboratory diagnostics. Lung infections resulted from a clinically significant NTM species should be treated with appropriate antimicrobial regimen.

Keywords: nontuberculosis mycobacteria, NTM, lung infections, NTM diagnosis, NTM infection

1. Introduction to genus *Mycobacterium*

The genus *Mycobacterium* was first proposed in 1896 by Lehmann and Neumann [1]. Currently, it contains about 160 species and it is likely that more will be discovered with recently developed more precise species identification techniques [2, 3]. Most species exist as free-living saprophytes and only minorities are successful as pathogens of higher vertebrates. The host-dependent mycobacteria are capable of reproducing *in vitro*. In contrast,

M. leprae and *M. lepraemurium* are uncultivable and require the intracellular milieu for survival and propagation [4].

The obligatory causative agents of the genus *Mycobacterium*, responsible for TB are classified into *Mycobacterium tuberculosis* complex (MTC). It comprises *M. tuberculosis*, *M.bovis*, *M. africanum, M. microti* [5], *M. canettii* [6], *M. caprae* [7], *M. pinnipedii* [8], *M. mungi* [9], *M. orygis* [10] and *M. suricattae* [11] species. *M. tuberculosis, M. africanum, M. canettii* and *M. orygis* cause TB primarily in human [4, 10], whereas *M. bovis* [12], *M. microti* [13], *M. caprae* [7] *M. pinnipedii* [8], *M. mungi* [9] and *M. suricattae* [11] infect cattle, domestic animals, goats, seals, mongooses and meerkats, respectively, and animal tuberculosis can also be zoonotic [14, 15]. However, geographical variation of the MTC species distribution has been identified. As an example, *M. africanum* is a common cause of human pulmonary TB (39%) as much as *M. tuberculosis* (55%) in West Africa [16]. In Ghana, 3% of pulmonary TB cases are represented by *M. bovis*, while 20% are *M. africanum* and 73% are *M. tuberculosis* [17].

Other medically important mycobacteria such as *M. avium, M. intracellulare* complex, *M. kansasii, M. marinum, M. fortuitum, M. chelonae* complex, *M. abscessus* and *M. scrofulaceum* are known as nontuberculosis mycobacteria (NTM) species or atypical mycobacteria or mycobacteria other than tuberculosis (MOTT). They are responsible for diseases including lymphadenitis in children, chronic pulmonary diseases, skin and soft-tissue diseases and infections of the skeletal system [18].

NTM are ubiquitous in nature and are widely distributed in water, soil and animals. Among prevailing NTM species, only a few species have a clinical impact on humans as opportunistic pathogens [19]. *M. avium* complex (MAC), *M. abscessus, M. kansasii, M. fortuitum, M. chelonae, M. szulgai, M. triviale* and *M. scrofulaceum* are common NTM species that cause pulmonary diseases in human [20]. Additionally, *M. riyadhense* was recently proposed as a causative agent of pulmonary NTM disease [21]. However, NTM are increasingly recognized as a significant cause of chronic human pulmonary infections in both immunocompromised and immunocompetent patients [3].

In contrast to TB, diseases caused by NTM have varied clinical manifestations, triggering a wide spectrum of infections with generally low virulence than TB [20]. Patients with underlying structural lung diseases such as chronic obstructive pulmonary diseases, cystic fibrosis, bronchiectasis, history of TB and chronic aspiration are more vulnerable to develop NTM lung disease [22]. Additionally, working in mining industry and advanced age are risk factors for NTM lung diseases. However, there is no evidence on animal-to-human (zoonosis) or human-to-human transmission of NTM, and human diseases are generally acquired from environmental exposure [22].

2. NTM species significant to lung diseases

NTM are emerging worldwide as significant causes of chronic pulmonary infection, while became a challenge for both clinicians and researchers in the past two to three decades [23]. However, isolation and discover of new NTM species from pulmonary clinical specimens have become frequent in the last years especially with the development of species identification techniques such as sequencing of 16S ribosomal DNA (rDNA) [24].

The pathogenicities of the different NTM species vary widely and show geographical heterogeneity. Commonly, most NTMPD infections are caused by the MAC, *M. abscessus*, *M. kansasii* [25, 26], *M. fortuitum, M. chelonae* [26], *M. szulgai, M. gordonae, M. vaccae and M. smegmatis* [20, 27].

MAC organisms are common in many environmental sites, including water and soil, and in animals as well as colonize in natural water sources, indoor water systems, pools and hot tubs [28]. Previously, MAC, a slow growing NTM species has been composed of *M. avium* and *M. intracellulare* but, with advance in genetic identification of species, MAC encompasses at least 10 species, i.e. *M. avium*, *M. intracellulare*, *M. arosiense*, *M. bouchedurhonense*, *M. chimaera*, *M. colombiense*, *M. marseillense*, *M. timonense*, *M. vulneris* and *M. yongonense*, as well as 4 subspecies, i.e. *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* [21, 25, 29]. MAC may cause progressive parenchymal lung disease and bronchiectasis in patients, particularly in middle-aged and elderly women without underlying lung diseases [30]. Fibrocavitary lung disease caused by MAC may associated with large cavities specially in late 1940s and early 1950s years, males who have a history of cigarette smoking and excessive alcohol use. Untreated form of this disease is generally progressive to extensive cavitary lung destruction and respiratory failure within 1–2 years. MAC lung disease also presents with nodular and interstitial nodular infiltrates frequently involving the right middle lobe or lingual, called as nodular bronchiectasis or nodular bronchiectatic disease [31]. Particular MAC species may have varying degrees of virulence and classifying MAC isolates into species level is important for identification of risk of clinical relapse/reinfection [32, 33].

Tap water is likely the major reservoir for *M. kansasii* causing human pulmonary disease [34]. Genotypic studies in Netherland and France have shown that isolates recovered from the patients have similar genotype to isolates from drinking water source and the environment [35, 36]. DNA sequencing of *M. kansasii* has confirmed the presence of seven subspecies, which are related to human infections [37], while subtype 1 is the predominant in human lung infections [37–39]. Clinical symptoms of *M. kansasii* lung disease are generally identical to those associated with pulmonary TB. Chest radiographic abnormalities are also very similar to reactivation of pulmonary TB, including cavitary infiltrates with an upper lobe predilection. Also, *M. kansasii* may show noncavitary or nodular/bronchiectatic lung disease [40], which is similar to clinical presentation of MAC.

Some studies have been strengthened that drinking water may be the source of infection of *M. abscessus* lung diseases [41]. The common clinical symptoms of *M. abscessus* and *M. fortuitum* infection are similar to other NTM respiratory pathogens, especially MAC, including cough and easy fatigability. Disease caused by *M. genavense* commonly has been recognized in acquired immunodeficiency syndrome (AIDS) patients, while observed also in HIV-negative patient with pulmonary nodules [42].

Although most NTM lung infections are caused by common organisms, other NTM species such as *M. flavescens, M. mucogenicum* [26, 43], *M. colombiense, M. genavense, M. holsaticum, M. kumamotonense, M. lentiflavum, M. mantenii, M. marseillense, M. monacense, M. neoaurum, M. parascrofulaceum, M. phocaicum, M. saskatchewanense, M. seoulense, M. septicum, M. setense, M. shimoidei, M. stomatepiae, M. szulgai, M. triplex* [44], *M. xenopi, M. malmoense, M. immunogenum* [45], *M. scrofulaceum, M. terrae complex, M. engbaeki, M. shimoidei, M. gilvum, M marinum, M. interjectum subspecies, M. heckeshornense, M. branderi* and *M. chromogen* [24] may cause pulmonary disease in both immunocompetent and immunocompromised patients.

Isolation of multiple NTM species from respiratory specimens has also been recorded. In Taiwan, two patients of 298, one had five isolates of MAC and one isolate of *M. fortuitum*, while another patient had 11 isolates of MAC and one isolate of *M. gordonae* [27]. Thus, the pathogenic significance of a NTM specimen must be determined in the context of a patient's clinical presentation.

3. Prevalence and current epidemiology of pulmonary NTM disease

In Western societies, most laboratories report a dramatically greater prevalence of NTM than TB [46]. However, the prevalence of NTM pulmonary infections, which based on laboratory records, should be coupled with clinical characteristics [47] as only approximately half of people with positive NTM cultures fulfilled clinical criteria for active infection [48]. Studies form North America, Australia, South Korea, Japan and Taiwan have shown the continued increase in NTM prevalence since 2000. The annual prevalence in North America and Australia ranges from 3.2 to 9.8 per 100,000 and is generally higher than in Europe. In Queensland, Australia, cases of pulmonary disease rose from 2.2 to 3.2 per 100,000 population [49] *during 1999–2005.* Furthermore, in Australia, the annual percent of NTM isolation has increased steadily every year, and the incidence rate of patients with NTM lung disease was 1.82 per 100,000 in 2006 and increased to 4.38 per 100,000 in 2010 [50], while the same changed from 9.4 per 100,000 in 2009 to 36.1 per 100,000 in 2016 [51]. In Africa and the Middle East, prevalence of NTM ranges from 4 to 15% among suspected TB cases and 18% to 20% among suspected multidrug-resistant TB (MDR-TB) cases [52]. The prevalence rate of NTMPD in Germany was increased from 2.3 to 3.3 cases per 100,000 population from 2009 to 2014, and this was strongly association with advanced age and chronic obstructive pulmonary disease [53]. The prevalence of NTM isolation approximately was doubled from 2005 (6%) to 2013 (11%) in Hawaii, USA [26], while in Oregon, USA, the estimated prevalence of NTMPD was 8.6 per 100,000 [48]. By 2014, in Japan, the incidence rate of NTMPD was 14.7 cases per 100,000 person, which was ≈2.6 times higher than the same reported in 2007 and current incidence rate of NTMPD may exceed that of TB in Japan [48]. The general prevalence of NTM was 477 per 100,000 in Zambia with the regional variation of rate of prevalence within the country [54]. In Korea, the rates of recovery of NTM from clinical specimens and the number of patients with NTM lung infections increased significantly between 2009 and 2015 [55].

While some species such as MAC and *M. abscessus* are commonly implicated worldwide, others (e.g., *M. malmoense, M. xenopi*) are regionally important [23]. Generally, MAC is predominant in North America and East Asia, whereas *M. kansasii, M. xenopi* and *M. malmoense* are more common in Europe [52]. In Hawaii, USA, the most prevalent species was MAC, *M. fortuitum* group and *M. abscessus* [26]. Even though isolation of slowly growing mycobacteria (SGM) is frequent in most of the European and Western countries, rapidly growing mycobacteria (RGM) species such as *M. fortuitum* and *M. abscessus* are more prevalent in Gulf Cooperation Council (GCC) except in few countries [56]. As examples, *M. fortuitum* was the predominant course of NTM lung disease in Middle East during 1984–2014 [57]. Furthermore, *M. fortuitum* and *M. abscessus* are predominant in Saudi Arabia, while MAC is the most common species in Oman [56]. However, it has been observed in Saudi Arabia that rare species are going to be prominent, alarming diversity of clinically relevant NTM's causing pulmonary infections [58].

Country (no. of infections tested)	NTM species (%)								
	M. abscessus	*M. avium*	*M. chelonae*	*M. fortuitum*	*M. gordonae*	*M. kansasii*	*M. triviale*	*M. scrofulaceum*	*M. szulgai*
India (15)	–	–	–	40	–	33	20	–	7
Hong Kong (28)	–	54	14	–	4	4	–	–	4
South Korea (131)	39	50	2	3	–	4	–	–	0
Japan (1064)	–	81	0.6	2	–	14	–	–	0.5
Thailand (132)	–	43	5	5	–	17	–	8	–
Singapore (15)	–	60	7	–	–	27	–	7	–
Taiwan (302)	19	43	10	10	–	9	–		–

Table 1. NTM species causing pulmonary infections in Asian region during 1971–2007 [20].

The most frequent NTM species were *M. intracellulare* followed by *M. avium* subspecies in South Africa by 2010 [59], while *M. kansasii* is the more frequently associated among definite or probable active TB patients. Also, *M. avium-intracellulare* complex was the prominent course of NTM lung infections in Greece during 2007–2013 [60].

Furthermore, clinical relevance of pulmonary NTM species shows not only the geographically heterogeneous but also the time-to-time variations. As examples, MAC was the main cause of pulmonary diseases in India during the period of 1971–2007 [20]. But, according to the recent publications, *M. abscessus* was the predominate species followed by *M. intracellulare* [61, 62] in India, even both of the species were not recorded till 2007 [20] (**Table 1**). *M. intracellulare* followed by *M. kansasii* were most common NTM species related to the NTMPD in China from 2004 to 2009 [63] and it changed in 2010–2015 period, as *M. kansasii* was replaced by *M. abscessus* [64, 65]. Similar observation had been in Japan where, dramatic increases of pulmonary *M. abscessus* incidence had been occurred [48] comparative to period of 2001–2007.

Furthermore, in Korea, *M. intracellulare* followed by *M. avium* was predominate species during 2009–2015, while it was *M. avium* followed by *M. abscessus* in earlier (**Table 1**) [55].

4. Host-pathogen interactions

Unlike TB, the mode of transmission of NTM to humans has not been defined. Bathroom showers have been implicated as a primary source of exposure to aerosolized NTM. Even though animals are potential reservoir for NTM infections, zoonosis is not properly evident yet. However, drinking untreated water and living in close contact with cattle or other domestic animals may lead of infection in human [66]. In USA, NTM diseases are more

associated with densely populated areas, suggesting the infective source as urban municipal water supply [67], while Japan suggesting the soil as the source for more patients who were farmers and gardeners [68]. Furthermore, characteristic gradient clustering of the ratios of *M. avium* and *M. intracellulare* has been observed in Japan, suggesting that environmental factors strongly affect the epidemiology of NTMPD [69].

The outcome of the respiratory NTM disease is a result of a complex interplay between microbial factors like particle size, number of organisms and duration of contact and host susceptibility factors such as immunity, genetic background, lung damages and chronic lung disease. The clinical presentation of NTM lung infections may be varied, including hypersensitivity pneumonitis (HP)-like granulomatous lung disease, cavitary (TB-like) disease and nodular bronchiectasis. A hypersensitivity pneumonitis (HP)-like granulomatous lung disease, with nontuberculous mycobacteria can be triggered by inhalation of NTM with hot water aerosols (hot-tub lung) from sources such as hot tubs/spas, showers and indoor swimming pools. This may have been the primary source of MAC infections in middle-aged women with subacute presentation of respiratory complaints and HIV patients in the United States [70]. While MAC is the most common NTM causing "hot tub lung," *M. fortuitum* has also been rarely implicated [71]. Physicians need to be alerted to the possibility of hot tub lung being caused by various NTM species other than MAC. Furthermore, a case study has been confirmed *M. gordonae* as a potential pathogen in humidifier lungs [72].

Rarely, with underlying lung disease or smoking or prior TB, cavitary disease could be caused by multiple NTM species especially by MAC. This condition is different from the typical presentation of MAC pulmonary infections as they may have upper lobe cavity, as well as TB-like symptoms [73, 74]. Nodular bronchiectasis, which is often present with older nonsmoking female, is associated mostly with MAC. Sometimes, mixed infections of MAC and *M. abscessus* may lead to nodular bronchiectasis [75, 76]. In really, solitary pulmonary nodules (SPN) due to MAC infection also have been identified in some studies [74, 77, 78]. However, clinical outcome of the NTM diseases basically depends on the interactions between NTM and the host (**Figure 1**).

4.1. Host factors

Immunosuppressed hosts who may be associated with immunosuppressive HIV infection, hematological and lymphoproliferative malignancy, stem cell and solid organ transplant and inflammatory disorders treated with biologicals are highly vulnerable for pulmonary infections caused by *M. avium* and other nontuberculous species. Defense against *Mycobacterium* species

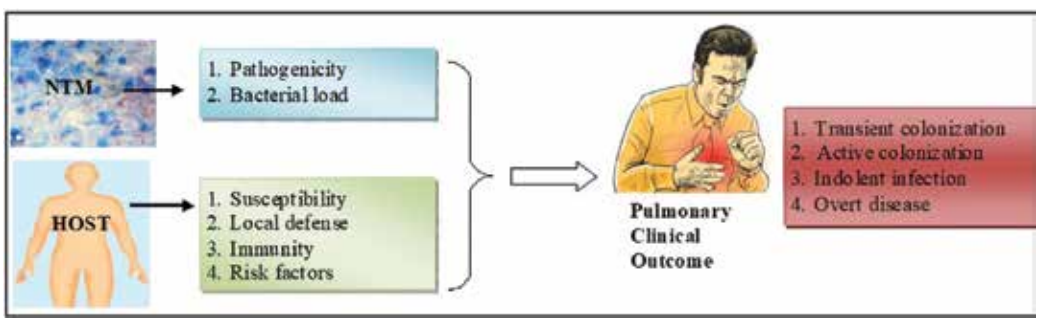

Figure 1. Interactions between NTM and the host that determine the clinical outcome.

is mediated by mononuclear phagocytes' ability to kill mycobacteria and secrete interleukin-12 (IL-12), augmented by interferon-gamma (IFNγ) secreting lymphocytes such as $CD4^{+}$T cells. Human natural killer cells (NK) are important in host defense against *Mycobacterium* as it secretes cytokines that induce macrophages to inhibit the growth of bacteria within macrophages [79, 80]. Cytokines that induce IL-32 (newly described pro-inflammatory cytokine), such as interferon-gamma, IL-18, IL-12, granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha, have considerable importance in mycobacterial immunity [81]. The alliance formed between IL-12 and IFN-gamma is essential for protective immunity against mycobacteria in human [82]. Therefore, genetic deficiencies in immunity mediated by IL-12 or IFN-gamma are highly susceptible to mycobacteria NTM infections in both individuals and familial clusterings of disease [79, 83].

IL-32 is expressed in multiple cell types in the lungs but particularly in the airway epithelial cells of patients with MAC pulmonary disease. Human airway epithelial cells (BEAS-2B) infected with *M. avium* produce IL-32 by a nuclear factor-kappa B-dependent mechanism. In both BEAS-2B cells and human monocyte-derived macrophages, exogenous IL-32 significantly reduced the growth of intracellular *M. avium* by increased apoptosis of infected cells. Thus, IL-32 not only facilitates host defense against MAC organisms but may also contribute to the airway inflammation associated with MAC pulmonary disease [81].

In immune evasion mechanism of *M. avium* subsp. paratuberculosis (MAP), bacteria are survived in macrophages by activation of mitogen-activated protein kinase (MAPK) pathway that leads to inhibition of antimicrobicidal activity of macrophages and over expression of IL-10. High levels of IL-10 in paratuberculosis promote the survival of MAP by reducing bactericidal activity of defense cells. Therefore, the pathways involved in the upregulation of IL-10 such as MAPK can be vital for developing a therapeutic strategy for the control of paratuberculosis [84]. A monogenic disorders conferring susceptibility to NTM infection are called as Mendelian Susceptibility to Mycobacterial Disease (MSMD) conditions, which are extremely rare and predominantly affecting children. Genetic disorders, which affect the immune response to mycobacterial infection, are known to result from disorders in genes of ISG15, IL-12B, IL12RB1, IFNGR1, IFNGR2, STAT1, IRF8, ISG-15, GATA2, NADPH and oxidase complex subunit genes such as CYBB [85].

Diseases and therapies that reduce cell-mediated immunity increase the risk of NTM disease. Acquired immunodeficiency virus (AIDS), cancer and organ transplants have been associated with NTM disease. The use of immunosuppressive drugs, including anti-TNF biologics, is also a risk factor for NTMPD [86]. NTM are often found in sputum cultures of patients with cystic fibrosis as they undergo lung transplantation followed by immunosuppressive medications. Therefore, effective medical treatment may need to control NTM after lung transplantation. The post-transplant infections can be associated with *M. abscessus*, which not affect for the survival of the patient in pre-transplantation stage. Therefore, sputum culture positivity for NTM before lung transplantation should not preclude transplantation, but should be treated in order to minimize the risk for recurrence after transplantation [87]. There is a possibility of co-existing pulmonary NTM infection in patients with lung cancer and disseminated NTM infection in patients with hematologic cancer [88, 89]. A study has suggested that anti-NTM therapy should be introduced only with worsening of symptoms under careful consideration as anti-NTM treatment is long and anti-mycobacterial drugs have extensive effects on anti-cancer drugs [90].

Also female sex, age, post-menopausal waning of endogenous estrogen levels, coeliac disease and exposure to use of dietary phytoestrogens can be risk factors for NTM lung diseases [91] while oral corticosteroids treatment in rheumatoid arthritis patients is also a comorbidity of NTM disease [92]. However, another study has showed that bronchiectasis and NTM lung disease are risk factors for breast cancer in women, and this phenomenon will open a new pathway for investigation of common pathophysiologic links of NTMPD [93].

4.2. Microbial factors

Aside from host factors, microbial factors such as virulence and microbial dose of exposed would be considerable factors for progression of NTM lung diseases. The critical exposure dose and relationship between quantitative mycobacterial exposure and disease are yet to be known. However, it may vary with the host susceptibility. Although exposure is common, disease is unusual, as most of NTM species are nonpathogenic and pathogenicities are varied according to the NTM species. Only few are highly pathogenic in human in descending order of pathogenicity, *M. malmoense*, *M. szulgai, M. kansasii, M. abscessus, M. Xenopi, M. avium* and *M. simiae*/*M. chelonae* and *M. intracellulare*. Even though MAC account for the plurality of pulmonary isolates as well as disease worldwide, the clinical relevance of NTM isolation from respiratory specimens appears to vary by geographic region, presumably due to variability in both environmental microbial distribution and the prevalence of host risk factors [23].

5. Diagnosis of NTM lung infections

Unlike TB, the isolation of NTM in pulmonary specimens does not equate with disease. The guidelines published in 2007 by American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) (ATS/IDSA) have specified that both clinical and microbiologic criteria must be met for the confirmation of diagnosis of pulmonary NTM disease [22]. Also, correct species identification is vital as NTM species differ in their clinical relevance. Correct diagnosis and choice of treatment regimen are needed as to prevent misdiagnosis, which direct chronic disease, antimicrobial resistance and death [94]. However, identification of all clinically obtained NTM isolates, especially from sputum, may not be needed. For instance, the exact identification of a pigmented rapidly growing mycobacteria isolated in low numbers from only one of multiple sputum specimens collected from patient undergoing therapy for MAC lung disease may not be necessary as it would not likely be clinically significant [22]. The diagnosis requires the presence of symptoms, radiographic abnormalities or chest high resolution computed tomography (HRCT) scan in the absence of cavitations, three or more sputum specimens for acid fast bacilli (AFB) analysis and exclusion of other disorders such as TB and lung malignancy. According to the ATS/IDSA guidelines, the criteria apply for definitive diagnosing of nontuberculous mycobacterial lung disease is following [22].

Clinical (both required)

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph or an (HRCT) scan that shows multifocal bronchiectasis with multiple small nodules.
2. Appropriate exclusion of other diagnoses.

Microbiologic.

1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.) (or)

2. Positive culture results from at least one bronchial wash or lavage (or)

3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.

5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.

6. Making the diagnosis of NTM lung disease does not, *perse*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients.

5.1. Clinical and radiographic based diagnosis

Delaying of diagnosis of NTM lung diseases is frequent due to the slow growing rate, misdiagnosed as TB or other AFB-positive bacilli and low index of clinical suspicion. The clinical symptoms, such as chronic cough, increased sputum production, dyspnea, low-grade fever, malaise and weight loss, are often nonspecific and overlapping clinical characteristics with pulmonary TB [95].

Radiological imaging and observing of radiological patterns, including miliary pulmonary pattern, nodular lesions, cavitary lesions, pleural effusion, abdominal adenopathy and splenic hypoechoic, is important when NTM lung disease is suspected in AIDS patients [96]. HRCT scanning allows early detection and better differentiation between colonization and invasive infection that are not visible on the chest X-ray [97]. In CT features; pleural effusion and nodules are significantly more common in patients with pulmonary TB (PTB) while bronchiectasis combined with cystic changes are significantly more common in patients with NTM lung infections [98]. Bronchiectasis in the right middle lobe or left lingual segment and thin-walled cavity with a diameter of more than 3 cm are the frequent chest CT features in patients with NTM-LD [99]. Furthermore, cavities associated with adjacent pleural thickening, ill-defined satellite tree-in-bud nodules or fewer noncavitary nodules in CT findings are highly suggestive of NTM disease rather than TB [58, 100].

Also, NTM lung infection can present itself with different radiological patterns, while two main patterns, fibrocavitary form and nodular bronchiectatic form, have been observed frequently [100]. The fibrocavitary form is usually characterized by upper lobe cavities with areas of increased opacity and with or without calcification (**Figure 2**) [98, 101].

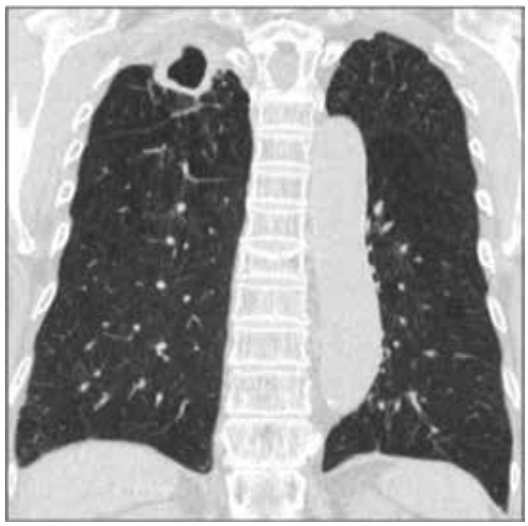

Figure 2. CT of fibrocavitary form of *M. intracellulare* pulmonary disease with a large cavity in the right upper lobe [101].

In the nodular bronchiectatic form: bilateral, multilobar bronchiectasis, especially in the middle and lower lung fields, with small nodules are the frequently observed chest CT features (**Figure 3**) [99, 101].

Even though there are no characteristic radiographic patterns for individual NTM species, centrilobular, peribronchovascular nodules, bronchiectasis, consolidation, tree-in-bud, pleural thickening and pleural adhesion are commonly observed CT findings in patients with MAC infection [102]. A recent study has shown that cavities are more common in patients with *M. malmoense*, while consolidations are mostly found among patients with an MAC and nodules are frequent in *M. kansasii* patients [103]. However, due to the presence of considerable overlap of the clinical symptoms and radiographic appearances of PTB and NTM lung diseases, the isolation and identification of causative organisms are mandatory for correct diagnosis of patients with AFB-positive sputum specimens [76].

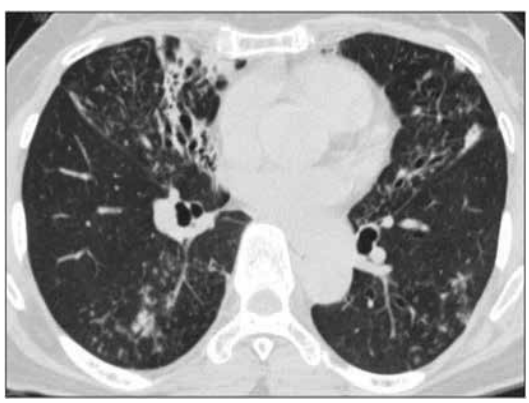

Figure 3. CT of nodular bronchiectatic form of *M. intracellulare* pulmonary disease with severe bronchiectasis in the right middle lobe and the lingular segment of the left upper lobe [101].

5.2. Laboratory diagnosis

The initial laboratory identification of the genus *Mycobacterium* can be made by microscopic observation for the presence of AFB. The definitive diagnosis demands the recovery of *Mycobacterium* species on a culture medium, followed by species identification tests. Although numerous novel, rapid and direct molecular methods have been developed, culture remains the gold standard for identification of *Mycobacterium* species from clinical specimens [104].

5.2.1. AFB smear microscopy

AFB staining, such as fluorochrome technique, Ziehl-Neelsen method or Kinyoun stain, which initially adopted for identification of *Mycobacterium tuberculosis* complex (MTBC), is satisfactory for NTM also. However, Smear microscopy cannot use for differentiation of MTBC form NTM, hence the presence of AFB can lead to a false-positive diagnosis of TB. The burden of organisms in clinical material is usually reflected by the number of organisms seen on stained smears. Since NTM are present in the environment, especially in water sources, the careful collection of high-quality respiratory specimens is necessary to avoid contamination. However, environmental contamination, which usually involves small numbers of organisms, rarely results in a positive smear examination. Semi-quantitative analysis of smears can be useful for diagnostic purposes and fluorochrome smears are graded from 1 (1–9 organisms per 10 high-power fields) to 4 (90 organisms per high-power field) [101, 105, 106].

5.2.2. Mycobacterium culture and species identification by conventional methods

Isolation of *Mycobacterium* by culturing is a primary requirement in conventional species identification and indirect drug susceptibility testing of NTM. The general microbiological measures of growing clinical material on a selective or differential culture media and subculturing to obtain pure cultures cannot be applied to *Mycobacterium*. Genus *Mycobacterium* will not grow on simple, chemically defined media and it requires special, enriched, selective media. Also, slow replication rate is a characteristic feature in culturing of *Mycobacterium, hence* culturing is time-consuming [107]. Generally, an AFB-positive sputum will require 3 weeks for producing visible colonies of *Mycobacterium* on solid medium [4]. However, NTM species, such as *M. fortuitum, M abscessus* and *M. chelonae,* are considered as rapid growers as they grow into visible colonies within 3–5 days of incubation [19, 22].

As per ATS/IDSA guidelines, both solid and liquid cultures are required for NTM species identification. Even though mycobacteria produce more rapid cultures with high yield in broth media than those on solid media, solid cultures need to proceed simultaneously as they allow observing of colony morphology, growth rates and mixed infections (more than one mycobacterial species), which are important factors in identification of the NTM species. Also, broth media cultures alone may not be sufficient for better diagnosis of NTM species due to the bacterial overgrowth and high chance for the contaminations from other bacteria and fungus [22].

In conventional culture techniques, Lowenstein-Jensen (LJ) media and agar-based Middlebrook media (7H10 and 7H11) are used as the common solid media, while 7H9 medium used as the liquid/broth media. BACTEC MGIT 960 system is a fully automated, nonradiometric system that is suitable for the detection of growth of TB and other mycobacteria with the shorter

detection time ~2 weeks [108]. The recently introduced, microchannel electrical impedance spectroscopy (m-EIS) has ability to detect *M. smegmatis* with initial loads of 1000 CFU/ml within 20 h, while commercial BACTEC MGIT 960 system need 41.7 h for the same [109].

Species, such as *M. haemophilum, M. genavense, M. avium* subsp. paratuberculosis (formerly *M. paratuberculosis*) and *M. ulcerans*, are required special supplementation for recovery on culture media. *M. haemophilum* grows only on media supplemented with iron-containing compounds such as ferric ammonium citrate, hemin or hemoglobin [110]. *M. genavense* and *M. avium* subsp. paratuberculosis require mycobactin J, and *M. ulcerans* may be optimally recovered with egg yolk supplementation [22].

Microscopic observation of ZN-stained smear prepared from culture will provide evidence only for the presence of mycobacteria, purity of the culture and cord formation. These basic characters are not sufficient for definitive species level identification. The conventional taxonomic differentiation of the genus *Mycobacterium* is based on phenotypic characters of the cultures and biochemical properties of bacteria. The characters of rapid growth, pigmentation (scotochromogens, photochromogens or nonchromogens), ability to grow in PNB incorporated media and creamy like watery colonies indicate the presence of NTM [107]. Several biochemical tests based on the properties of the genus *Mycobacterium*, including nitrate reductase, niacin production, catalase activity, production of arylsulfatase and urease, tween 80 hydrolysis, growth in the presence of 5% NaCl and MacConkey agar without crystal violet and the use of mannitol, inositol and sorbitol, may adequate to identify majority of clinically relevant mycobacterial species [107].

5.2.3. Molecular-based identification methods

Biochemical analysis and phenotypic characters may occasionally fail to arrive at a definitive identification. Because of differences in antimicrobial susceptibility at species level that determine treatment options, precise species identification of the NTM is required and only determination of merely as groups, such as *M. chelonae* (or/and *M. abscessus*) group, is not recommended [22]. To fulfill this requirement, rapid accurate and cost-effective molecular-based techniques, both *in-house* and commercial kits, with satisfied sensitivity and specificity were developed during last years. Currently, molecular methods especially assays based on the principle of nucleic acid amplification which allows a speedy and precise identification of the *Mycobacterium* species in <24 h have been developed.

Real-time PCR, DNA sequencing, probe hybridization, multiplex PCR and polymorphism analysis of restriction fragments (PCR-RFLP) are commonly used for differentiation of NTM species related to lung infections [111, 112]. The real-time PCR assays are advantageous because of its rapidity and high sensitivity. Furthermore, the specificity of the real-time PCR can be enhanced by combination with HPLC, which is a useful tool to discriminate NTM at the species level, although it requires specific equipment and technical expertise [113]. Furthermore, multiplex real-time PCR assay combined with melting curve analysis is also an accurate, rapid and effective tool for the mycobacterial identification from cultures [114]. The commercial form of real-time PCR Light cycler® *Mycobacterium* detection assay, which based on the 16S ribosomal RNA (rRNA), has shown 100% sensitivity and 99% specificity for differentiation of MTBC and *M. avium* from sputum samples [115, 116].

Several commercial kits, which are based on PCR amplification of selected fragment of 16S or 23S rRNA gene or 16S–23S rRNA spacer region, followed by reverse hybridization on nitrocellulose membrane strips such as GenoType *Mycobacterium* CM/AS (Hain Lifescience, Nehren, Germany) [117–120] and INNO-LiPA Mycobacteria (LiPA; Innogenetics, Zwijnaarde, Belgium) [121, 122] are available for identification of common pathogenic NTM species with high sensitivity and specificity. Genus *Mycobacterium*, MTBC and 16 NTM species are identified by INNO-LiPA mycobacteria assay, and it is based on the nucleotide variations in the 16S–23S rRNA spacer region (**Figure 4**).

Mixed populations easily identified with this assay and fully automated processing of the strips is possible using TENDIGO™ and Auto-LiPA 48. GenoType *Mycobacterium* CM kit identifies

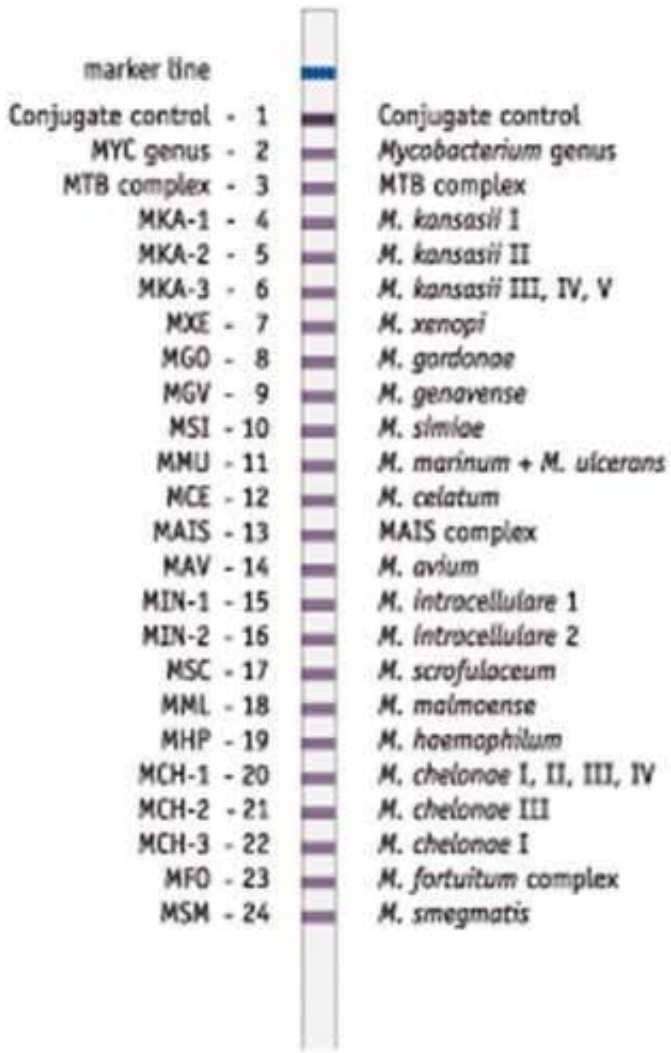

Figure 4. Location of the different probes on the INNO-LiPA Mycobacteria v2 strip.

the MTBC and differentiates of 27 clinically relevant NTM, while GenoType *Mycobacterium* AS kit enables the differentiation of 19 additional NTM species (**Figures 5** and **6**).

Direct sequence analysis of amplified 16S rRNA gene is a promising rapid and accurate method for species determination of nontuberculous mycobacteria [123], and in last decades, novel NTM species related to pulmonary infections were identified by this technique. In addition to that, several gene targets, including *rpoB* gene [124–126], *secA1* gene [127] and *hsp65*

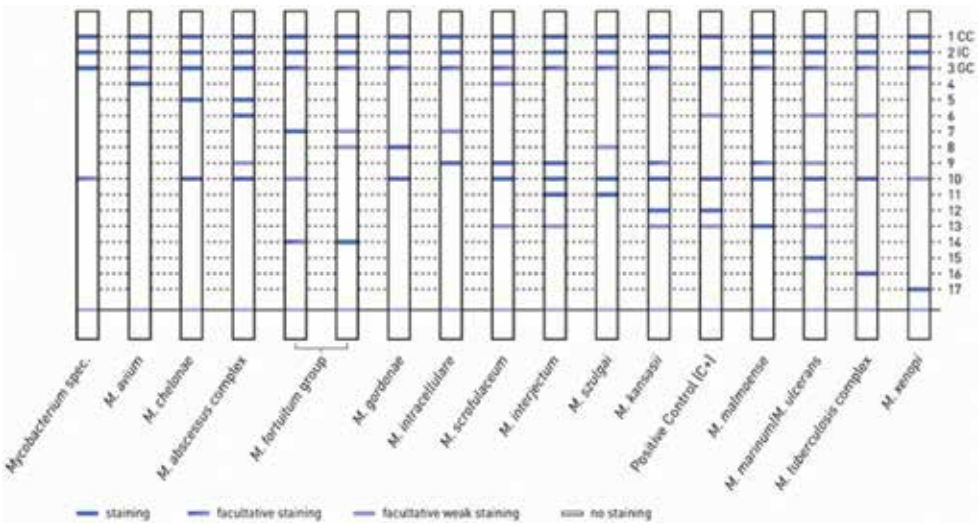

Figure 5. Location of the different probes on the GenoType *Mycobacterium* AS strip.

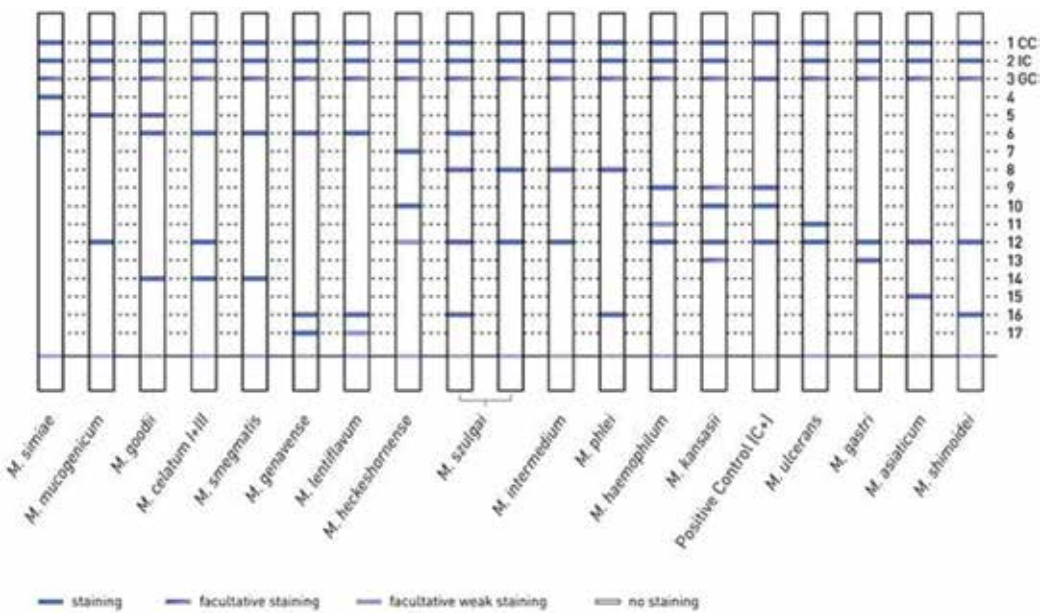

Figure 6. Location of the different probes on the GenoType *Mycobacterium* CM strip.

gene [128], have used for NTM species identification by DNA sequencing. Also, *gyrB*-based microarray [129], mycobacteria mobility shift assay (MMSA) [130], biochip assay system [131] and multiplex SNaPshot assay [132] have been proven as rapid detection methods to identify closely related mycobacterial species with satisfied level of sensitivity and specificity, which may be useful in the diagnosis and effective management of NTM lung disease [129–132].

6. Antimicrobial susceptibility testing for NTM

The laboratory susceptibility testing of pulmonary infective NTM species are based on the ATS/IDSA and Clinical and Laboratory Standards Institute (CLSI) guidelines. CLSI has recommended broth microdilution method as the gold standard for laboratories where antimicrobial susceptibility testing of NTM is performed [133]. There are no current recommendations for a specific method of *in vitro* susceptibility testing for fastidious NTM species and some less commonly isolated NTM species. Validation and quality control should be in place for susceptibility testing of antimicrobial agents with all species of NTM. According to the diagnostic guidelines for nontuberculous mycobacteria which are recommended by the ATS [22], only the Clarithromycin should be tested for susceptibility for new, previously untreated MAC isolates and susceptibility testes for other drugs are not recommended. Also, MAC isolates from patients who fail macrolide treatment or prophylaxis regimens should be tested to clarithromycin susceptibility. Isolates of *M. kansasii* that show susceptibility to rifampin will also be susceptible to rifabutin. Therefore, previously untreated *M. kansasii* strains should be tested *in vitro* only to rifampin. The rifampin resistant of *M. kansasii* isolates should be tested against a panel of secondary agents, including rifabutin, ethambutol, isoniazid, clarithromycin, fluoroquinolones, amikacin and sulfonamides. Unless the patient fails treatment after several months, *M. marinum* isolates do not require susceptibility testing.

The *in vitro* susceptibility patterns of some NTM such as *M. kansasii*, *M. marinum* and *M. fortuitum* are closely parallel to the clinical response to therapeutic agents. But, MAC, *M. abscessus* and *M. simiae* have limited evidences for the correlation between *in vitro* susceptibility results and clinical response in the treatment of pulmonary disease caused by these agents [134]. Furthermore, antimicrobial susceptibility patterns of rapidly growing mycobacteria (RGM) including isolates of the *M. fortuitum* group, *M. chelonae* and *M. abscessus* provide taxonomical value also in addition to the evidence of drug resistance [135].

According to the recent publications, the microplate Alamar Blue assay [136] and tetrazolium Microplate Assay [137] have also shown reliable results to the recommended microdilution method. However, molecular assays have not yet been able to replace time-consuming culture-based susceptibility methods in the mycobacteriology laboratory.

7. Treatment of NTM lung infections

After determination of the clinical significance of a NTM species, patient should be treated with appropriated antimicrobial regimen. The duration of treatment for most pulmonary NTM pathogens is based on treatment recommendations. Frequently encountered species

such as MAC and *M. kansasii* are treated 12 months of negative sputum cultures while on therapy. For disseminated disease, treatment duration for most NTM pathogens is the same as for disseminated MAC infection.

Treatment recommendations for infrequently encountered NTM are made on the basis of only a few reported cases. As recommendations for routine *in vitro* susceptibility testing of NTM isolates are limited, the clinician should use in vitro susceptibility data with an appreciation for its limitations. Empiric therapy for suspected NTM lung disease is not recommended. Furthermore, there are no widely accepted criteria for choosing patients with NTM lung disease for resectional surgery. In generally, surgery could be considered based on risk/benefit perspective in case of NTM infections that are more difficult to treat medically [22].

7.1. *M. avium* complex (MAC)

Drug therapy for MAC lung disease should be a combination of several antibiotics (**Table 2**), and the optimal therapeutic regimen has yet to be established [22].

Special recommendations of drug regimens are needed for patients with intolerance to first-line agents, a macrolide-resistant MAC cases or failed prior drug therapy. The macrolides should never be used as monotherapy for treatment of MAC lung disease. The duration of the treatment is 12 months of negative sputum cultures while on therapy; hence continuous observation of AFB in sputum of the patient is required throughout the treatment [22, 138]. The addition of intramuscular streptomycin to standard regimen for the first 3 months of treatment for MAC pulmonary disease improves the rate of culture conversion, even though clinical response and radiological outcome are not significantly improved. An intermittent (3× per week) oral antibiotic regimen should not be used in individuals with severe MAC pulmonary disease or in individuals with a history of treatment failure [138].

Major risk factors for macrolide-resistant MAC disease are inappropriate prescription patterns and deviations from the standard treatment due to adverse drug reactions [139]. More

Drug	Initial therapy for nodular/ bronchiectatic disease	Initial therapy for cavitary disease	Advance or previously treated disease
Macrolide	Clarithromycin 1000 mg TIW or azithromycin 500–600 mg TIW	Clarithromycin 500*–1000 mg/d or azithromycin 250–300 mg/d	Clarithromycin 500*–1000 mg/d or azithromycin
Ethambutol	25 mg/kg TIW	15 mg/kg/d	15 mg/kg/d
Rifamycin	Rifampin 600 mg TIW	Rifampin 450*–600 mg/d	Rifabutin 150*–300 mg/d or rifampin 450*–600 mg/d
IV aminoglycoside	None	Streptomycin or amikacin or none	Streptomycin or amikacin

Notes: IV = intravenous; TIW = three times weekly.*Lower dose for weight 50 kg.

Table 2. Recommended antimicrobial combination [138].

effective therapy is essential to treat and prevent macrolide-resistant with MAC lung disease [140]. Antibiotic treatment associated with rifampicin, ethambutol and isoniazid or a quinolone with streptomycin or amikacin and surgical resection of disease can be used in macrolide-resistant MAC diseases [31, 101, 138, 140]. Furthermore, the addition of moxifloxacin can improve the outcomes of patients with macrolide-resistant [141]. However, recent study has shown that continuation of macrolides or the addition of a new quinolone or injectable aminoglycoside to therapy with rifampicin and ethambutol would not improve clinical outcome

Species	Drug regimen	Duration
M. kansasii – Rifampicin-sensitive	Rifampicin 600 mg daily + Ethambutol 15 mg/kg daily + Isoniazid 300 mg (with pyridoxine 10 mg) daily or Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily	12 months after culture conversion.
M. malmoense	*Non-severe disease:*	
	Rifampicin 600 mg daily + Ethambutol 15 mg/kg daily + Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily	Minimum of 12 months after culture conversion
	Severe M. malmoense-pulmonary disease:	
	Rifampicin 600 mg daily +Ethambutol 15 mg/kg daily + Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily + consider intravenous amikacin for up to 3 months or nebulized amikacin	Minimum of 12 months after culture conversion
M. xenopi	*Non-severe*	
	Rifampicin 600 mg daily + Ethambutol 15 mg/kg daily + Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily + Moxifloxacin 400 mg daily or Isoniazid 300 mg (+ pyridoxine 10 mg) daily	Minimum of 12 months after culture conversion
	Severe	
	Rifampicin 600 mg daily + Ethambutol 15 mg/kg daily + Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily + Moxifloxacin 400 mg daily or Isoniazid 300 mg (+ pyridoxine 10 mg) daily + consider intravenous amikacin for up to 3 months or nebulized amikacin	Minimum of 12 months after culture conversion
M. abscessus Clarithromycin sensitive isolates or inducible macrolide-resistant cases	*Initial phase*:	
	≥1 month iv amikacin 15 mg/kg daily or 3× per week + iv tigecycline 50 mg twice daily and where tolerated iv imipenem 1 g twice daily and where tolerated oral clarithromycin 500 mg twice daily or oral azithromycin 250–500 mg daily	
	Continuation phase:	
	Nebulized amikacin + oral clarithromycin 500 mg twice daily or azithromycin 250–500 mg daily +1–3 of the following antibiotics guided by drug susceptibility results + patient tolerance: oral clofazimine 50–100 mg daily, oral linezolid 600 mg daily or twice daily (with pyridoxine 50 mg daily), oral minocycline 100 mg twice daily, oral moxifloxacin 400 mg daily, oral co-trimoxazole 960 mg twice daily	Minimum of 12 months after culture conversion

Species	Drug regimen	Duration
M. abscessus Constitutive macrolide-resistant cases	*Initial phase*:	
	≥1 month iv amikacin 15 mg/kg daily or 3× per week and iv tigecycline 50 mg twice daily + where tolerated iv imipenem 1 g twice daily	
	Continuation phase:	
	Nebulised amikacin and 2–4 of the following antibiotics guided by drug susceptibility results + patient tolerance: oral clofazimine 50–100 mg daily, oral linezolid 600 mg daily or twice daily (with pyridoxine 50 mg daily), oral minocycline 100 mg twice daily, oral moxifloxacin 400 mg daily, oral co-trimoxazole 960 mg twice daily	Minimum of 12 months after culture conversion

Note: iv = intravenous.

Table 3. Recommended treatment regimen for *M. kansasii, M. malmoense, M. xenopi and M. abscessus* pulmonary diseases [138].

after the emergence of chloramphenicol-resistant MAC [142]. If microbiologic, clinical or radiographic improvements are not shown after 6 months of appropriate therapy or achieved conversion of sputum to AFB culture negative after 12 months of appropriate therapy, patients are considered as treatment failures [22].

In addition to antibiotics, for patients with MAC lung infection, adjunctive therapies may also be given. Patients whose disease is predominantly localized to one lung, poor response to drug therapy, the development of macrolide-resistant MAC disease or the presence of significant disease-related complications such as hemoptysis might be considered for surgery. Although adjuvant pulmonary resection is complicated, it provides high level of treatment success rate in selected patients [143, 144]. Successful treatment of disseminated MAC in persons with AIDS is based on treatment of both the mycobacterial infection and the HIV infection. Both clarithromycin and azithromycin have been shown to be effective in combination regimens for the treatment of disseminated MAC. But, treatment of these cases may be complicated by adverse drug effects [22, 145].

Recommended treatment regimen for *M. kansasii, M. malmoense, M. xenopi and M. abscessus* is described in **Table 3**. The treatment for *M. abscessus* pulmonary disease should comprise an initial phase antibiotic regimen followed by a continuation phase antibiotic regimen. However, individuals with a history of treatment intolerance or treatment failure should be managed in collaboration with a physician experienced in managing NTMPD.

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Chapter 14

Unique Biochemical Features of the Cytokinetic Protein FtsZ of Mycobacteria

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Additional information is available at the end of the chapter

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Abstract

FtsZ, the bacterial cytokinetic protein, a structural homologue of mammalian β-tubulin, is present in bacteria of diverse genera, including mycobacteria. The FtsZ protein of *Mycobacterium tuberculosis* (*M. tuberculosis* FtsZ), the causative agent of tuberculosis, is the most studied among the mycobacterial FtsZ proteins as it is a potential anti-tuberculosis drug target. *M. tuberculosis* FtsZ possesses many unique biochemical features, which include slow polymerisation kinetics, presence of charged amino acids in the C-terminal domain that interacts with a variety of other cell division proteins, and the presence of specific amino acids at unique locations that makes it distinct from the FtsZ of other mycobacterial species and of other bacterial genera. On the other hand, although the FtsZ of *Mycobacterium leprae* (*M. leprae* FtsZ), the causative agent of leprosy, shows high level of conservation with *M. tuberculosis* FtsZ, it has biochemical properties that are very different from those of *M. tuberculosis* FtsZ due to the difference in specific amino acid residues at critical locations on the protein. The present review focuses on these structural features of *M. tuberculosis* FtsZ and *M. leprae* FtsZ, as studied by others and by us, in comparison to those of the FtsZ of other mycobacterial species and of other bacterial genera.

Keywords: mycobacteria, FtsZ, biochemical features, polymerisation, GTPase, GTP binding, bacterial cytokinesis

1. Introduction

Bacterial cell division involves karyokinesis and cytokinesis. The replication and segregation of genetic material occur in karyokinesis. In cytokinesis, the cytoplasm and its contents increase in quantity and get partitioned between two daughter cells with the development of the septum between them and formation of new cell membrane and cell wall in the sacculus.

The process of septation is guided by the cytokinetic protein, FtsZ [1, 2], which is the bacterial structural homologue of mammalian β-tubulin [3]. Polymeric FtsZ, as the inner membrane bound FtsZ ring at the mid-cell site [1, 2], guides septation. A large number of cell division proteins interact with FtsZ at the median (reviewed in [4, 5]). The duration of the whole process of cell division varies from bacterium to bacterium, with different strains of *Escherichia coli* completing cell division in 18–55 min [6], *Bacillus subtilis* taking 120 min [7], whereas *Mycobacterium smegmatis* taking 3 h [8], *M. tuberculosis* 18 h *in vivo* [9] and 24 h in vitro [10], and *M. leprae* completing it in 13.5 days once [11]. Accordingly, since the septation process takes only a part of the whole cell division duration, the polymerisation and depolymerisation dynamics of FtsZ of each bacterium need to be different in different bacteria to suit the septation duration in the bacterium. The required modifications in the polymerisation and depolymerisation dynamics of the cytokinetic protein to suit the time span of septation need to be effected through specific changes in the amino acid residues of FtsZ. Such changes, which can influence polymerisation and depolymerisation dynamics of FtsZ, can be vividly discerned from the evolutionarily purposeful placement of specific amino acid residues at specific structural locations on the protein. Here, we have tried to point out this particular aspect of the structure-function correlation of FtsZ from mycobacteria with that of FtsZ from other mycobacterial species and bacterial genera.

2. Basic common structural features of FtsZ of other bacteria

The biochemical identity of FtsZ was first established through the demonstration of the GTP binding and GTPase activities of *Escherichia coli* FtsZ (*E. coli* FtsZ) [12, 13]. Both the authors showed the presence of a peptide motif (105 GGGTGTG 111), similar to the tubulin signature motif (140 GGGTGSG 147), and postulated that it would form a phosphate-binding loop like in β-tubulin [14]. The GGGTGTG motif is crucial for GTP binding as its change to SGGTGTG in the FtsZ84 mutant of the temperature-sensitive *E. coli* (*ftsZ84*) strain markedly reduced GTP binding ability (by crosslinking) [13] and converted FtsZ into an ATPase *in vitro* [12]. Tubulin and FtsZ share poor primary sequence homology of 10–18%, except in two sequence regions (amino acids 95–175 in β-tubulin and 65–135 in *E. coli* FtsZ, and 305–350 in β-tubulin and 255–300 in *E. coli* FtsZ) where they show clear sequence homology of 85–87 and 51–78%, respectively [15]. The first region contains β-tubulin/FtsZ signature motif, flanked by identical looking secondary structural elements [15]. However, the overall three-dimensional structures of FtsZ and β-tubulin are almost similar [16, 17], which is reflected in the GTP-dependent polymerisation of *E. coli* FtsZ *in vitro* [18, 19] like β-tubulin polymerisation [20].

The crystal structures of the FtsZ proteins of *Methanococcus jannaschii* [16], *Thermotoga maritima* [21], *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Aquifex aeolicus* [22] essentially showed independently folding N- and C-terminal domains arranged around a central helix. The N-terminal domain contains GTP-binding site and an incomplete GTPase active site. During the course of polymerisation, the T7 loop of one monomer is supplied in *trans* to the GTP-binding pocket of the next, thus forming the active site for GTP hydrolysis [23–28]. From the co-crystal structure of SulA-FtsZ, it was found that SulA, a cell division inhibitor [29], binds

the T7 loop surface of FtsZ, thereby blocking polymer formation as a part of SOS response [30]. Thus, the T7 loop seems to be critically required for FtsZ polymerisation, thereby carrying the potential to be an antibacterial inhibitor target.

In all the solved structures of FtsZ of *M. jannaschii* [16], *P. aeruginosa* [22], *A. aeolicus* [22], *T. maritima* [21], and of *B. subtilis* [31], the end part of the C-terminus has been found to be unstructured and containing variable residues. It has been found to be a platform for the interaction of a large number of cell division proteins, ZipA [32], FtsA [33], EzrA [34], SepF [35, 36], ZapD [37], and with FtsZ itself [4, 5, 38].

2.1. Specific residues required for the biochemical activities of FtsZ

GTP-binding residues are best conserved between FtsZ and β-tubulin [39]. Monomeric FtsZ binds GTP tightly with a K_d of 5 μM [40]. The FtsZ84 (G105S) mutant protein has an impaired GTPase activity *in vitro* [12]. In other examples, while the mutant FtsZ6460 (G109S) showed nil GTPase activity, FtsZ9124 (P203L) possessed reduced GTPase activity, and both the mutants failed to polymerise *in vitro* [41]. Extensive work has been carried out on the characterisation of FtsZ mutants *in vitro* and *in vivo* [42–44]. Based on a model structure of *E. coli* FtsZ, which was built on the crystal structure of *M. jannaschii* FtsZ [16], the mutants were found to be primarily of three classes [43]. (i) On the front and back surface of protofilament and few on the top and bottom surfaces not in contact with GTP, which were termed as benign mutants as all of them could complement *ftsZ84* strain at non-permissive temperature. Few examples of these mutants are A70T, A81V/F268C (Z100), D158A, D158N, D187A, F268C (Z114), D269A, and D299A. (ii) GTP contact mutants, e.g., N43D, D45A, D45N, D209A, did not complement *ftsZ84* and could polymerise only in the presence of DEAE-dextran. (iii) Mutants, D86K, D96A, E238A, S245F, and E250A, where the mutations were thought to be on the lateral surface of protofilaments and therefore believed to play role in inter-protofilament interactions. These mutants could polymerise *in vitro*, but none of them could complement *ftsZ84* at non-permissive temperature.

Further studies showed that the mutations on the top surface of *E. coli* FtsZ model (e.g., G21K, L68C\D\W, F182C) have far less disturbing effect on the complementation (in plate and liquid culture) and GTPase activity of FtsZ compared to those of the mutations on the bottom surface (e.g. D96A, N207C, D209A\C\K, F210A) [44]. Interestingly, one top surface mutant Q47K that binds GTP at more than 1:1 ratio and having around 30-fold less GTPase activity, compared to that of wild-type FtsZ, failed to complement cell division, both in plate and in liquid culture [44]. The E83 and R85 in the helix H3 bend and lateral residues of *E. coli* FtsZ were found to be important for the polymerisation, GTPase activity, and cellular viability [45]. E93R substitution in *E. coli* FtsZ induces bundling of protofilaments, reduces GTPase activity, and impairs bacterial cytokinesis [46]. Similarly, R191 of *B. subtilis* FtsZ was found to be required for polymerisation [47].

The residue D212G of the T7 loop is conserved among all FtsZ sequences known [48] and all the mutations D212A\N\C, D212G (FtsZ2) impaired GTPase activity [42–44]. The location of FtsZ2 mutation was later validated by the crystallography data that SulA binds to T7 loop

region of FtsZ and a mutation in T7 loop might prevent binding of SulA to block FtsZ polymerisation [30]. There was no other structural abnormality of FtsZ2 to bind GTP and therefore could polymerise in the presence of DEAE-dextran *in vitro* [19] or co-polymerise with wild-type FtsZ [25].

The C-terminal variable (CTV) region residues in *E. coli* FtsZ and *B. subtilis* FtsZ were found to mediate electrostatic interactions to facilitate lateral association of the FtsZ protofilaments to form polymers *in vitro* and *in vivo* [39] and for interaction with other proteins such as ZapD ([49], reviewed in [4, 5]).

2.2. Polymerisation properties of FtsZ *in vitro* and *in vivo*

FtsZ has been found to exist mostly as dimers [50, 51]. FtsZ assembly process leading to polymerisation has also been found to involve a dimer nucleus [52]. Among the wide variety of FtsZ structures visualised by various methods till date, the simplest one is the protofilament constituted by FtsZ subunits stacked one above the other with a diameter of 5 nm, with each subunit placed at 4.3 nm apart [53]. While straight protofilaments were favoured by high concentration of GTP or by GTPase inhibition, curved conformation was triggered by GTP hydrolysis or in the presence of GDP [54]. Examination of the FtsZ polymers using atomic force microscopy (AFM) showed that individual protofilaments can fragment and re-anneal on a surface [55]. Also, individual protofilaments were found to have tendency to form bundles in the presence of GTP and singular curved protofilaments were observed in the presence of GDP-AlF_3 [55]. This observation might have implications on the assembly dynamics of FtsZ ring on the inner cell membrane during bacterial cell division.

The *E. coli* FtsZ-Q47K mutant, which does not support cell division [44], formed bundles and rings in yeast cytoplasm [56]. They found that the double mutant *E. coli* FtsZ-Q47K-**D86K**, which carries a lateral mutation (indicated in bold letters) formed long linear cables but did not assemble into a ring. In a separate observation, the authors have reported that FtsZ cables laterally assemble to form bundles in yeast cytoplasm. Thus, these observations indicated that the lateral contacts in FtsZ are important *in vivo* for FtsZ polymeric ring formation. Interestingly, it was found that the C-terminal unstructured tail, or region equivalent to it, is completely dispensable for *in vitro* and/or *in vivo* polymerisation of *E. coli* FtsZ [57, 58], *P. aeruginosa* FtsZ [30], and *B. subtilis* FtsZ [34].

In *in vivo* studies, FtsZ rings constituted by FtsZ polymers have been observed at the mid-cell site using fluorescence microscopy [1, 2] and super resolution 3D-structured illumination microscopy (3D-SIM) [59]. *In vivo* studies in *E. coli* [60] and *B. subtilis* [61] have shown oscillation of FtsZ in a helical pattern throughout the length in the presence of FtsZ ring in *E. coli* [60] and later constricting to the median as the Z-ring form in *B. subtilis* [61].

3. Unique structural features of mycobacterial FtsZ and their role in biochemical activities

Like in the case of FtsZ from other bacterial systems [50–52], *M. tuberculosis* FtsZ exists as a dimer [62] and the assembly process leading to polymerisation has also been found to involve

a dimer nucleus [63]. Like in the case of FtsZ from other bacterial systems, the T7 loop of one monomer is supplied in *trans* to the GTP-binding pocket of the next, thus forming the active site for GTP hydrolysis [64]. In a FRET-based system, *M. tuberculosis* FtsZ was found to take about 60–100 s to reach polymerisation saturation, about 10 times slower compared to *E. coli* FtsZ [63]. At steady state also, subunit turnover and GTPase activity were about 8–10 times slower than those of *E. coli* FtsZ [63]. FRAP experiments showed that *M. tuberculosis* FtsZ has a slower recovery than *E. coli* FtsZ in yeast cytoplasm also [56], which is consistent with the slower polymerisation of *M. tuberculosis* FtsZ [65]. Thus, the FtsZ polymerisation and assembly dynamics of FtsZ of *M. tuberculosis*, which divides once in 18–24 h [9, 10], are much slower than those of the FtsZ of *E. coli*, the different strains of which divide once in 18–55 min [6].

3.1. The role of N-terminal domain residues

The N-terminal domain of the FtsZ proteins of all mycobacterial species is highly conserved (**Figure 1**). In spite of this conservation, conspicuous drastic differences exist at specific amino acid locations. A typical example is the glaring presence of T172 in *M. leprae* FtsZ as the lone exception in lieu of A172 in the FtsZ proteins of all the other mycobacterial species. *M. leprae* FtsZ was found to be polymerisation-lethargic *in vitro*, even in the presence of DEAE-dextran and under a variety of other conditions [66]. However, interestingly, change of T172 to A172, as it exists in *M. tuberculosis* FtsZ, showed dramatic polymerisation as in the case of *M. tuberculosis* FtsZ [66]. Conversely, the reciprocal replacement of A172 with T172 in *M. tuberculosis* FtsZ, as it exists in *M. leprae* FtsZ, completely abolished the polymerisation potential of *M. tuberculosis* FtsZ. These observations showed the crucial nature of A172 residue for polymerisation of FtsZ of all mycobacterial species that have generation time of 24 h or less. On the contrary, the change of A172 to T172, exclusively in *M. leprae* FtsZ, seems to be an evolution-driven modification, probably to dramatically tone down FtsZ polymerisation rate, as *M. leprae* divides only once in 13.5 days [11]. The positioning of T172, which can wield such dramatic influence on FtsZ polymerisation, is well justified to be in the N-terminal domain that is known to be important for polymerisation, as found in *E. coli* FtsZ [21, 57]. Modelling of *M. leprae* FtsZ and *M. tuberculosis* FtsZ showed that probably the presence of Thr, which is a hydrogen-bonding and branched residue, at 172 position in the T6 loop might have imposed rigidity on the T6 loop-H10 helix-T7 loop segment and thereby affecting the polymerisation potential of *M. leprae* FtsZ [66]. Conversely, at position 172, the presence of the non-branched residue Ala that does not engage in hydrogen bonding might not impose rigidity on the T6 loop-H10 helix-T7 loop, thereby facilitating polymerisation of *M. tuberculosis* FtsZ. The link of T6 loop, which contains the 172 residue, to the T7 loop, which might be crucial for polymerisation *in vitro* [66], via the H10 helix, supports this possibility. In view of these observations, it is needless to state that there have to be cellular factors that might enable polymerisation of *M. leprae* FtsZ in a very slow manner suiting the slow generation time of the bacterium when *M. leprae* divides inside human cells. The D84 and D94 of *M. tuberculosis* FtsZ are equivalent to D86 and D96 of *E. coli* FtsZ, respectively [42], and both the residues form salt bridge at the dimer interface [62]. The D94 and N22 form salt bridge with R181 and E136, respectively, in the other subunit [62]. Such interactions might be necessary for the protofilament association during *M. tuberculosis* FtsZ polymerisation as found necessary for the *E. coli* FtsZ polymerisation [42]. While G103 was found to be involved in GTP binding [67], C155 has also been found to play an important role in the assembly of *M. tuberculosis* FtsZ into protofilaments during polymerisation [68].

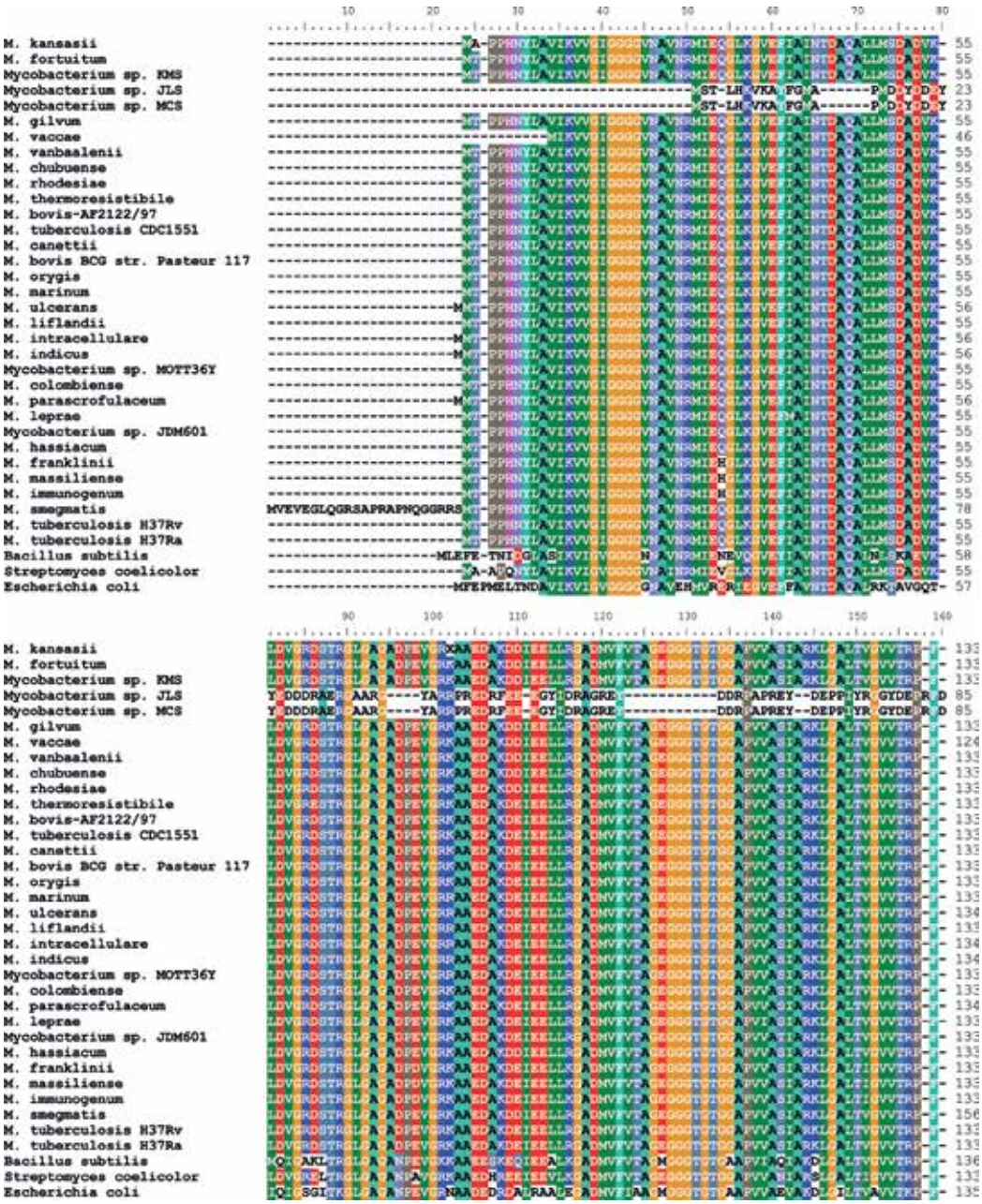

Figure 1. Homology comparison of the amino acid sequences in the N-terminal domain of FtsZ of various mycobacterial species.

3.2. The role of C-terminal domain residues

The C-terminal stretch of FtsZ protein has been found to have structural and functional roles in different bacterial systems (reviewed in [4, 5]). The crystal structure of *M. tuberculosis* FtsZ

did not contain the co-ordinates for the 66 residues at the C-terminal portion [62], indicating its unstructured nature like in the case of the C-terminal stretch of the full-length FtsZ of *M. jannaschii* [16], *Pseudomonas aeruginosa* [22], *Aquifex aeolicus* [22], *Thermococcus maritima* [21], and *B. subtilis* [31]. On the tubulin dimer template, the molecular model for the complete structure of *M. tuberculosis* FtsZ, inclusive of the extreme C-terminal 66 residues [69], the co-ordinates of which were not visible in the crystal structure of *M. tuberculosis* FtsZ [62], also showed an unstructured C-terminus [69].

Like in the case of *E. coli* FtsZ and *B. subtilis* FtsZ, the C-terminal region of the FtsZ proteins of all the mycobacterial species contain charged residues towards the C-terminal end. FtsZ of diverse mycobacterial species also shows wide variations in the nature of the residues in the C-terminal region (**Figure 2**). These residues show high levels of divergence from the residues on the FtsZ of *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) and even from the FtsZ of *Streptomyces coelicolor*, which is also an Actinobacteria like mycobacteria. Many mycobacterial species have an insertion of GGIAD in the C-terminal variable region, the function of which is under investigation.

The *M. tuberculosis* FtsZ dimer model showed a possible role for the C-terminal Arg residues (R378 and R379) on the stability of the dimer and hence on the polymerisation of the protein. In fact, biochemical studies showed that the deletion of the extreme C-terminal residues, R378 and R379 in the C-terminal extreme stretch of $_{373}$PPFMRR$_{379}$ of *M. tuberculosis* FtsZ, completely abolished polymerisation *in vitro* [69]. Besides the deletion of R379, the deletion of the R378 or its replacement with Lys, His, Ala, or Asp completely abolished polymerisation activity, indicating the crucial nature of the residues in the unstructured region of the protein for polymerisation. However, the polymerisation potential of the protein was not affected by the deletion of the single R379 residue alone [69]. The C-terminus of most of the mycobacterial FtsZ ends with $_{377}$MRR$_{379}$ and very few with $_{377}$MRH$_{379}$, with the conservation of M377 (**Figure 2**).

M. tuberculosis FtsZ has been found to interact with *M. tuberculosis* FtsW *in vitro* [70] and *in vivo* [71]. The presence of three of the four aspartate residues, D367 to D370, was found to be critically required for the interaction of *M. tuberculosis* FtsZ with a cluster of positively charged residues in the C-terminal tail of *M. tuberculosis* FtsW *in vitro* [70] and *in vivo* [71] (**Figure 2**). Similarly, PknA-dependent phosphorylation of T343 in *M. tuberculosis* FtsZ is required for FtsZ function during oxidative stress [72]. Like in the case of FtsZ-SepF interaction in *B. subtilis* [35, 36], the C-terminal tail of *M. tuberculosis* FtsZ was found to be required for the interaction of SepF with FtsZ [73]. These and other studies showed that *M. tuberculosis* SepF is found to be an essential part of the mycobacterial cell division machinery [74], probably in assisting FtsZ localisation at the mid-cell site [73].

3.3. The role of other residues in the protein

Like in the case of the mutations D212A\N\C, D212G (FtsZ2) in *E. coli* FtsZ, which impair GTPase activity [42–44], the equivalent mutant D210G of *M. tuberculosis* FtsZ also showed impaired GTPase activity [67] (**Figure 3**). Although *M. tuberculosis* FtsZ-D210G polymerised *in vivo* to the mid-cell ring in a merodiploid *Mycobacterium smegmatis* background, it failed

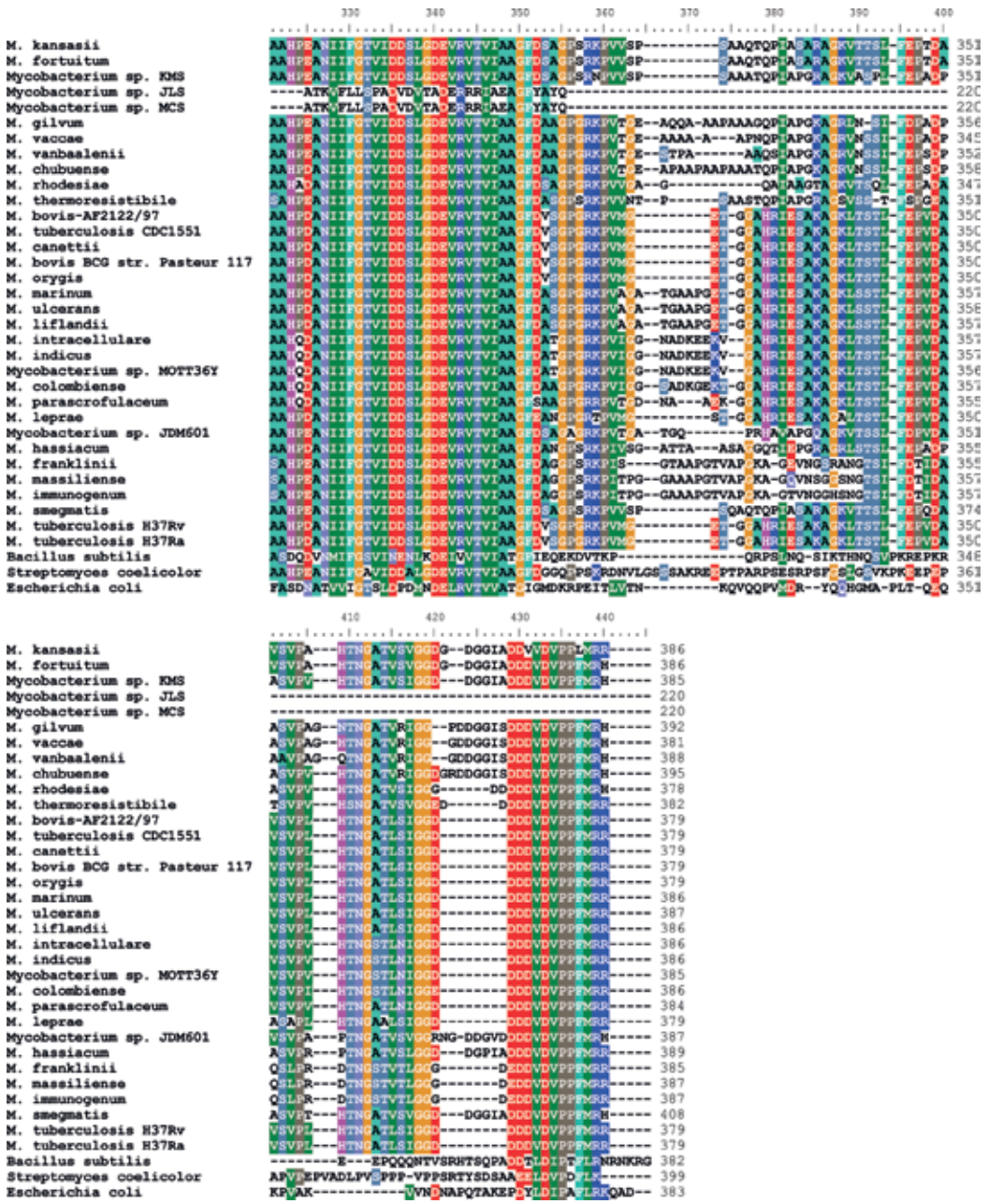

Figure 2. Homology comparison of the amino acid sequences in the C-terminal domain of FtsZ of various mycobacterial species.

to act as the sole source of FtsZ *in vivo* and showed 100-fold impaired GTPase activity *in vitro* [67]. The authors suggested that the mutation on the T7 loop might not prevent FtsZ self-association or association with the wild-type protein but rather specifically affect GTP

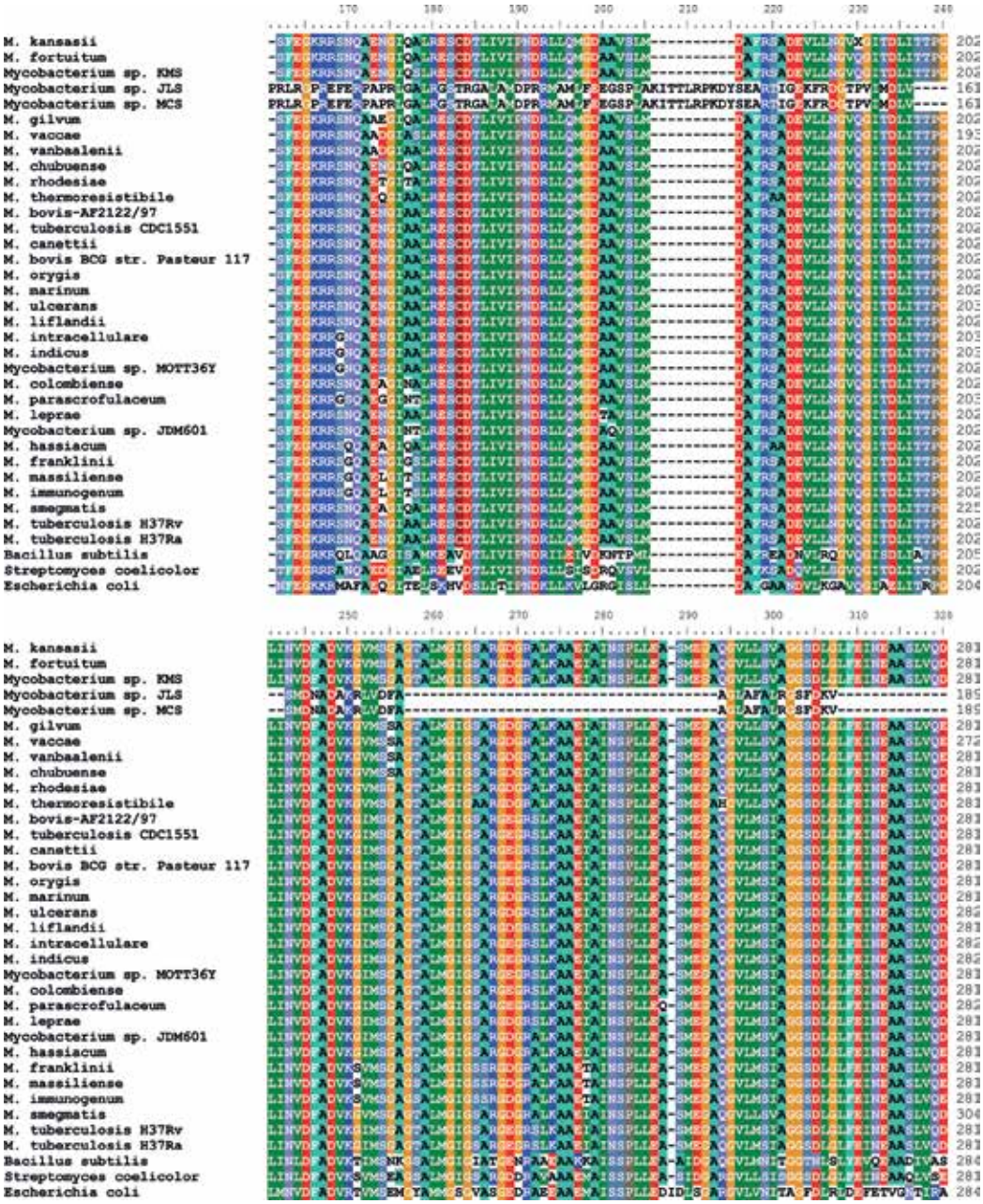

Figure 3. Homology comparison of the amino acid sequences at other parts of FtsZ of various mycobacterial species.

hydrolysis, thereby uncoupling GTPase property from polymerisation. The specific residues, which have been found to be involved in the polymerisation or interaction of *M. tuberculosis* FtsZ with itself or with other cell division proteins, are listed in **Table 1**.

Residue in *M. tuberculosis* FtsZ	Interacting protein of *M. tuberculosis*	Functional role of the interaction	Reference
N22	E136 of FtsZ	Polymerisation	[62]
D94	R181 of FtsZ	Polymerisation	[62]
G103	FtsZ	GTP binding	[67]
C155	FtsZ	Polymerisation	[68]
A172	FtsZ	Polymerisation	[66]
D210	FtsZ	GTPase	[67]
T343	PknA	FtsZ function*	[72]
D367-D370	FtsW	FtsZ function*	[70]
R378, R379	FtsZ	Polymerisation	[69]
C-terminal tail	SepF	FtsZ function*	[73]

*The exact biochemical activity of the FtsZ, in which these residues participate, is not known.

Table 1. Residues in ***M. tuberculosis*** FtsZ that interact with residues in another subunit or with other cell division proteins.

4. Correlation between FtsZ polymerisation kinetics and bacterial generation time

Mycobacterium leprae is one of the slowest growing bacteria with a generation time of 13.5 days *in vivo* [11]. The generation time of *M. tuberculosis* is 18 h *in vivo* [9] and 24 h *in vitro* [10]. Similarly, while *M. smegmatis* divides once in 3 h [8], *S. coelicolor* A3(2), which is classified under Actinobacteria like mycobacteria, has a generation time of 2.31 h, except that some of the strains grow as slow as 28.9 h depending upon growth conditions [75]. Meanwhile, different strains of *E. coli* show generation time of only 18–55 min [6]. In agreement with the slower generation time of *M. tuberculosis*, compared to that of *E. coli*, *M. tuberculosis* FtsZ showed slower polymerisation *in vitro* [63, 65]. Interestingly, the FtsZ of *M. leprae*, which divides once in 13.5 days [11], did not polymerise at all *in vitro* even in the presence of DEAE-dextran [66]. Comparatively, the time taken by *M. smegmatis* and *S. coelicolor* to reach steady state of FtsZ polymerisation is about 4 min [76]. Similarly, the time taken by the FtsZ of *Caulobacter crescentus*, which has a generation time of 3 h [77], to reach steady state of polymerisation is 5 min [78]. On the contrary, while *E. coli* FtsZ takes only 1–6 s to reach steady state of polymerisation [18, 79], FtsZ of *B. subtilis*, which divides once in 120 min [7], takes approximately 200 s [47]. These observations on the comparative polymerisation kinetics of FtsZ proteins of *E. coli*, *M. tuberculosis*, *S. coelicolor*, *M. smegmatis*, and *M. leprae* and the generation time of the respective bacterium (**Table 2**) probably allude to the existence of a correlation between the generation time of the bacterium and the time taken by the respective FtsZ to reach steady state kinetics *in vitro*, which may hold true *in vivo* as well. Mechanistically, such a correlation needs to be necessarily in-built so that kinetics of FtsZ polymerisation during division of the cell keeps pace with the overall generation time of the bacterium. The presence of A172 in the FtsZ of

Bacterium	Time taken to reach steady state of FtsZ polymerisation (from light scattering data)[a] [with reference]	Generation time of the bacterium[a] [with reference]
M. smegmatis	~4 min [76]	3 h [8]
S. coelicolor	~4 min [76]	2.31 h [75]
M. tuberculosis	~6–10 min [65]	24 h [10]
M. leprae	ND[b] [66]	13.5 days [11]
E. coli	~1–6 s [18]	18–55 min [6]
B. subtilis	~200 s [47]	120 min [7]
C. crescentus	~3 min [78]	3 h [77]

[a]The studies from which the respective values were taken are given in the parenthesis.
[b]Not determined as *M. leprae* FtsZ does not polymerise *in vitro* [66].

Table 2. Correlation between the time taken to reach FtsZ polymerisation steady state and generation time of the bacterium.

all mycobacterial species, except in *M. leprae* where it is T172, is a typical example for such a correlation. The homology comparison of mycobacterial FtsZ sequence vividly shows the existence of several such minor differences in terms of specific amino acid residues at crucial positions that may play significant role in conferring differences in the polymerisation kinetics of the FtsZ protein of the respective bacterium. Structure-function studies on the polymerisation kinetics of a large number of FtsZ molecules from diverse bacterial genera differing widely in their generation time might establish such correlation.

5. Application of the FtsZ structure-function studies in public health

FtsZ being the principal essential cytokinetic protein in bacterial systems, it has been examined as a potential target for the design of inhibitory compounds that could be used as antibacterial drugs against diverse pathogenic bacteria (reviewed in [80]). Although the overall sequence conservation between FtsZ and β-tubulin is only 10–18% except at two stretches [15], it is important to ensure that anti-FtsZ compounds do not inhibit β-tubulin in humans. Owing to the overall conservation of the three-dimensional structure of FtsZ proteins from diverse bacterial genera, developing broad spectrum antibiotics, which are equally effective against the FtsZ of pathogenic bacteria of diverse genera, by designing inhibitor against the FtsZ molecule of a single bacterium, seems to be an attractive possibility [81].

6. Perspectives and challenges

The vast regions of homology and overall conservation of the three-dimensional structure of FtsZ proteins of diverse bacterial systems may seem attractive to design common inhibitors directed against these regions expecting them to be effective against the FtsZ proteins.

However, the structure-function studies on FtsZ revealing the subtle differences among the primary, secondary, and tertiary structures of FtsZ proteins from diverse bacteria give the hint that an anti-FtsZ inhibitor designed against the FtsZ of a select pathogenic bacterium may not act effectively with the same MIC/MBC against the FtsZ proteins of other pathogenic bacteria, as found [81]. Thus, the design of a common inhibitor that may be expected to act against FtsZ proteins from diverse pathogens remains a big challenge in the development of inhibitors against bacterial cytokinetic protein, FtsZ. Secondly, identification of the residues contributing to the structure that is required for the generation of force by FtsZ for cell wall/membrane constriction [82] during the physical division of the mother cell also remains a challenge for future studies.

7. Conclusions

The essential cytokinetic protein, FtsZ, of different Mycobacterial species and of other bacteria has evolved to possess specific amino acid residues at crucial positions on the protein to suit the polymerisation kinetics that befit cell division duration. Thus, each FtsZ protein is unique in terms of the specific types of amino acid residues at crucial positions on the protein in spite of the regions of homology and overall conservation of the three-dimensional structure. It is these unique differences in the residues at specific crucial positions on the FtsZ protein that confer subtle differences on the FtsZ structure and hence on the cytokinetic function of the protein in the respective bacterium.

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Chapter 15

Mycobacteria-Derived Agents for the Treatment of Urological and Renal Cancers

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Additional information is available at the end of the chapter

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Abstract

Mycobacteria are the unique group of bacteria that are currently used in antitumoral immunotherapy. Specifically, intravesical instillation of viable cells of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), after transurethral resection of non-muscle invasive bladder cancer, is the most efficacious treatment for avoiding recurrence and progression of the disease. BCG has been used for the last 35 years for bladder cancer treatment, but other mycobacteria or mycobacteria components are currently under preclinical and clinical studies for the immunotherapeutic treatment of non-invasive bladder cancer and also of other types of tumors located at the urinary system. Those are, for instance, cell wall extracts or heat-killed forms from BCG or other mycobacteria such as *Mycobacterium phlei* or *Mycobacterium indicus pranii* (MIP) or even viable cells from non-pathogenic mycobacteria such us *Mycobacterium brumae*. A review of the literature in which mycobacteria components, non-viable mycobacteria, and viable mycobacteria have been used for these different cancers will be performed. In this chapter, the function of mycobacteria as antitumor agents will be then analyzed, awarding the audience a broad knowledge of one of the beneficial applications of mycobacteria, which are usually introduced as dangerous microorganisms.

Keywords: BCG, bladder cancer, immunotherapy, *Mycobacterium brumae*, *Mycobacterium phlei*

1. Introduction

Since Science journal chose cancer immunotherapy as the breakthrough of the year in 2013, with only a few evidence of its efficacy and consequences, this field is now in fashion [1]. Immunotherapies follow extremely diverse strategies, and the only point they have in common is that all of them activate somehow the cancer patient's immune system to attack tumor

cells. In fact, tumor cells are supposed to be recognized by the immune system as foreign, however, in the cases in which cancer progresses because the tumor and the immune system reach equilibrium that drives to immunotolerance. Immunotherapies include antibodies against tumor epitopes, cytokines, checkpoint inhibitors that break the equilibrium, oncolytic virus, T cell therapy using T cells removed from the patient and modified with chimeric antigen receptors (CARs), and finally, therapeutic vaccines and adjuvants which are the most ancient immunotherapies that exist.

1.1. Prechemotherapeutic era: the first association between mycobacteria and cancer

The first thoughts about a possible intervention of the immune system in the clearance of tumors were made at the beginning of the nineteenth century (**Figure 1**). It was observed that in cancer patients who underwent a gas gangrene, caused by *Clostridium*, their tumors regressed [2]. Later three different physicians independently made the same observations: first Busch in 1868, then Fehleisen in 1882, and, finally, Coley in 1891 observed tumor shrinkage in patients suffering from erysipelas. From their observations, all three had the same idea: exposing their cancer patients to the infectious agent. At that time, Busch ignored that erysipelas was an infectious disease caused by *Streptococcus pyogenes* (Fehleisen would describe this later [3]) which resulted in the death of his first patient due to the infection despite the

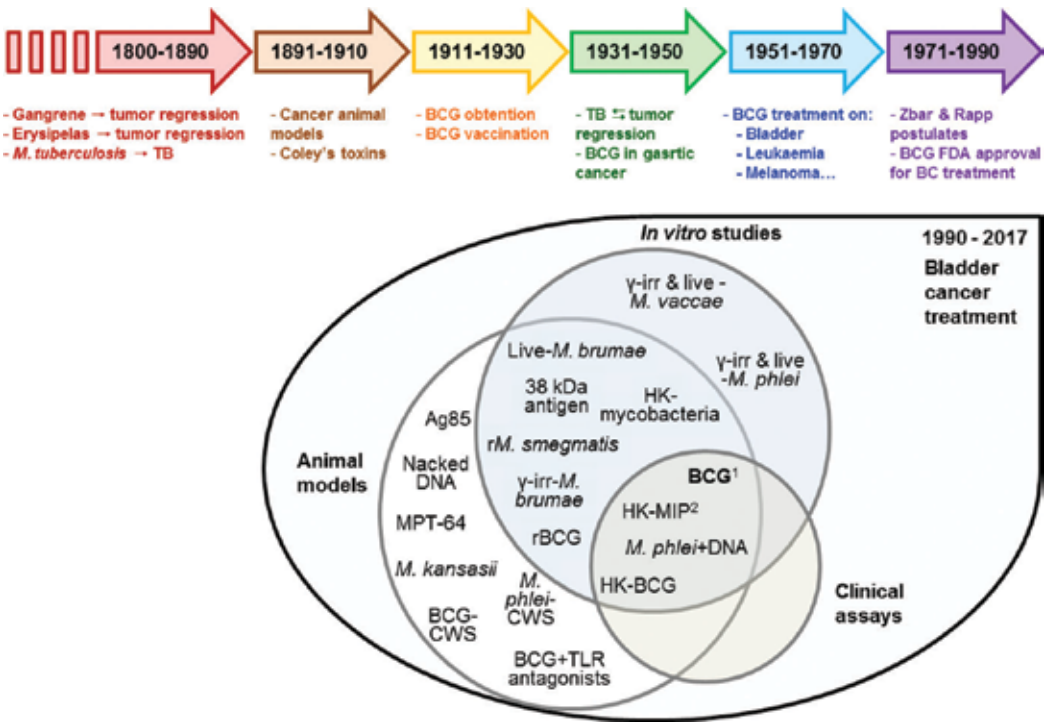

Figure 1. Time course of the development of BCG immunotherapy and studies using mycobacteria-derived agents carried out for BC treatment. [1]Alone or in combination with chemotherapy. [2]In combination with chemotherapy and radiotherapy.

regression of the tumor. Coley was the first one who systematically exposed their patients to infectious agents; he treated more than 1000 patients. The combination of the bacterial products of *S. pyogenes* and *Serratia marcescens* became the well-known Coley's toxins [3]. Although the efficacy of Coley's toxins became later controversial, nowadays it is correctly considered based on an immunotherapeutic effect [4]. Another relevant scientist who extensively contributed to cancer understanding and cure through immunotherapy was Clowes, who systematized the methodology to monitor tumor size in transplantable animal cancer models. He dedicated part of his life in seeking the immunomodulating agent responsible for tumor clearance [5]; nevertheless, as all we know, this "magic" antigen has not still been described.

The phenomenon described in the case of erysipelas disease was also observed for tuberculosis (TB) patients. In 1929, Pearl published a large series of studies describing the inverse relationship between patients suffering from cancer and TB based on the evaluation of hundreds of autopsies [6]. Almost in parallel, 8 years before the publication of Pearl's studies, the first girl was being administered with three doses of an attenuated strain of *Mycobacterium bovis* during the first week of her life. After the discovery of *Mycobacterium tuberculosis* as the causative agent of TB in humans by Koch, scientists raced to find a vaccine against this big killer. Nocard isolated the highly virulent species *M. bovis*, and in 1904, he transferred the strain to Albert Calmette and Camille Guérin who obtained the attenuated strain by subculturing *M. bovis* over bile-potato medium every 3 weeks for 13 years. They demonstrated the avirulence without reversion in a guinea pig model, and the strain received the name of *M. bovis* Bacillus Calmette-Guérin (BCG) [7]. In 1932, the BCG was approved as safe vaccine against TB.

Therefore, studies on the use of BCG for cancer treatment started right after, and in 1935, Holmgren was the first scientist to report success in cancer patients [8]. As a continuation of the experiments he had begun in 1913 in which he evaluated the sensitivity to tuberculin in more than 600 gastric cancer patients, he intravenously injected repeated BCG doses in 28 cancer patients, most of them were gastric cancer patients [8]. In the 1950s, Rosental observed specifically lower incidence of leukemia in people who had received BCG at birth [9]. However, once the patient developed the tumor, there was no chance to previously immunize the patient, so the key point was to know whether the BCG had a curative role [10]. In the following years, many authors proved the efficacy of BCG in several cancer animal models including the bladder [11]. However, the spreading of the modern chemotherapy and radiotherapy for the treatment of cancer weakened the investigations on BCG as tumor treatment.

1.2. Post-chemotherapeutic era: consolidation studies

During the 1970–1980s, hundreds of articles were published in the field of immunotherapy using of BGC or BCG components for the treatment of cancer following different strategies. First, as was done until that moment, BCG was directly used intratumorally, and many studies in different cancers during these decades continued in this line of investigation. Following another strategy, some other scientists assayed the antitumoral effect of BCG as adjuvant in patients undergoing chemotherapy or radiotherapy for the treatment of lung [12], melanoma

[13], cervical [12], head and neck [12], or ovarian cancers [14], for instance. Another completely different strategy was the use of BCG as an adjuvant administering it together with tumor cell lysates [15]. In that period there were some authors who evaluated the antitumor efficacy of some mycobacterial fractions or other mycobacterium species in different cancer models, for instance, of hepatoma or sarcoma [16].

In 1974, Zbar and Rapp established the favorable conditions for obtaining a positive outcome using a guinea pig model [17]. They determined the amount of bacilli that should be administered, that it was a mandatory a close contact between BCG and the tumor, and that BCG worked better against small tumors and immunocompetency of the host which permit to mount an immune response against the BCG [17]. Thanks to this postulates, in 1976, the urologist Morales and collaborators published the results of a successful small clinical trial in which they evaluated the intravesical administration of BCG in bladder cancer (BC) patients. It was not until 1990 that BCG was finally approved by the Food and Drug Administration (FDA) for the treatment of superficial BCG (**Figure 1**).

2. Mycobacteria as antitumor agents in the last 25 years

Although more than a century of research led finally only to the standard use of live BCG for BC treatment, the research trying to use mycobacteria components for cancer treatment has not been abandoned. In fact, in the last 25 years, several attempts for using different mycobacteria as immunotherapeutic agents for bladder cancer treatment have been carried out (**Figure 1**).

Despite the molecule or molecules of BCG responsible for its antitumor effect are still unknown, several genus-specific antigens have been described in mycobacteria cells, and most of them are known stimulators of the immune system. Specifically, the mycobacteria cell wall is rich in a variety of exclusive lipids, glycolipids, lipoproteins, glycans, and proteins that are recognized by immune receptors. Zlotta et al. [18] demonstrated that not only mycobacterial cell wall components are responsible for the antitumor effect but other fractions also triggered the production of Th1 cytokines and stimulated the cytotoxic activity against T24 BC cells by peripheral blood cells. Antigens from different cell fractions are recognized by surface-located receptors present in antigen-presenting cells.

Molecules such as lipoarabinomannan (LAM), phosphatidylinositol mannosides, or heat-shock proteins (HSP) are recognized by Toll-like receptors (TLR) 2 and 4 or mannose receptors; or antigens like trehalose mono- and dimycolate are agonist of C-type lectin receptors. Other mycobacteria antigens interact to specific intracellular immune receptors after being internalized to the cell, such as unmethylated cytosine-guanosine nucleotide (CpG)-rich DNA motifs, muramyl dipeptide (MDP), or cytosolic DNA which bind to TLR-9, NOD2, or cyclic GMP-AMP synthase (cGAS), respectively. After being processed inside the cells, some antigens are presented to T cells via CD1 receptors such as mycolic acids (MA) or trehalose and glucose mycolates. Signaling through these receptors can induce the production of cytokines and/or chemokines favoring a desirable pro-inflammatory profile in tumor microenvironment [19].

Nevertheless, not all mycobacteria possess all the mentioned antigens, and even the structure of each antigen can vary between different species. Although some of these molecules such as LAM, trehalose dimycolate (TDM), or MA are present in all mycobacteria, the structure, for example, the presence or not of mannose residues in LAM structure, the length or the presence of unsaturations or oxygenated groups in the lipidic chains of TDM or MA, etc., determine the interaction to the corresponding immune receptor, and in consequence, the immune response is generated. The complexity of mycobacteria antigenicity is enormous leading even to the fact that different strains of the same species can have different antigenic pattern. The case of the antigenic profile of BCG is a good example. As it is known, from the seed strain originated in France by Albert Calmette and Camille Guérin, different strains were originated after subculturing the original BCG in different countries. Before being preserved by freezing, decades of subculturing originate deletions of some genome regions or even the duplication of some other genome regions. Today, we count about a decennium of different BCG strains [20] that are used broadly for BC treatment as well as for TB vaccination. Each different BCG strain possess or not some immunogenic antigens like phenolglycolipids, phthiocerol dimycocerosates, and MBT64 protein antigen, or even they have two different MA profiles: some strains possess alpha, methoxy- and keto-MA, while some other possesses only alpha- and methoxy-MA. The relevance of these differences has tried to be related to BCG efficacy as antitumor agent or TB vaccine, but until nowadays the critical antigens are still not known.

In the following sections, the use of different mycobacteria or their components in the last 25 years for urinary tract cancers will be reviewed. We will mainly focus on the use of these components as unique antitumor agents, although the inclusion of studies in which mycobacterial antigens are used as adjuvants for tumor antigens or other therapies will also be mentioned.

3. Urinary tract cancers and mycobacteria

3.1. Bladder cancer and live BCG: a fruitful relationship

The immunotherapeutic ability of mycobacteria against cancer has the most successful example in the case of the use of BCG for the treatment of high-risk non-muscle invasive bladder cancer (NMIBC) patients. All the conditions described by Zbar and Rapp are accomplished in intravesical BC treatment: a close cavity where mycobacteria can be loaded and being in close contact to tumor cells triggering also an immune response [17]. BC is one of the most common malignances in urology. The number of new cases and deaths of BC was 20.1 and 4.4 per 100,000 men and women per year, being in 2013 estimated 587,426 people living with BC in the USA [21]. In 2016, 76,960 new cases of BC were estimated, and around 70% of them present as NMIBC. Whether NMIBC patients are not treated after transurethral resection (TUR) of tumor, as much as 80% will experience disease recurrence and/or progression. Therefore, after TUR, the standard treatment for high-risk NMIBC patients consists in the intravesical instillation of live BCG. The bladder cavity allows a closed contact between the possible remaining tumor cells and the bacilli. For a short period of time (approximately 2 h), a high concentration of microorganisms (between 10^7 and 10^9 bacilli depending on the commercially

available preparations of BCG strains), besides being in contact to possible remaining tumor cells, initiates an immune cascade of events. Although the detailed chronogram and magnitude of these events are not totally understood, numerous studies have provided information about the immune cells and signals implicated in the action of BCG. Several excellent reviews have covered this field, explaining what is known until today, see [22, 23]. In summary, BCG firstly interacts with remaining bladder tumor cells inducing apoptosis and/or cell cycle arrest as demonstrated in *in vitro* experiments [24–26]. Moreover, internalization of the mycobacteria triggers the production of cytokines and chemokines [27] that work as initial signals for the host immune system, leading to the infiltration of multiple immune cells into the bladder lumen, as observed in in vivo models of the disease [28, 29] as well as in BCG-treated patients [27]. Although the role of each infiltrated immune subsets—T cells, B cells, natural killer (NK) cells, macrophages, γδ cells, etc.—is still unknown, their presence is critical for an appropriate antitumor response [30]. This BCG-triggered immune response is finally able to fight against the presence of new tumors. BCG significantly reduces the risk of recurrence in treated patients, compared to NMIBC patients to whom only TUR is applied [31]. Moreover, in a recent meta-analysis of the literature, BCG has been confirmed as the only agent associated with decreased progression risk versus TUR alone [32].

Intravesical BCG therapy is then successful. In fact, the same protocol of instillations has been used in high-risk NMIBC patients for the last 30 years. But, although efficacious, BCG is not the perfect treatment. On the one hand, a percentage of patients do not respond to BCG for unknown reasons. On the other hand, a high percentage of patients suffer adverse events during the treatment.

3.2. Increasing efficacy

Several strategies are tried to solve the problem of unresponsive BCG patients. The main strategy consists of improving the immune response triggered by BCG, by combining BCG and immunomodulators or modifying genetically the bacterium for expressing these immunomodulators. For instance, BCG plus an optimized interleukin (IL)-15 mutant significantly increased immune activation and reduced tumor burden and angiogenesis compared to the single agents in the carcinogen-induced rat NMIBC model [33]. The list of modified BCGs is long and comprises the expression of cytokines and chemokines—IL-2, interferon (IFN)-γ, GM-CSF, etc.—or immunodominant mycobacteria antigens like alpha-crystallin antigen (fibronectin-binding protein) complex (Ag85) [9, 22, 34]. These strategies seem to be promising for improving the efficacy of BCG alone as *in vitro* and *in vivo* experiments using animal models of the disease have shown. In that way, intravesical instillation of recombinant BCG strain expressing the fusion protein of IL-15 and Ag85B (BCG-IL-15) in tumor-bearing mice leads to a high neutrophil infiltration, increased presence of chemokines into the bladder, and prolonged survival rates compared to mice treated with BCG alone [35]. Recombinant BCG expressing human interferon-alpha 2b (hIFNα-2b) showed higher antiproliferative capacity on EJ BC cells after infection compared to wild-type BCG. Furthermore BCG-hIFNα-2b-stimulated lymphocytes trigger the production of higher levels of IFN-γ, tumor necrosis factor (TNF)-α, and IL-12, together with higher cytotoxic activity against BC cells, compared to those treated with BCG [36].

The study of the immune response triggered by BCG inside the bladder has revealed that an excess of IL-10 production in tumor microenvironment is detrimental for BCG efficacy. Another successful strategy is then the use of anti-IL-10 antibodies in combination to BCG for an improved effect. To block IL-10 receptor together with intravesical BCG reach high tumor regression rates in the murine orthotopic model of BC, enhancing also a systemic specific antitumor immune response compared to BCG alone [37].

Another strategy is the combination of BCG with TLR agonists. BCG together with TLR4 agonist such as polyporus polysaccharide triggers the expression of activation molecules like CD80 and CD40. In rat BC models, this combination therapy showed a synergic effect reducing invasiveness of cancer together with reduced adverse events originated by BCG alone [38]. Similarly BCG plus a TLR3 agonist (polyinosinic:polycytidylic, acid poly(I:C)) showed to be more efficacious on reducing MBT-2 bladder tumor growth in treated mice than the monotherapies [39].

Finally, it has been also demonstrated that the combination of live BCG together with chemotherapeutic treatments improves disease survival compared to BCG alone. Although some works showed no impact on the progression or survival rates of the patients [16, 17], recent study has demonstrated the beneficial effect of sequential intravesical treatment with BCG and mitomycin C. While in mice experiments an augmentation of beneficial M1 tumor-associated macrophages on tumor-bearing treated mice was observed, in treated patients increasing IL-2, IL-8, IL-10, and TNF-α urine levels during treatment and increased efficacy over BCG treatment alone were observed [40].

3.3. Reducing adverse events

The most critical adverse event regarding intravesical instillations of BCG is the possibility of the patient to be infected. Numerous cases of BCGosis have been described in the literature in the last 5 years [41, 42]. The main reason is a traumatic instillation that can lead to the dissemination of BCG throughout the systemic circulation. As soon as BCG showed to be an excellent option for BC treatment, researchers tried to compare its effect to non-viable mycobacteria, cell wall extracts, or even purified antigens from mycobacteria.

3.3.1. Purified mycobacteria antigens and non-viable mycobacteria

The majority of mycobacteria antigens, which are able to stimulate the immune system, have been widely studied in order to find epitopes for vaccines to prevent TB infection and also to develop immunodiagnostic tests for TB infection or disease. Researchers have taken advantage of the knowledge of these molecules for trying to use them as antitumor agents for BC. In this line of research, MPT-64 antigen, 38 kDa protein, Ag85 antigen, or mycobacterial DNA have been evaluated for their ability to treat BC. For MPT-64 a dose-dependent response in survival rates was observed when instilled intravesically. The higher MPT-64 dose administrated provided higher survival rates in tumor-bearing treated mice than non-treated mice, triggering also a favorable IFN-γ systemic response [43]. When the 38 kDa antigen was studied, a cytotoxic activity against T24 BC cells was observed in 38 kDa antigen-activated peripheral blood mononuclear cells (PBMC), also in a dose-dependent

manner. Again, 38 kDa antigen-intravesically treated tumor-bearing mice survived longer than non-treated tumor-bearing mice [44], triggering a systemic response observed when splenocytes from treated mice respond specifically to the instilled antigen. Different works have evaluated the antitumor capacity of Ag85. Initially, the generation of cytolytic CD8 and an antitumor response was observed when cDNA from *M. kansasii* Ag85 antigen was transferred to MBT-2 BC cells and injected into mice [45]. Dendritic cells (DC) expressing Ag85 have also shown to activate T cells triggering them to be cytotoxic to bladder tumors [46]. Ag85-dendritic cells induce the infiltration of T cells into tumor site triggering also the reduction of BC tumors in in vivo experiments [46]. Finally, mycobacterial DNA, specifically from BCG and from *Mycobacterium phlei*, has proven to have antitumor activity. DNA from BCG activates NK and triggers the production of IFN-α, IFN-β, and IFN-γ in splenocytes [47–49]. DNA from *M. phlei* is the responsible for the induction of cytokine release by monocytes and macrophages and to inhibit BC-cell proliferation by triggering apoptosis [50, 51].

Apart from purified antigens, complex extracts of mycobacteria have also been evaluated for BC treatment. The two principal assayed compositions have been a cell wall extract from BCG (composed by the cell wall skeleton or also called SPM-105) [52] and a mixture of cell wall plus DNA of *M. phlei* (MCNA) mentioned above. The main problem to work using cell wall preparations is the huge hydrophobic character of the mycobacteria cell wall. Thus, in both cases, although early *in vitro* studies showed promising results [51, 53], a reformulation of the cell wall extract was needed in order to optimize the solubility and stability of the preparation and facilitate the contact with target cells.

On the one hand, the BCG-cell wall skeleton (CWS) has been formulated into liposomes in which their surface was modified by an octaarginine (R8) anchor, an efficient cell-penetrating peptide [54–56]. Researchers demonstrated that R8-liposome-BCG-CWS binds to MBT-2 murine BC cells inhibiting its growth in a syngeneic subcutaneous tumor model [57], being also efficacious in an intravesical BC rat model of the disease [58]. Furthermore, the formulated extract is able to inhibit human BC growth *in vitro* [55], activated immune cells to a Th1 profile, and leads to their cytotoxic activity against T24 and RT112 BC tumor cells [58].

On the other hand, *M. phlei* cell wall extract was initially formulated in mineral oil-in-water emulsion to be effective. After showing to induce BC apoptosis *in vitro*, the first clinical trial demonstrated a similar rate of response in NMIBC patients who previously were refractory to BCG treatment and those without previous BCG treatment, indicating a possible role of *M. phlei* as a second-line drug after BCG failure [59]. From the initial composition evaluated, the formulation was improved by considering the inclusion of *M. phlei* DNA that, as previously explained, showed antitumor effect [50, 60]. The new composition, denominated MCNA, triggers both a direct effect by inducing apoptosis on cancer cells and an indirect effect by triggering an immune response [60]. MCNA was therefore evaluated in phase II and phase III clinical trials [61]. Both studies had some drawbacks: the number of patients finally considered was relatively small and, among them, a small percentage (around 2%) of patients showed serious adverse events, and around 60–70% of patients in each study showed mild drug-related adverse events. In the phase III study, there was no signal of cancer in 25% of patients after 1 year and in 19% of patients after 2 years. Thus, MCNA could be a therapeutic option for patients in which the only therapeutic option is cystectomy after being refractory to BCG treatment, although further studies are needed [62].

Finally, whole non-viable mycobacteria have also been evaluated for BC treatment. Contrary to purified antigens or cell extracts, the use of the whole bacteria warrants the presence of the whole antigenic profile, but depending on the inactivation method, some of the possible crucial antigens can be altered or lost. On the one hand, heat-killing form has been the most studied although mycobacteria cells are damaged [63, 64], and on the other hand, γ-irradiation is the treatment which preserves better the integrity of the mycobacteria cell and maintains some metabolic activity [63].

Regarding *in vitro* BC cell growth inhibition, works using T24, J82, RT4, RT112, EJ28, or HT1376 demonstrated that both heat-killed (HK) and γ-irradiated BCG, *M. brumae*, *M. vaccae*, or *M. phlei,* or HK MIP, inhibit BC proliferation similarly to live mycobacteria [63, 65–67], while other works found less growth inhibition of 253J and T24 cells significantly when HK BCG was used [68]. Unlike the observations made by the above mentioned authors, other authors found that HK BCG had no inhibitory effect in MGH BC cells [69]. Moreover, lower cytokine levels were produced by HK and γ-irradiated mycobacteria-infected cells than those infected by live bacteria [63, 69]. Between both treatments, γ-irradiated mycobacteria trigger cytokine levels closer to those induced by live mycobacteria, than those induced by HK mycobacteria. Regarding immune activation: induction of cytotoxicity by activated cells, induction of cytokine production, and expression of activation markers on immune cells, again the immune response induced by live mycobacteria was higher than that triggered by non-viable mycobacteria [63, 66, 70, 71].

Using the orthotopic murine intravesical model of BC, HK BCG-treated mice survived similarly to non-treated tumor-beating mice [72], together with lower production levels of Th1 cytokines [72] that has also been related to inability to trigger T-cell infiltration into bladder cavity [28]. Interestingly, when live BCG or *M. brumae* was instilled in the first week of treatment and subsequent instillations were done using γ-irradiated mycobacteria, significant prolonged survival rates were observed in tumor-bearing mice compared to untreated mice [71].

In BC patients, HK BCG instilled to previously live BCG nonresponders showed reduced toxicity and no increase in the risk of tumor recurrence [73]. Whole HK MIP has been instilled intravesically in five BC patients undergoing radiation therapy [74], maintaining 100% survival rates and recurrence-free rates. It has been also instilled in the treatment of BCG-refractory patients [75].

3.3.2. Live non-BCG mycobacteria

Concluding for the whole work compiled in the literature, it seems that live BCG provides the best option compared to the non-viable mycobacteria or mycobacteria fractions. Therefore, another feasible option as alternative to BCG for BC treatment is to consider the use of live non-pathogenic mycobacteria. However, few studies have considered them for BC treatment. As explained above, the majority of mycobacterium species is saprophytic and potentially share immunomodulatory antigens with BCG.

In a recent work of our research group [76], several non-pathogenic-considered mycobacteria (*M. confluentis*, *M. chitae*, *M. chubuense*, *M. fallax*, *M. gastri*, *M. hiberniae*, *M. mageritense*, *M. obuense*, *M. phlei*, and two strains of *M. vaccae*) were evaluated for their capacity to inhibit BC cell proliferation *in vitro*, compared to the effect of BCG Connaught. Among all the species

studied, *M. brumae* effect highlighted over the rest of results. Among the mycobacteria tested on 7 BC cell lines (T24, J82, RT112, SW780, HG-MG3, 5637, and RT4, belonging to different grades), *M. brumae* stood out for inhibiting BC-cell proliferation at a similar extent to BCG in high-grade cell lines and for showing an improved effect than BCG in low-grade cell lines. *M. brumae* triggered the expression of activation markers on macrophages at higher degree than *M. phlei* or *M. vaccae* did. Moreover, *M. brumae* was able to activate human PBMC *ex vivo* to kill BC cells by both direct contact and using only the soluble factors released by the activated PBMC. *M. brumae* also was able to activate a murine macrophage cell line [66, 76]. Using the murine orthotopic model of BC, a high percentage of live *M. brumae*-treated mice survived compared to BCG-treated and non-treated tumor-bearing mice, being the differences significant only in comparison to non-treated-bearing mice [76, 77].

In vitro experiments have demonstrated that *M. brumae* cells did not persist alive inside both macrophages and BC cells after 72–96 h after infection [76, 77]. Besides *in vivo* experiments in the murine BC model showed that *M. brumae* do not persist neither in the spleen of mice treated intravesically with *M. brumae*, contrary to BCG, which is found in splenocytes after finishing the intravesical treatment [76, 77]. Nevertheless further security studies are needed to confirm the safety of this mycobacterium when it is used in its live form.

Apart from *M. brumae*, only *M. vaccae*, *M. smegmatis*, and *M. kansasii* have been studied in their live forms for BC treatment. According to our results [76], Baran and collaborators show that any of the three different strains of *M. vaccae* they studied was able to inhibit BC proliferation *in vitro* in a better way than BCG [78]. Regarding *M. smegmatis*, a recombinant strain expressing human TNF-α was studied. This strain inhibited EJ18, MGH-U1, RT4, and RT112 human BC-cell growth and triggered higher release of cytokines than the parental strain [79]. Later, inoculated in the heterotopic syngeneic mouse model, TNF-α-expressing *M. smegmatis* induced higher survival rates than the parental strain or BCG [80]. The authors also demonstrated a systemic immune response obtaining higher IFN-α levels in mycobacteria-stimulated spleen cultures from TNF-α-*M. smegmatis*-treated mice. Finally, only one study showed that live *M. kansasii* triggers higher tumor reduction in the orthotopic murine BC model than a range of BC strains [81].

Although antitumor effect of mycobacteria is not considered for muscle-invasive BC, MIP plus radiation therapy in a small number of patients (5) showed disease-free survival more than 2 years [74].

3.4. Other renal and urinary tract cancers

Immunotherapy using mycobacteria components has been also applied in urinary tract cancers other than NMIBC.

For prostatic cancer, studies using BCG, *M. phlei* cell wall extract, MCC (a previous version of MCNA), SLR-172, and HSP65 have been carried out. Intravenous injection of BCG, but not subcutaneous injection, demonstrated to avoid metastasis of prostate adenocarcinoma (PA-III) cells in rats [82]. Intraperitoneal injection of BCG, however, avoids only propagation of PA-III cells in the peritoneal cavity. Thus, BCG and tumor cells have to be in the same compartment to prevent systemic propagation of the tumor [83]. Both live-attenuated BCG and

SRL172 plus autologous whole tumor cell vaccination were effective in the prevention of MAT-LyLu tumors and increase survival rates in the rat model of prostate cancer. SRL172 alone did not provide any effect [84]. In this way, regular vaccination with tumor cells plus SRL172 in hormone-refractory prostate cancer patients demonstrated to be safe and induce cytokine production, specific antibody levels, and evidence of T-cell proliferation in response to the vaccinations [85]. Regular intradermal infection of only SRL172 in patients with advanced hormone-refractory prostate cancer demonstrated its safety and the ability to modulate cytokine changes in these patients. Serum PSA diminished in 5 out of 10 patients, and an increase in IL-2 secreting PBMC was also shown [86]. Finally, MCC also showed an antitumor effect on prostatic cancer. *In vitro*, MCC induced both a dose-dependent proliferation reduction of LNCaP prostate cancer cells and the production of IL-12 and GM-CSF [87]. When administrated in the Dunning R3327-H adenocarcinoma of the prostate in rats, no effect was seen after intraperitoneal administration, but up to 50% of animals showed complete tumor regression after intratumor administration in small-volume tumors [88]. Finally, immunization with a recombinant GnRH vaccine conjugated to *M. bovis* HSP65 prolonged significantly survival, triggering suppression of local tumor growth, strong lymphocyte proliferative responses, and high IFN-γ levels in the orthotopic prostate cancer mouse model [89].

In renal cancer, SRL172 has been administrated in patients suffering from metastasis demonstrating low toxicity and similar survival rates compared to patient treatment with cytokines (IL-2 or IFN-α) [82] and increased rates when synergized both treatments: SRL172 and antibodies [90]. In stage IV renal cell cancer, patients treated with BCG plus irradiated autologous tumor cells, and later infused with autologous activated T cells, showed durable tumor responses [91].

Few studies have evaluated the efficacy of BCG in the treatment of upper tract urothelial cancer, but the conclusions driven from them indicate that there is no role for BCG in these cases [92, 93].

4. Future perspectives: a long history with room for improvement

The potential beneficial effect of mycobacteria as antitumor agents has been clearly demonstrated after almost a century of observations and experimentation. But even in the case of BCG treatment for NMIBC patients, many issues remain under question: the appropriate schedule to reduce recurrence and progression without increasing adverse events [94–96]; the reason why a proportion of patients do not respond to BCG treatment; the detail description of immune mechanism which could permit to predict the response to the treatment; the possibility of using other mycobacteria species that have shown similar or increased effective than BCG but with potential increased safety; etc. In fact, the experience using mycobacteria in BC is permitting novel approaches for improving its efficacy. In this sense, a critical point for mycobacteria efficacy is the delivery of mycobacteria or mycobacteria antigens into the tumor site. The optimization of mycobacteria formulation could be critical for reducing adverse-associated events [97] and/or improving mycobacteria antitumor efficacy [77]. Moreover, the possibility to manipulate genetically mycobacteria for being vehicle for delivery antigens could lead also a chance to get more potent antitumor tools.

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Chapter 16

Application of Integrated Translational Research as Leprosy Problem Solution in Indonesia

Cita Rosita Sigit Prakoeswa

Additional information is available at the end of the chapter

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Abstract

In Indonesia, leprosy remains a health problem because its elimination has not achieved. This shows the high *Mycobacterium leprae* transmission as a result of difficulties in the early detection, termination of the transmission chain, and management evaluation. Integrated translational research has been carried out as a solution for the problem. Dissemination of the various results of the research is conducted by the educational aspects tiered with a variety of learning methods including a textbook based on research findings, scientific papers at various scientific meetings, and published journals, as well as aspects of community service through electronic media, newspapers, and management and counseling with leprosy patients and their contact person, especially in endemic pockets area.

Keywords: leprosy, *Mycobacterium leprae*, translational research

1. Introduction

Leprosy (Morbus Hansen) is a chronic infectious disease caused by bacteria *Mycobacterium leprae*, which primarily affects the peripheral nerves, and secondary attacks the skin and other organs. World Health Organization (WHO) classified leprosy into two types: paucibacillary (PB), which is relatively not contagious, and multibacillary (MB), which have infectious potential. Complications that occur in leprosy [erythema nodosum leprosum (ENL)] can cause disability in patients resulting in decreased quality of life.

In Indonesia, leprosy remains a health problem. Indonesia has the third-highest number of leprosy patients in the world after India and Brazil, the discovery of new cases is relatively stable from year to year, the dominance of the type of MB (potentially infectious) causes disability (9.9%) and can strike children (7.8%) thus affecting the future of the nation buds. This

shows ongoing leprosy transmissions as a result of difficulties in the **early detection, management evaluation, and termination of the transmission chain**. Integrated translational research is done to resolve leprosy problems both in the community and clinics during the process of medical education.

1.1. Methods featured achievement

The data of leprosy problems in community studies were obtained from Department of Health. In addition, the process of medical education with a unique learning method, not only through lectures but also plunge the management of patients in the clinic (bed side teaching), provides an opportunity to discover a wide range of issues that require to handle leprosy patients.

Research that has been carried out involving all of the Members of the Division of Leprosy, Dermatology and Venereology Department, Faculty of Medicine, Airlangga University—Dr. Soetomo Teaching Hospital Surabaya along with the Leprosy Study Group—Institute of Tropical Disease Airlangga University, students of the Faculty of Medicine, Universitas Airlangga, residents of Dermatology and Venereology Department, Faculty of Medicine, Airlangga University—Dr. Soetomo Teaching Hospital, and postgraduate students, Airlangga University. In addition not only involving the faculty and students of Airlangga University, but also wide range universities such as Indonesia University, Padjadjaran University, Diponegoro University, Hassanudin University, and Sam Ratulangi University to conduct collaboration researches. The cooperation is also carried out by various local and foreign communities, among others Leprosy Research Centre of Tokyo, the Netherlands Leprosy Relief, and the Royal Tropical Institute, Amsterdam.

Working closely with various communities and doing some research setting requires a good strategy. The success of team work was supported by the dedication, discipline, and clear job description. At the time of this research, education and community service aspects are not left behind. By involving medical students, the educational process can still take the time to do some research studies, and to perform community service through health services and counseling.

Broadly speaking, research of **diagnosis**, **management,** and **mode of transmission** has been conducted to overcome the problem of leprosy that includes **early detection**, **management evaluation**, and **termination of the transmission chain**.

2. Review of Research Collaboration by Faculty of Medicine Universitas Airlangga, Dr. Soetomo Teaching Hospital, and Leprosy Study Group, Institute of Tropical Disease, Universitas Airlangga

2.1. Diagnosis of leprosy

Diagnosis of leprosy using cardinal sign only detects the clinical leprosy due to the limitation of this method. Acid-fast bacilli test for detection of *M. leprae* has a limitation in the sensitivity

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and specificity. Detecting *M. leprae* by biotechnology based on biomolecular aspect requires laboratories facility and analysts. This method has been performed only in research areas in Indonesia. The advantage of this method is more accurate than the routine method. Its accuracy is important for early detection of leprosy.

2.1.1. Detection of DNA M. leprae using PCR

The presence of *M. leprae* in the blood of patient with subclinical leprosy is still a question mark [1]. The answer should be taken into account by considering the management of subclinical leprosy, because subclinical leprosy has the potency of manifest leprosy and be the source of transmission. *M. leprae* DNA in the blood of subclinical leprosy patient, was investigated using polymerase chain reaction (PCR) test. This study took place in two leprosy endemic villages named Kombang and Poteran in Talango Island of Sumenep District, Madura, East Java, Indonesia. After dermatologic examinations of 122 people with leprosy contact, venous blood was collected to estimate the seropositivity to various mycobacterial antigens. The antiphenolic glycolipid (antiPGL-1) IgM antibody was measured by indirect ELISA.

In those 122 patients with leprosy contact, we found 29 people who had >600 U/ml antiPGL-1 IgM antibody refer to subclinical leprosy (**Figure 1**). From 29 subclinical leprosy patients, we collected 2 ml of venous blood and extracted *M. leprae* DNA using TaKaRa GenTLE methods (**Figure 2**) followed by the PCR test using nested primer Lp1–Lp4 from RLEP repetitive sequence (**Figure 3**).

The result of this study is expected to be important for the management of patients with subclinical leprosy. Considering the potential to manifest into leprosy and become a source of transmission, therefore, we suggest that using new preventive measures such as chemoprophylaxis for high risk groups is important to control the spread of leprosy.

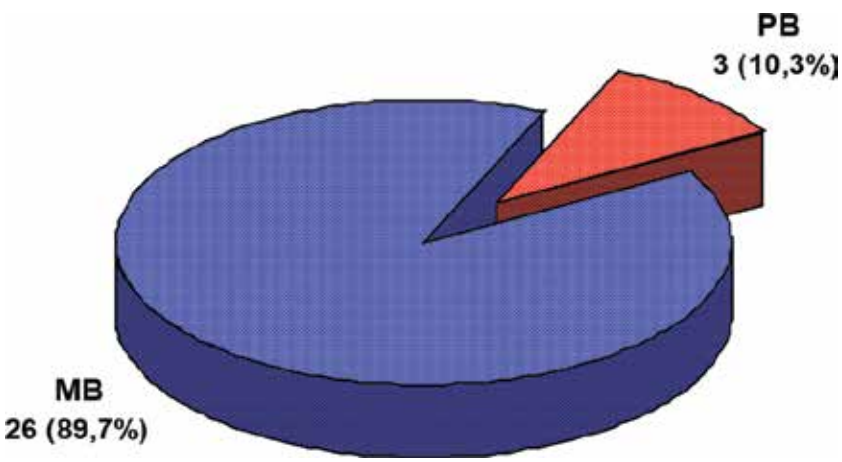

Figure 1. Leprosy type in contact distribution.

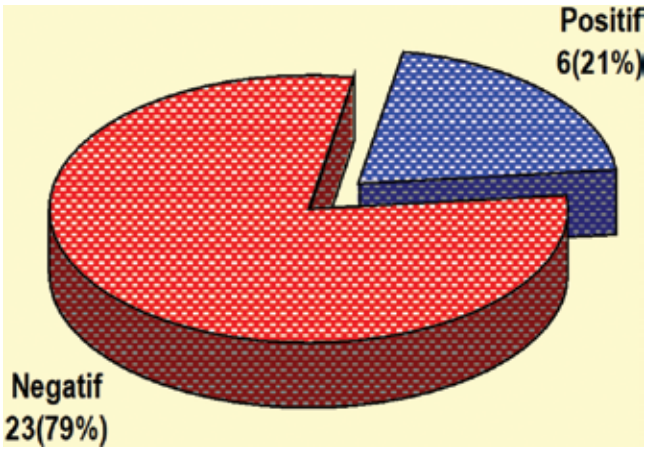

Figure 2. Distribution PCR result: 6 samples positive (21%) and 23 samples negative (79%).

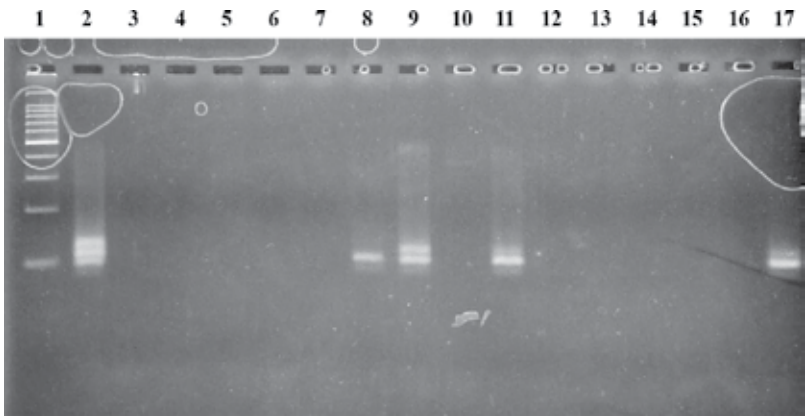

Figure 3. LP1-LP4 PCR product. Lane 1: 100 bp ladder; lanes 2–15: samples; lane 16: NC; lane 17: PC.

2.1.2. Detection of RNA M. leprae (viable) using RT-PCR and quantification of DNA M. leprae using real-time PCR

Detecting viable *M. leprae* is still a problem due to the uncultivable characteristics of the bacilli [2]. A new biomolecular technique of RNA isolation is now used for the detection of viable *M. leprae,* since RNA is rapidly degraded upon cell death. The reverse transcriptase polymerase chain reaction (RT-PCR) method for detecting 16 ribosomal RNA subunit (16S rRNA) *M. leprae* can be used as viable sign of *M. leprae*. 16S rRNA *M. leprae* denoting housekeeping gene containing specific and unique 1170 nucleotide sequence having specific structure variants and were relatively abundant with 1000–10,000 copies in one bacteria with specific characteristic and will be soon degraded after the death of *M. leprae*. Thus, 16S rRNA study by RT-PCR could reflect *M. leprae* viability with high sensitivity and specificity. We detected 16S rRNA *M. leprae* from skin biopsy and blood of new leprosy patients and to

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improve the weakness of skin tissue biopsy sample which was invasive and did not take into account the comfortability of the patient. Skin biopsy and peripheral blood mononuclear cells (PBMCs) were obtained from 24 newly diagnosed (14 male and 10 female) untreated leprosy patients in Dr Soetomo Hospital, Surabaya. Diagnosis is based on clinical and AFB examinations. Informed consent was signed beforehand by all of the patients. RNA isolation, cDNA synthesis, conventional PCR, and real-time PCR using primer set P2 (*forward*, 69–91) and P3 (*reverse*, 218–239) were performed in all samples (**Figures 4** and **5**).

In skin biopsy and blood from both MB and PB leprosy, 16S rRNA *M. leprae* can be detected, showing a systemic process that occurred. Reverse transcription methods using conventional PCR and real-time PCR has better sensitivity than AFB staining (**Figure 6**). Detecting viable *M. leprae* by reverse transcription methods may prove to be useful in early detection of leprosy and the potency of transmission, also assessing the efficacy of treatment and potency source of relapse.

2.1.3. The failure of phagolysosome process as a marker viability

Phagolysosome process in macrophage of leprosy patients is important in the early phase of eliminating *M. leprae* invasion [3]. This study was done to clarify the involvement of Rab5, Rab7, and trytophan aspartate-containing coat protein (TACO) from host macrophage and leprae lipoarabinomannan (Lep-LAM) and phenolic glycolipid-1 (PGL-1) from *M. leprae* cell wall as the reflection of phagolysosome process in relation to 16 subunit ribosomal RNA (16S rRNA) *M. leprae* as a marker of viability of *M. leprae*. Skin biopsies were obtained from 47 newly diagnosed and untreated leprosy at Dr Soetomo Hospital, Surabaya, Indonesia and used cross-sectional as the study design (**Figures 7–11**). RNA isolation and complementary DNA synthesis were performed. Samples were divided into two groups of 16S rRNA *M. leprae*-positive and 16S rRNA *M. leprae*-negative. The expressions of Rab5, Rab7, TACO, Lep-LAM, and PGL-1 were assessed with an immunohistochemistry technique. Mann-Whitney U analysis (**Table 1**) showed a significant difference in the expression profile of Rab5, Rab7, Lep-LAM, and PGL-1 ($p < 0.05$), but there was no significant difference of TACO

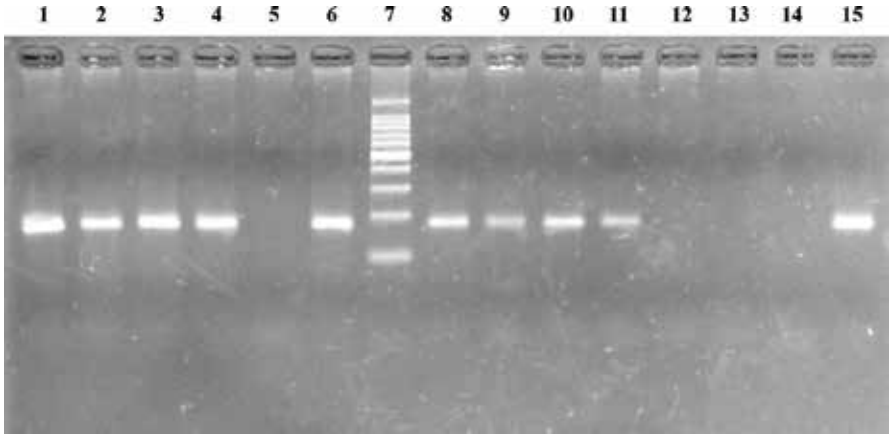

Figure 4. LP1-LP4 PCR product: lanes 1–6 samples; lane 7: 100 bp ladder; lanes 8–13: samples; lane 14: NC; lane 15: PC.

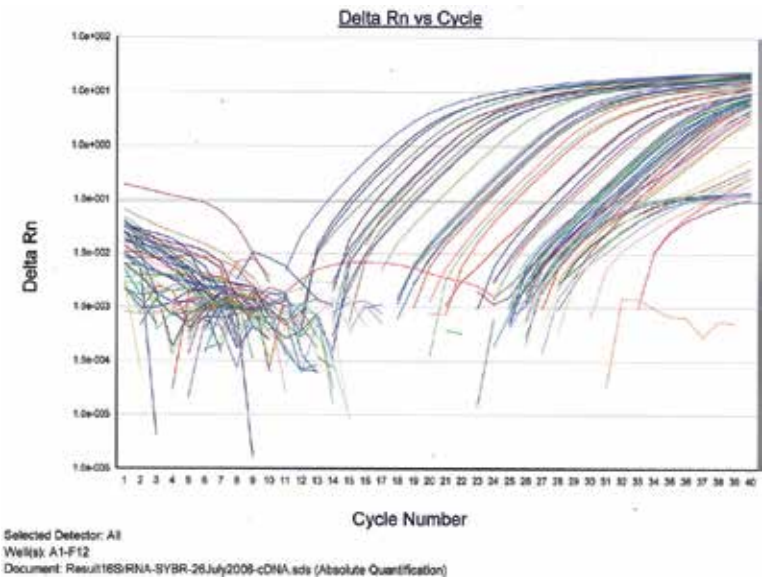

Figure 5. Amplification plot showing increases in fluorescence from each sample.

Figure 6. Sample (from skin biopsy and PBMC) showing positive result at leprosy type, AFB examination, 16S rRNA *M. leprae* examination by conventional PCR and teal-time PCR.

between the two groups ($p > 0.05$). Spearman analysis (**Table 2**) revealed that there was a significant correlation between the score of Rab5, Rab7, Lep-LAM, and PGL-1 and the score of 16S rRNA *M. leprae* ($p < 0.05$).

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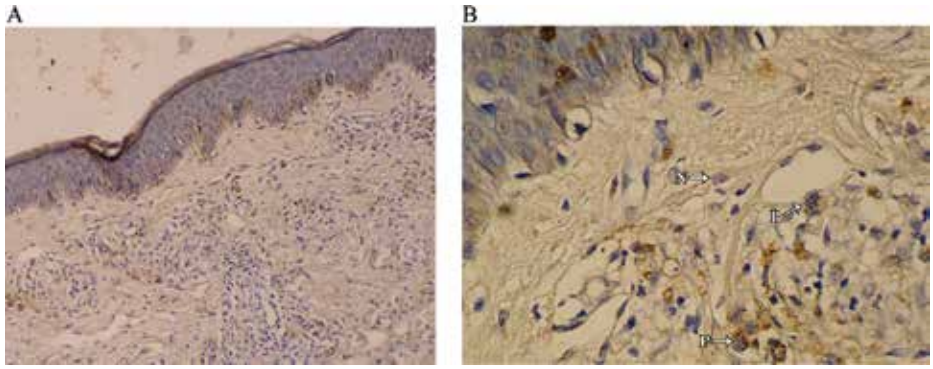

Figure 7. Expression of Rab5 in the skin biopsy section of sample number 14. (A) With the enlargement of 100; and (B) with the enlargement of 450. Note: N = negative reaction (any colors other than brown); P = positive reaction (brown).

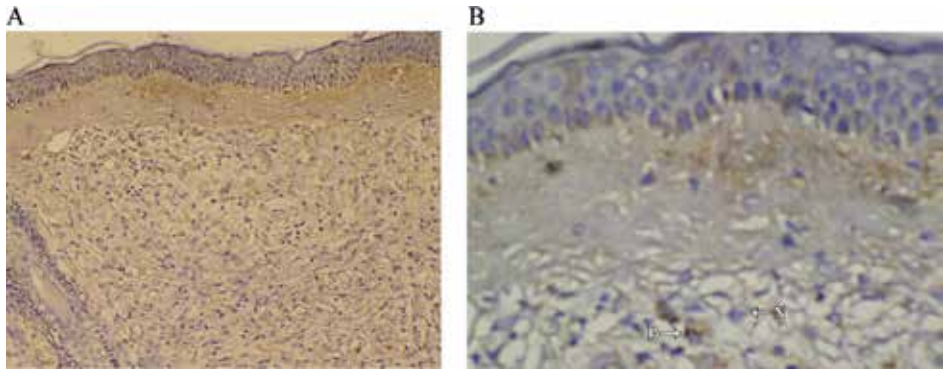

Figure 8. Expression of tryptophan aspartate-containing coat protein in the skin biopsy section of sample number 3. (A) With enlargement of 100 and (B) with enlargement of 450. Note: N = negative reaction (any colors other than brown); P = positive reaction (brown).

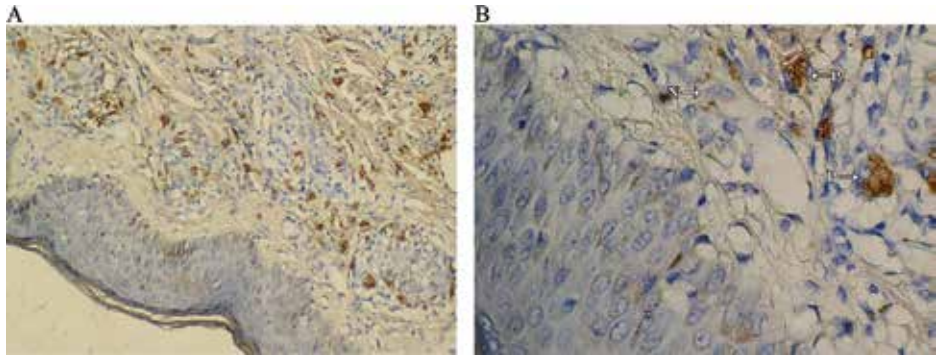

Figure 9. Expression of leprae lipoarabinomannan in the skin biopsy section of sample number 8. (A) With a magnification of 100 and (B) with a magnification of 450. Note: N = negative reaction (any colors other than brown); P = positive reaction (brown).

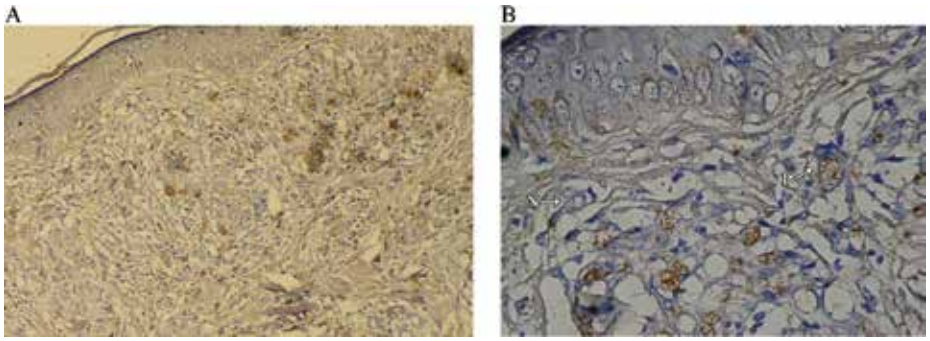

Figure 10. Expression of phenolic glycolipid-1 in the skin biopsy section of sample number 16. (A) With the enlargement of 100 and (B) with the enlargement of 450. Note: N = negative reaction (any colors other than brown); P = positive reaction (brown).

Membrane trafficking in phagolysosome failure is deemed as an important discovery in the study and it is represented by two compounds derived from the host (Rab5 and Rab7) and from the agent (Lep-LAM). PGL-1 role in the inhibition of lysosomes activation pathway in

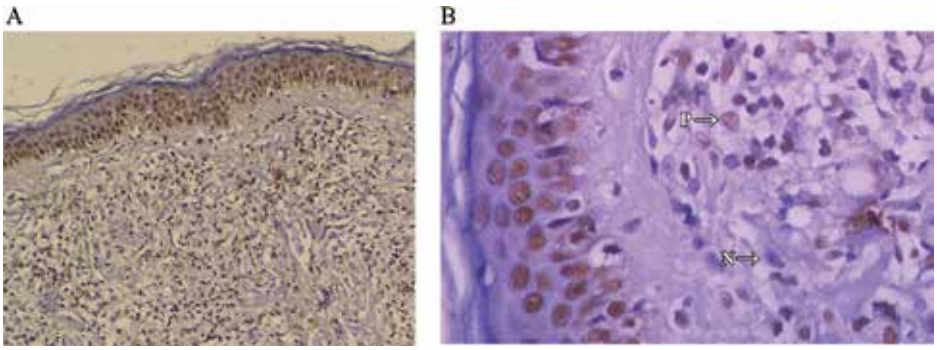

Figure 11. Expression of Rab7 in the skin biopsy section of sample number 6. (A) With enlargement of 100 and (B) with enlargement of 450. Note: N = negative reaction (any colors other than brown); P = positive reaction (brown).

Variables	*p*
Rab5	0.002
Rab7	<0.001
TACO	0.584
Lep-LAM	<0.001
PGL-1	<0.001

Table 1. Mann-Whitney U test results of the differences in the expression of Rab5, Rab7, tryptophan aspartate-containing coat protein (TACO), leprae lipoarabinomannan (Lep-LAM), and phenolic glycolipid-1 (PGL-1) based on positive and negative viability with $p = 0.05$.

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Variables	Correlation coefficient	*p*
Rab5	0.483	0.001
Rab7	0.682	<0.001
TACO	0.065	0.662
Lep-LAM	0.608	<0.001
PGL-1	0.491	<0.001

Table 2. Spearman correlation test between combined scores between cell number and staining intensity of the expression of Rab5, Rab7, tryptophan aspartate-containing coat protein (TACO), leprae lipoarabinomannan (Lep-LAM), and phenolic glycolipid-1 (PGL-1) with the score of 16 subunit ribosomal RNA *M. leprae* quantity ($p = 0.05$).

phagolysosome failure was also found from the study. Hence, the expression profiles of Rab5, Rab7, Lep-LAM, and PGL-1 can be used as markers of *M. leprae* viability. From these discoveries, an early diagnostic method for leprosy based on expression pro-files of Rab5, Rab7, Lep-LAM, and PGL-1 is possible. Early diagnosis in leprosy cases is very useful to prevent the occurrence of disability or transmission. In addition to the above discoveries, it is important to study the expression profiles of Rab5, Rab7, Lep-LAM, and PGL-1 in peripheral blood mononuclear cells (PBMCs). Based on the research done by the Leprosy Study Group of the Institute of Tropical Disease, Universitas Airlangga (Prakoeswa, 2011), the results showed no significant differences between expression profiles of 16S rRNA of *M. leprae* in skin biopsy tissue and PBMCs using real-time PCR. Therefore, from the study, the expression profiles of Rab5, Rab7, Lep-LAM, and PGL-1 in PBMCs can be explored to be used as a base. Blood tests without skin biopsy may be sufficient enough and used to create a simplified and noninvasive early diagnostic marker tool for leprosy viability.

Based on the three studies above, early diagnostic method for leprosy can be performed suited to the condition of the facility involved. In a highly qualified laboratorium with skilled analysts, DNA and RNA tests can be performed whereas in a laboratorium with limited facility and analysts, Immunohistochemistry (IHC) test may be used instead based on the phagolysosome failure process.

2.2. Management of leprosy

There has been a decrease in the number of new leprosy patient after MDTL era. However, the number of new cases found is still relatively stable. This means that interventions are still needed to evaluate, and measures to be taken to manage leprosy cases.

2.2.1. Dapsone and rifampicin resistance

Drug resistant cases can be tested by using the biomolecular method as an alternate solution as it is relatively simpler and less time consuming [4]. Based on the detection of mutation in *folp* and *rpoB* gene, we conducted a study about the prevalence of *M. leprae* drug resistance to dapsone and rifampicin in East Java. DNA templates from 153 isolates obtained from MB

leprosy patients from East Java were processed. Polymerase chain reaction (PCR) test was initiated using Lp1–Lp4 primer to show the presence of *M. leprae*. The *folP* and *rpoB* genes were amplified using folP1–folPR and rpoBF-rpoBR primers to obtain the DNA sequence target with 59 isolates for *folP* gene study and 94 isolates for *rpoB* gene study. After purification of PCR product, DNA sequencing was initiated to analyze the mutation on nucleotide sequence.

All isolates showed positive PCR results by Lp1–Lp4. From 59 isolates, 50 isolates showed positive PCR results by folP1–folPR (**Figure 12A**) and the same result goes by rpoBF-rpoBR from 77 out of 94 isolates (**Figure 12B**). In *folP* gene examination, no mutation was found in *rpoB* gene (**Figure 13A**). There are 3 isolates out of 53 that were found to have mutation in amino acid at codon 53; two cases where threonine (ACC) became alanine (GCC) (**Figure 13B** and **C**), and in one case threonine (ACC) became arginin (AGA) (**Figure 13D**). This mutation held responsible for the resistance of *M. leprae* to dapsone. The result suggested that three isolates (6%) from East Java-Indonesia in this experiment are resistant to dapsone and all isolates (100%) are sensitive to rifampicin.

Surprisingly, from three cases that show mutations in the *folP* gene, one of them is a new case with 1 month of multidrug treatment (MDT) duration time. The electropherogram of this sample can be seen in **Figure 13D**. The mutation was detected in amino acid at codon 53 (157–159 nucleotides that are from "ACC" (threonine) to "AGA" (arginine). The isolate is regarded to as a primary diaminodiphenyl sulfone (DDS) resistant. Another mutation in amino acid at codon 53 (157–159 nucleotides) that was detected in two samples is from "ACC" (threonine) to "GCC" (alanine). Based on the clinical data, these two samples are suspected resistant to DDS.

2.2.2. Methylsulfonylmethane treatment in erythema nodosum leprosum

Erythema nodosum leprosum (ENL) is a complex reaction found in the immune system [5]. It causes antibody-antigen complexes to be deposited within various tissues and may cause vasculitis. This condition may occur in MB leprosy patients. While the immunopathology of this disease is not fully understood, the reaction is known as a tumor necrosis factor-α (TNF-α)-mediated process. The severity of the condition, possible complications, limited treatment choices, and recurrent nature of the disease makes it complicated to manage. Research studies to find choices of treatment options for ENL are imperative as the current treatment options

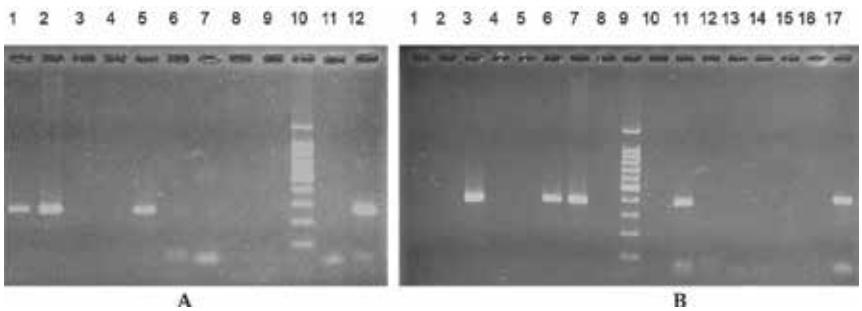

Figure 12. (A). *folP* PCR product, lanes 1–9: samples, lane 10: 100 bp ladder, lanes 11 and 12: NC and PC; (B) *rpoB* PCR product, lanes 1–8 and 10–15: samples, lane 9: 100 bp ladder, lane 16: NC; lane 17: PC.

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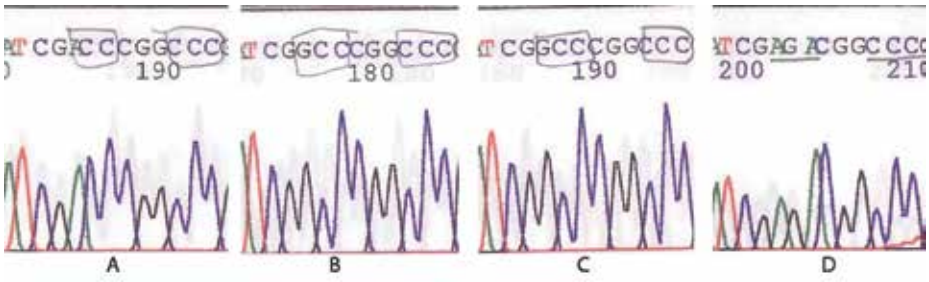

Figure 13. Four *folP* gene sequencing results, (A) No mutation; (B and C). Mutation at codon 53 ACC→GCC; (D) Mutation at codon 53 ACC→AGA.

are limited with high level of morbidity and chronicity. Previous study showed that methylsulfonylmethane (MSM) has strong capability as antiTNF-α properties in vitro. This means that MSM might be useful for treating TNF-α-mediated conditions, such as ENL reaction. Hence, the objective of this study is to establish a correlation whether MSM is effective to treat the clinical signs and symptoms of recurrent ENL reaction in MB leprosy patients.

In this study, patients eligible for the study were all those with MB and admitted for at least a second episode of severe ENL reaction. A total of 10 patients who were eligible for the inclusion and exclusion criteria were enrolled for the study. A standardized history taking using a checklist was recorded from all of the patients chosen for the study. Thorough physical examination was done to look for skin signs, motoric or sensory neuropathy signs, and other possible complications of ENL. After each examination, ENL reaction severity scale was performed and included the basic neurological examination.

Nerve function assessment includes sensory nerve function using the Semmes-Weinstein monofilament test (MFT) and motor nerve function using voluntary muscle tests (VMTs) and all impairments will be recorded. Blood (10 ml) was taken for laboratory assessments on day 1, 7, and 63 for TNF-α examination and routine blood assessment on day 1, 7, 14, 56, and 112. MSM was given to the patients in the study with a dose of 0.1 g/kg bodyweight daily in two divided doses in addition to the World Health Organization's (WHO) multidrug treatment (MDT) and/or additional clofazimine, if a patient has already taken it when the new reaction occurred. If the patient shows clinically significant improvement, the dose would be tapered off by 1 g every 2 weeks, starting from 1 week from the start of MSM treatment. Treatment will be stopped completely in 2 weeks after reaching 1 g/day level.

Graphic of TNF-α in 10 patients is shown in **Figure 14**. Two out of 10 patients showed improvement from ENL reaction. These patients revealed high level of TNF-α, and this value decreased along with lessening of ENL severity scale. First, patient showed increased ENL severity scale within MSM tapering off (full dose of MSM repeatedly and tapered off). The second patient was still in a good condition during follow-up. Eight other patients were dropped on day 3 and 5. In those eight patients, the value of TNF-α showed to be normal and was excluded from the study due to the increase of ENL severity scale. MFT and VMT examinations showed no changes during the study.

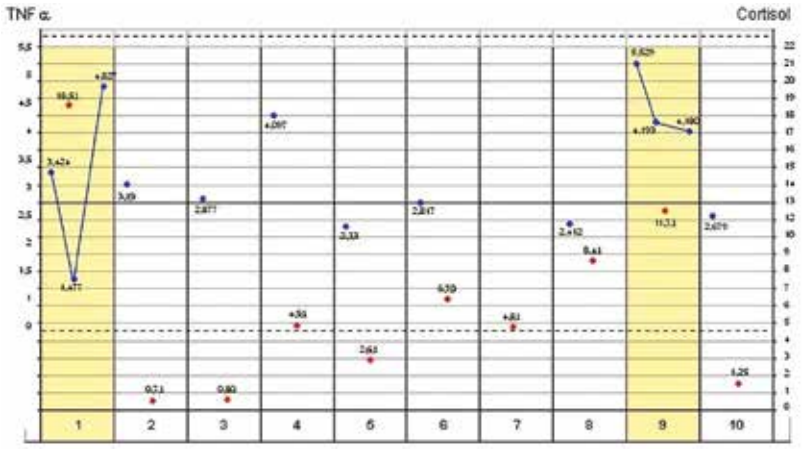

Figure 14. Graphic of TNF-α in 10 patients. Yellow: improving patients; white: dropped out patients; blue: level of TNF-α.

This finding proves that MSM treatment modality is possible as drug of choice for ENL patients with high level of TNF-α in concordance to its mechanism of action as an antiTNF-α.

2.2.3. Chemoprophylaxis in subclinical leprosy

Subclinical leprosy is a person who has high titer of IgM antiPGL-1 without clinical manifestation of leprosy that manifests after several years [6]. Preventive treatment of this leprosy is required especially in children in order to prevent manifest toward leprosy and prevent it from spreading. We evaluate the result of 2 years preventive treatment to subclinical leprosy in elementary school children using special regiment rifampicin and clarithromycin in Raas Island and Nguling, East Java, Indonesia.

Serological surveys for leprosy were conducted and involved a total of 5066 school children, who were screened in 2 leprosy endemic locations in East Java. About 302 elementary school children [109 from Nguling (**Figure 15**) and 193 from Raas Island (**Figure 16**)] were positive for sero (+++) with high IgM antiPGL-1 antibody titer (>3000 U/ml ELISA). Rifampicin 300 mg daily with 250 mg clarithromycin daily for 10 days was given as a preventive treatment, continued with the same drugs administered intermittently every 2 weeks for 3 months. Every year, clinical and serological examination was evaluated.

After 2 years evaluation, none of the children showed any manifestation of leprosy clinically. IgM antiPGL-1 antibody level showed to decrease between these 2 years of evaluation (Raas Island and Nguling: $p = 0.00$). The majority of the children (Raas Island 96.46%; Nguling 94.83%) showed a decrease in IgM antiPGL-1 antibody level, but some of these children (Raas Island 3.54%; Nguling 5.17%) also showed an increased level of IgM antiPGL-1 antibody. All the medications were well tolerated by these children and only a few side effects due to these drugs were reported.

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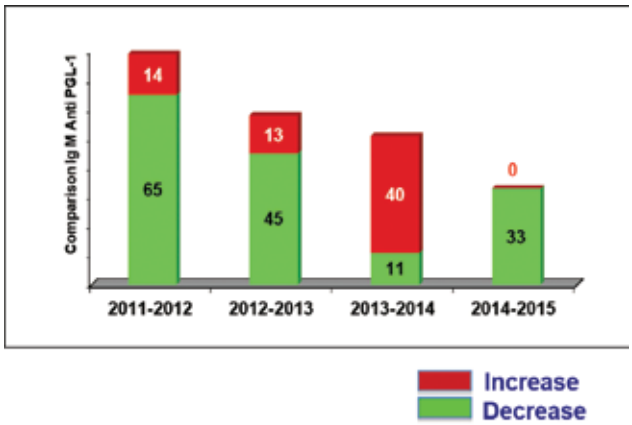

Figure 15. Evaluation of IgM antiPGL-1 in Nguling. Green = decrease; red = increase.

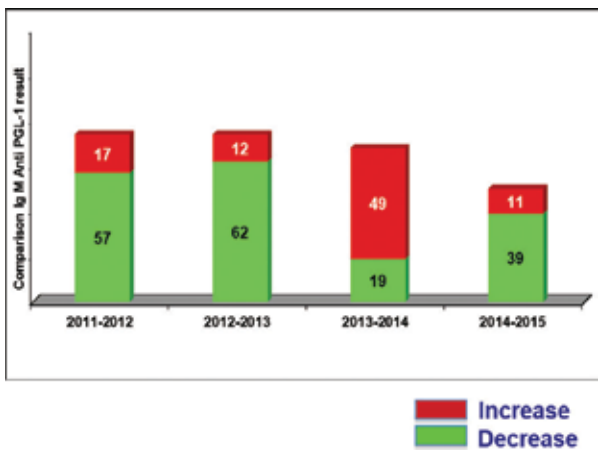

Figure 16. Evaluation of IgM antiPGL-1 in Raas Island. Green = decrease; red = increase.

Chemoprophylaxis for subclinical leprosy in children showed a promising good result after 2 years of evaluation. Further evaluation will be conducted for the next 3 years ahead. Our research about subclinical leprosy in children may support the clinical importance of it in exploring the disease transmission and how we can prevent it.

From these three studies, evaluation of resistance for suspected cases, MSM administration for refractory cases to steroid and administration of chemoprophylaxis for subclinical leprosy, can be implemented on routine leprosy management. Ongoing research is expected to prevent the onset of leprosy. Study on the impact of chemoprophylaxis in household and neighbor contact person with a grant from the Netherlands Leprosy Relief is also being carried out in collaboration with the Ministry of Health and local government.

2.3. Mode of the transmission

During this time, it is believed that the only source of transmission is leprosy MB type, but as we have not achieved elimination, we begin to think about another source of the transmission.

2.3.1. Strain local study in endemic area using PCR sequencing

Multiple locus variable number of tandem repeat (VNTR) analysis has been proposed as a mean of genotyping for tracking leprosy transmission [7]. Many tandem repeats have been reported to be polymorphic with the potential as genetic markers to differentiate strains of *M. leprae*. However, depending on the population, the characteristics of polymorphism may vary. We measured the copy number of repeat in four genetic markers, which are TTC, AC 8a, AC 9, and 6–7 (**Figures 17–23**) in leprosy patients. A number of 23 patients were recruited from outpatient clinic in Department of Dermato-Venereology of Dr Hasan Sadikin Hospital, Bandung. Multiple locus VNTR analysis at four loci was applied using total DNA extracts from skin biopsies.

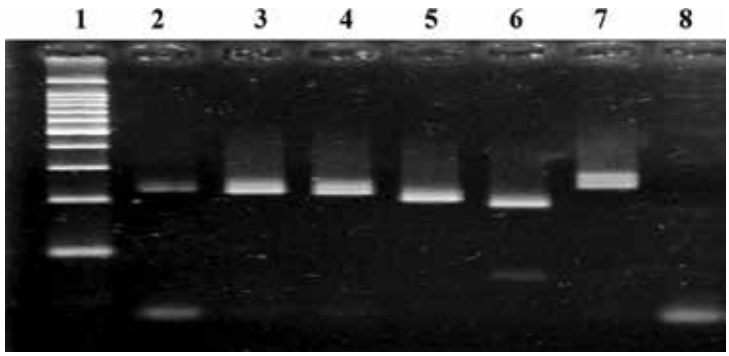

Figure 17. TTC PCR product: lane 1: 100 bp ladder, lane 2: PC, lanes 3–7: samples, lane 8: NC.

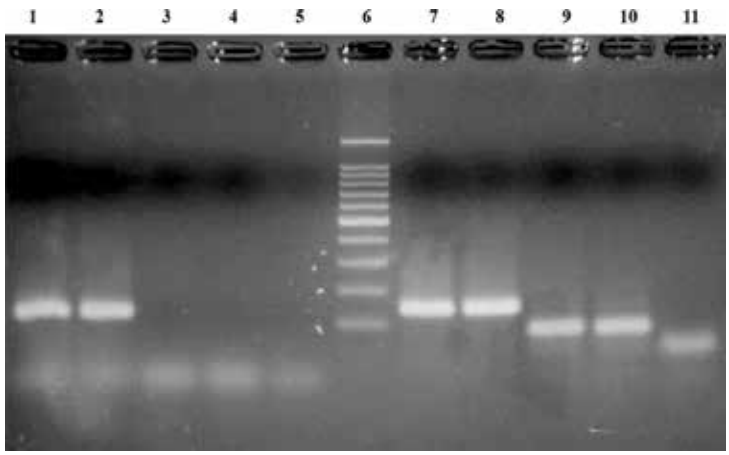

Figure 18. AC 8A and AC 9 PCR product: lane 1: PC AC 8a, lanes 2–4: samples, lane 5: NC AC 8a, lane 6: 100 bp ladder, lane 7: PC AC 9, lanes 8–10: samples, lane 11: NC.

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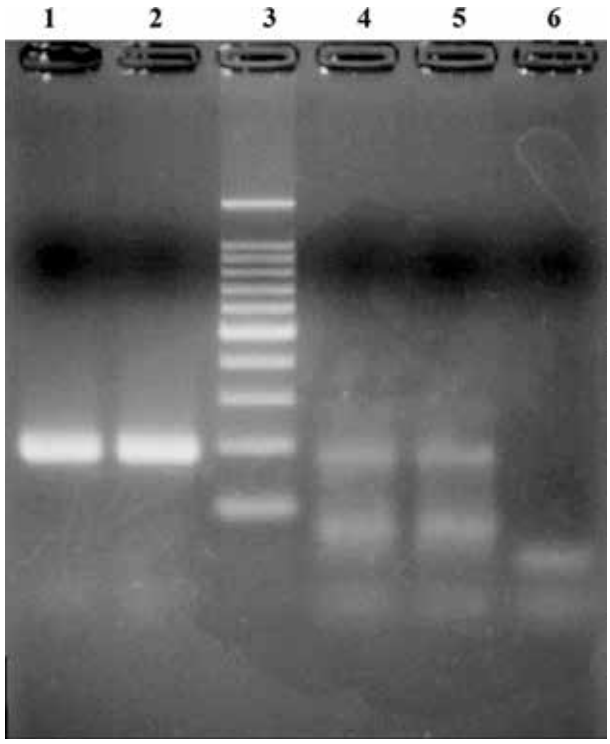

Figure 19. PCR product: lane 1: PC, lanes 2,4,5: samples, lane 3: 100 bp ladder, lane 6: NC.

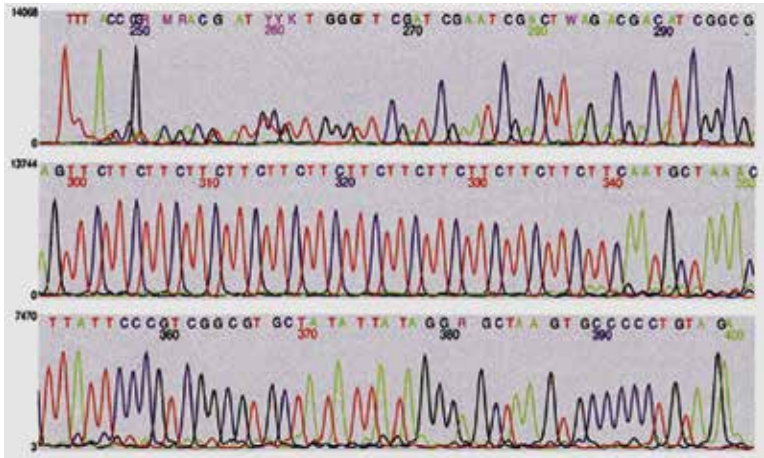

Figure 20. PCR sequencing result: primer TTC.

As shown in **Table 3**, there were five samples showing the same copy number of four genetic marker: TTC = 15; AC 8a = 10; AC 9 = 10, and 6–7 = 6. Two samples showing the same copy

number of four genetic marker: TTC = 16; AC 8a = 10; AC 9 = 11, and 6–7 = 6. The multiple locus VNTR analysis shows two identical *M. leprae* VNTR profiles from Bandung. These attributes support the use of VNTR loci for transmission studies and VNTR analysis can use for multicases family study.

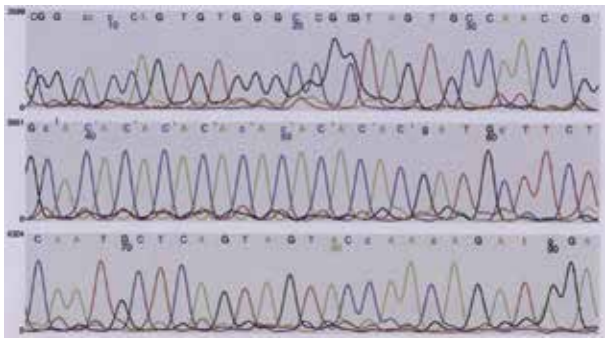

Figure 21. PCR sequencing result: primer AC 8A.

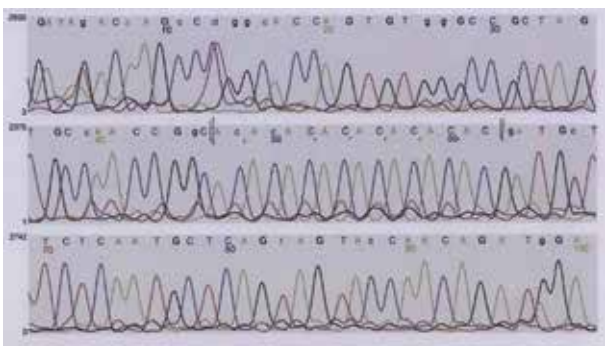

Figure 22. PCR sequencing result: primer AC 9.

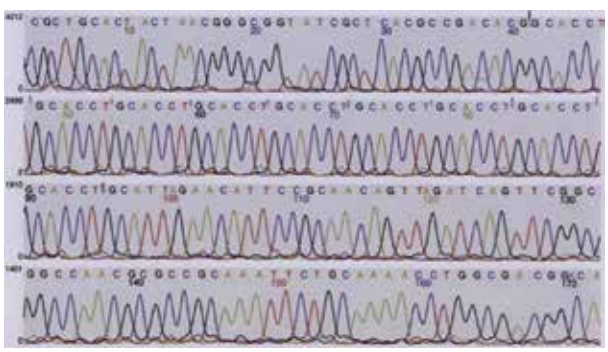

Figure 23. PCR sequencing result: primer 6–7.

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Table of VNTR analysis				
No	**TTC**	**AC 8a**	**AC 9**	**6–7**
1	12			
2	13	11		6
3	14			
4	15	10	10	6
5	15	10	10	6
6	15	10	10	6
7	15	10	10	6
8	15	10	10	6
9	15	10	10	6
10	15	10	10	6
11	16	10	11	6
12	16	7	10	
13	16	10	11	6
14	16	10	11	6
15	17	9		6
16	17	8	8	6
17	18			
18	21	8	10	8
19	21	8	9	8
20	22			
21	23			
22	25			8
23	37			

Table 3. VNTR analysis in clinical sample from Bandung isolates.

2.3.2. Environment study and multicase family study using PCR sequencing

East Java is a province in Indonesia that has few endemic areas for leprosy [8]. In order to comprehend this increasing incidence of leprosy, molecular typing will make it feasible to study geographical distributions of *M. leprae*. Genotyping analysis was done by using *variable number tandem repeats* that found in the *rpoT* gene which was followed by the recognition of the TTC triplet in a region of the *M. leprae* genome. The aim of this study is to analyze the number variation of TTC repeats and their distributions in leprosy endemic areas. Poteran

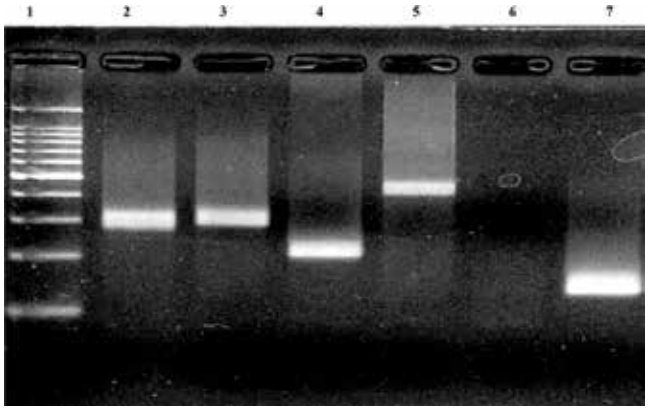

Figure 24. TTC PCR product lane 1: 100 bp ladder; lanes 2–5: sample, lane 6: NC, lane 7: PC.

Island in Madura was chosen for its high prevalence for leprosy and the number has remained stable for the last 5 years. Samples were collected and divide into 3 groups: 91 water sources; 42 nasal swabs of household contact, and 68 skin tissues of leprosy patients. All samples were analyzed by using PCR (**Figure 24**) and the numbers of TTC repeats were confirmed by direct sequencing. From all collected samples, 24 isolates from water resources were positive (26.4%); 26 nasal swabs were also positive (61.9%); and also 24 skin tissues (35.3%).

No. of repeats	Slit-skin specimens	Nasal swabs	Water sources
TTC-9	0	0	4
TTC-10	20	10	8
TTC-11	**32**	**42**	**36**
TTC-12	4	4	0
TTC-13	8	4	12
TTC-14	16	28	32
TTC-15	0	0	4
TTC-16	4	0	0
TTC-17	0	4	0
TTC-24	4	0	0
TTC-28	4	0	0
TTC-40	4	0	0
TTC-44	0	8	0
TTC-49	0	0	4
TTC-60	4	0	0
Total	100% (24 cases)	100%(26 cases)	100% (24 cases)

Table 4. Genotypes TTC frequency (%).

Location	Family member	Relationship	TTC repeat	
			Nasal swab	Slit-skin spec.
House 1	MB patient	Husband	10	10
	Family contact	Wife	11	
	Family contact	Child	-[a]	
	Suspect leprosy	Child	-	11
	Family contact	Mother	10	
	Family contact	Sister in law	11	
	Family contact	Mother in law	10	
	Family contact	Father in law	-	
House 2 (Neighborhood)	MB patient	Child	11	11
	Family contact	Mother	11	
	Family contact	Father	10	
	Family contact	Child	-	
	Family contact	Child	11	

Table 5. Variation of TTC repeats in multicase family.

The copy number of TTC repeats in Talango Island varied from 9 to 60 copies (**Table 4**). The 11-copy TTC genotype was the most frequent in all samples.

The copy number of TTC repeats in Talango Island varied from 10 to 11 copies (**Table 5**). The 11-copy TTC genotype was the most frequent in all samples. There were no differences were found statistically in the pattern distribution of TTC repeats between nasal swab of households contacts and skin tissues of patients (p = 0.594); skin tissues of leprosy patients and water resources (p = 0.441); nasal swab of households contact with water resources (p = 0.906). It can be concluded in endemic areas, transmission of *M. leprae* has strong ties with these three aspects: agent, host, and environment.

2.3.3. Mother-baby transmission in leprosy

Lucio phenomenon is a rare type of reaction in untreated lepromatous leprosy type with diffuse infiltrative form, characterized with ulcerative type of skin lesions [9]. In this study, a case of 29-year-old Indonesian female, 7th month primigravida with lucio leprosy, without prior treatment using WHO-multidrug therapy (MDT). Laboratory examinations reported bacterial index 6+ and morphological index 7% from slit-skin smear; histopathology revealed lucio phenomenon; PCR examination found *M. leprae* DNA on amniotic fluid and skin lesion: positive; umbilical cord membrane and umbilical cord: negative (**Figure 25**). AntiPGL-1 IgM and IgG from patient: 4854 and 1061 U/mL, respectively; from 5-month-old baby: 5 and 1724 U/mL, respectively; from 1-year-old baby: 0 and 3 U/mL, respectively.

Figure 25. PCR examination: 1: skin lesion; 2: amniotic fluid; 3: umbilical cord membrane; 4: umbilical cord; 5: negative control; 6: positive control *M. leprae* Thai53; 7: DNA ladder.

In **Table 6**, patient's antiPGL-1 IgM and IgG titers collected during caesarian section were way over the cutoff limit, whereas the antiPGL-1 IgM and IgG titers from the umbilical cord blood were below the limit. Hence, placenta is regarded as a protective barrier against fetomaternal transmission of *M. leprae*. The placenta has multiple innate defense properties against pathogens. Only few pathogens can pass through these barriers at low frequencies. DNA of *M. leprae* was found in the amniotic fluid. About 5% of babies born from

Subject	ELISA anti PGL-1 (U/mL)		Cut off (U/mL)	
	IgM	IgG	IgM	IgG
Patient during the caesarian section	4854	1061		
Umbilical cord blood during the caesarian section	0	516		
Patient (after 7 months of therapy)	l912	1505	605	630
Baby (5 month-old)	5	1724		
Baby (1 year-old)	0	3		

Table 6. Results of serological examination using ELISA.

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mothers with active leprosy had self-limited indeterminate leprosy before 2 years old and also antiwill have *M. leprae* antibodies of class IgA, IgG, and IgM. The presence of IgA and IgM anti*M. leprae* antibodies in the cord blood of newborn babies from mothers with leprosy might shows an intrauterine immunologic stimulation process that happened due to transplacental transmission of *M. leprae* antibodies. Titers for antiPGL-1 IgM and IgG were reviewed again after the babies reached 5 months old and 1-year-old. On assessment, the titers were found to drop drastically, especially antiPGL-1 IgG titer. Based on these facts, we assume that passive antibody to *M. leprae* from the babies had been acquired from their mothers' blood and transmitted through the umbilical cord blood as shown by the presence of antiPGL-1 IgG antibody.

Studies in genotyping of patients and contact person proved that genotyping is not always appropriate; there is still the possibility of environmental transmission source. From these three studies above, there can be further potential research on transmission of leprosy from nonhuman sources. In the transmission pattern from mother to baby, it shows the importance of the role of placenta as a barrier, therefore, the health of the expecting mother needs to be optimized so as to prevent fetomaternal transmission and to treat the mother as early as possible and closely monitored the baby during incubation phase.

3. Conclusions

Dissemination of an integrated translational research above is done through aspects: education: lectures, case discussions, bed side teaching, and writing research-based textbook for medical students, undergraduates, postgraduates, and dermatology and venereology residency program. Besides, presentations are done at local and international scientific meetings as well as publication of journals. Research: an integrated translational research is a continuous activity with the ultimate goal to eliminate leprosy in Indonesia. Community services: improving services and counseling at social events as well as when we are doing research in endemic pockets area. Besides, counseling is also done through electronic media. Community services can be done in practice of the research results of what they learned through lectures and case discussions in accordance with the level of competence they need to accomplish.

Various educational activities, research, and community service above increase knowledge about leprosy with the ultimate goal of achieving elimination through improvements in the field of preventive, curative, promotive, and rehabilitative. Here, the role of educational institutions is very important in helping to resolve the national problem. In the future, we hope more collaboration research of diagnosis, management, and mode of transmission will be conducted to overcome the problem of leprosy include early detection, management evaluation, and termination of the transmission chain. Integrated translational research is important to be done to resolve leprosy problems both in the community and clinics during the process of medical education.

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Chapter 17

Mycobacterium as Polycyclic Aromatic Hydrocarbons (PAHs) Degrader

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Additional information is available at the end of the chapter

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Abstract

The genus *Mycobacterium* has the ability to degrade various environmental pollutants including polycyclic aromatic hydrocarbons (PAHs). *Mycobacterium* has an ability to withstand adverse environmental conditions and it has been considered for future bioremediation applications for the removal of PAH contaminants from crude oil–polluted sites. The degradation of PAHs using a cost-effective laboratory microcosm system was discussed. The various conditions such as environmental habitat, degradation behavior, enzymatic mechanisms, and ecological survival are thoroughly discussed in this chapter. Based on the above study, *Mycobacterium* has proved to be a better candidate in bioremediation of PAH-contaminated sites.

Keywords: *mycobacterium*, PAHs, microcosm, bioremediation

1. Introduction

As a result of anthropogenic activities, toxic chemicals have become ubiquitous contaminants of soils and groundwater worldwide. Thus, they are omnipresent in the environment due to rapid industrialization, urbanization, and modernization. This type of pollution is now being taken seriously by various industries, governments, environmental agencies, and non-governmental organizations. They are now always looking for an eco-friendly and cost-effective approach toward the removal of emerging environmental contaminants. Consequently, biodegradation is recognized as an efficient, economic, and versatile alternative to physico-chemical treatment of oil contaminants. Hence, microbial biodegradation plays a crucial role in the removal of polycyclic aromatic hydrocarbons (PAHs) specifically actinobacteria, which are a group of diverse bacteria, having the ability to degrade a wide range of organic compounds particularly hydrophobic compounds as PAH polychlorinated biphenyls (PCB), BTEX, pesticides, and so on [1].

Members of the genus *Mycobacterium* are of great interest due to their multiple PAH degradation capability, specifically high molecular weight (HMW), especially polycyclic aromatic hydrocarbons (PAHs) containing four or more fused benzene rings [2].

These compounds are persistent in environment due to high hydrophobicity and high stereochemical stability. They are known to possess mutagenic, genotoxic, and carcinogenic properties, causing deleterious effects on plants, aquatic organisms, animals, and humans. In contrast to low molecular weight (LMW) PAHs that can be degraded by various microorganisms (bacteria, actinobacteria, etc.), enrichment culture methods with HMW PAHs as sole sources of carbon and energy often lead to the isolation of *Mycobacterium* spp.

The goal of this chapter is to provide an outline of the current knowledge about biodegradation of PAHs using *Mycobacterium*. Moreover, various conditions as physiology of mycobacteria, environmental habitat, degradation behavior, enzymatic mechanisms, and ecological survival strategies toward organic compounds such as PAHs have also been discussed.

2. Calligraphy

2.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that are widely distributed in the environment. PAHs is a predominant term describing hundreds of individual chemical compounds containing two or more fused aromatic rings and are known to persist or accumulate in the environment. PAHs in the soil have recently become a matter of great concern due to their potential toxicity, mutagenicity, and carcinogenicity. Therefore, 16 PAH compounds have been identified by the United States Environmental Protection Agency (USEPA) as priority pollutants [3, 4]. They are ubiquitous compounds that are formed either naturally during thermal geological reactions, fossilization, and biological reactions or anthropogenically during mineral production, combustion of fossil fuels, refuse burning, forest and agricultural fires, and so on. On the basis of physical and chemical properties of PAHs, they are classified into two groups: low molecular weight (LMW PAHs, including 2–3 rings) and high molecular weight (HMW PAHs, including four or more rings). **Table 1** shows the physico-chemical properties of 16 PAHs as a number of benzene rings, vapor pressure, aqueous solubility, and octanol-water partitioning coefficient (K_{ow}) values. Therefore, LMW PAHs are greatly more soluble and volatile as compared to HMW PAHs due to their higher hydrophobicity than the LMW PAHs [5]. The K_{ow} values also reflect the hydrophobicity of the PAHs. These properties regulate the environmental behavior of PAHs. Therefore, HMW PAHs are persistent in the environment specifically in soil due to their high hydrophobicity.

Generally, the rate of degradation of PAHs is inversely proportional to the number of rings in PAH molecule [6]. LMW PAHs, such as naphthalene, fluorene, phenanthrene, and anthracene, are more easily degraded and usually utilized as the model PAHs for further understanding the degradative mechanisms on the HMW PAHs. HMW PAHs are more persistent in the environment as they exhibit higher hydrophobicity and toxicity [7] than LMW PAHs.

Sr. no.	PAH	No. of rings	M_r	Melting point (°C)	Boiling point (°C)	Water solubility (mg L^{-1})	Vapor pressure (Pa)	K_{ow} value
1	Naphthalene	2	128.17	80.6	218	31	10.4	3.37
2	Acenaphthene	3	154.21	95	279	3.47	3.0×10^{-1}	3.92
3	Acenaphthylene	3	152.20	93.5–94.5	265	3.93	8.93×10^{-1}	4.07
4	Fluorene	3	166.22	116	295	0.190	8.0×10^{-2}	4.18
5	Anthracene	3	178.23	217.5	340	0.0434	1.0×10^{-3}	4.54
6	Phenanthrene	3	178.23	99.5	340	1.18	2.0×10^{-2}	4.57
7	Fluoranthene	4	202.26	110.8	375	0.265	1.23×10^{-3}	5.22
8	Pyrene	4	202.26	156	404	0.013	6.0×10^{-4}	5.18
9	Benz[a]anthracene	4	228.29	159.8	437.6	0.014	2.8×10^{-5}	5.91
10	Chrysene	4	228.29	255.8	448	0.0018	5.70×10^{-7}	5.86
11	Benzo[k]fluoranthene	5	252.31	215.7	480	0.00055	7.0×10^{-7}	6.04
12	Dibenz[a,h]anthracene	5	278.35	266	524	0.0005	1.33×10^{-8}	7.16
13	Benzo[a]pyrene	5	252.31	176.5	495	0.0038	1.40×10^{-8}	6.25
14	Indeno[1,2,3-cd]pyrene	6	276.34	162.5	536	0.0620	1.0×10^{-10}	6.58
15	Benzo[b]fluoranthene	6	252.31	167	357	0.0012	6.67×10^{-5}	6.57
16	Benzo[g,h,i]perylene	6	276.34	278.3	500	0.00026	1.39×10^{-8}	7.10

Table 1. Physico-chemical properties of 16 PAHs as classified by USEPA.

With increase in the number of benzene rings, PAH solubility decreases while hydrophobicity increases. The K_{ow} values of the 16 PAH priority pollutants are in the range from 3.37 to 6.5, which is generally considered moderate-to-higher lipophilic (**Table 1**). Thus, PAHs tend to adsorb onto organic fractions in soil sediment and biota and are also accumulated in the food chain [8].

2.2. Characteristics of *Mycobacterium*

The genus *Mycobacterium* comprises aerobic, rod-shaped, acid-fast, mycolic acid (lipid moieties)-containing bacteria; they are common saprophytes, distributed in different environmental pools. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in many other bacteria, being hydrophobic, waxy, and rich in mycolic acid content. As per the Floyd et al. [9] data collection, the abundance of *Mycobacterium* genera accounted for 2.6% of total soil microbial diversity present in the environment. On the basis of growth cycle, they are divided into two categories such as slow and fast growers exhibiting growth within seven and after seven days, respectively. These phenomena are further supported by the difference in 16S rRNA sequences. Fast growing strains have two copies of the 16S rRNA gene, whereas slow growing strains normally have a single copy of the gene [10].

Moreover, properties of one or two 16S rRNA genes are assumed to be comparatively slow growth and lower metabolic activities, which require more time for adaptation into the environment [11].

Mycobacteria are high G + C-containing genera; they possess many properties that make them good candidates for application in bioremediation of soils contaminated with organic pollutants. *Mycobacterium* sp. is frequently found in environmental habitats including PAHs-contaminated soil. Nocardio-forming Actinobacteria has a unique enzymatic mechanism that degrades a wide range of complex organic compounds and their spores are resistant to desiccation. In addition, these groups of microorganisms have the ability to degrade a wide range of hydrophobic compounds and produce biosurfactants. Biosurfactant is useful for the adhesion of microbial cells to the hydrophobic compound. Therefore, many mycobacterial stains have the capability to degrade organic compounds as pesticides, PAHs, polychlorinated biphenyls (PCB), and so on. The nocardio-forming actinomycetes such as *Mycobacterium* sp., *Rhodococcus* sp., *Gordonia* sp., and so on have been reported to possess hydrocarbon degradation capability in PAHs-contaminated soil. Many *Mycobacterium* species have been reported

Strain	Compound degraded	Source	Reference
Mycobacterium spp. NJS-1 and NJS-P	pyrene	PAH-contaminated farmland soil, China	Zeng et al., [19]
Mycobacterium sp.	Phenanthrene, pyrene, fluoranthene	PAHs-contaminated soil	Johnsen et al., [38]
Mycobacterium sp. AP1	Phenanthrene, pyrene, fluoranthene	Crude oil-contaminated sand, Spain	Vila et al., [21]
Mycobacterium sp.	benzo[a]pyrene, pyrene, fluoranthene, and phenanthrene		Hennessee et al., [39]
Mycobacterium sp. CP1/CP2/CFt2/CFt6	Naphthalene, phenanthrene, anthracene, acenaphthene, fluorene, pyrene, fluoranthene,	Creosote-contaminated soil, Spain	López et al. [32]
Mycobacterium sp. S65	Phenanthrene, pyrene, fluoranthene	Soil contaminated with jet fuel, Quebec	Sho et al. [40]
Mycobacterium sp. SNP11	pyrene, fluoranthene, phenanthrene, and fluorene		Pagnout et al., [20]
Mycobacterium sp. 1B	Phenanthrene, pyrene, fluoranthene	Manufactured gas plant-contaminated soil, Australia	Dandie et al. [41]
Mycobacterium sp. MHP-1	Pyrene	Contaminated soil sample, Japan	Habe et al. [42]
Mycobacterium austroafricanum GTI-23	Pyrene, Fluoranthene, Phenanthrene	Manufactured gas plant site, Iowa	Bogan et al., [15]
Mycobacterium litorale	Fluoranthene, Phenanthrene	Oil-contaminated soil, India	[3, 4]

Table 2. Global scenario of PAH degradation in different environments by *Mycobacterium* strain.

as high molecular weight (HMW) PAH degraders, specifically pyrene, fluoranthene, benzo[b] pyrene, and so on. Thus, they are promising candidates for environmental bioremediation because of their ubiquitous presence in soils and their ability to catabolize aromatic compounds. *Mycobacterium* sp. has an ability to operate the unique catabolic pathway of HMW PAHs as compared to gram-negative bacteria. Cerniglia [12] has reported the *Mycobacterium* sp. PYR-l in enhanced degradation of four aromatic rings of PAHs when inoculated into microcosms-containing sediment. The scientific community worked on biodegradation of PAHs in different habitats as marine sediment, agriculture soil, and soil with alkaline or acidic conditions [1, 4, 13, 14] as listed in **Table 2**.

3. PAH biodegradation using *Mycobacterium*

3.1. *Mycobacterium* degradation ability

Our laboratory has worked on degradation of HMW PAHs as pyrene and fluoranthene using *M. litorale* on solid agar and liquid medium. Multiple PAHs-degrading bacterial strains were isolated from the PAHs-contaminated site near Bhavnagar. Preliminary culture was enriched in Bushnell Haas (BH) broth and further isolated on PAH-coated BH agar plate. Isolate showed a zone of clearance on PAH (fluoranthene)-coated BH plate and growth of bacteria in liquid culture (BH broth) supplemented with PAHs as the carbon source (**Figure 1**), which indicated that *Mycobacterium litorale* had the ability to utilize fluoranthene, a four-ring HMW PAH, as the sole source of carbon and energy [3]. Similar results have also been reported by Bogan et al. [15] who reported that *M. austroafricanum* utilized phenanthrene, pyrene, and fluoranthene as the sole source of carbon and energy.

Many *Mycobacterium* strains have been isolated from different environmental habitats (**Table 2**). Recently, culture-independent molecular techniques and PCR-based amplification of 16S rRNA gene were used to compare the diversity and abundance of indigenous *Mycobacterium* populations in different historically contaminated soils [16]. A wide variety of *Mycobacterium*

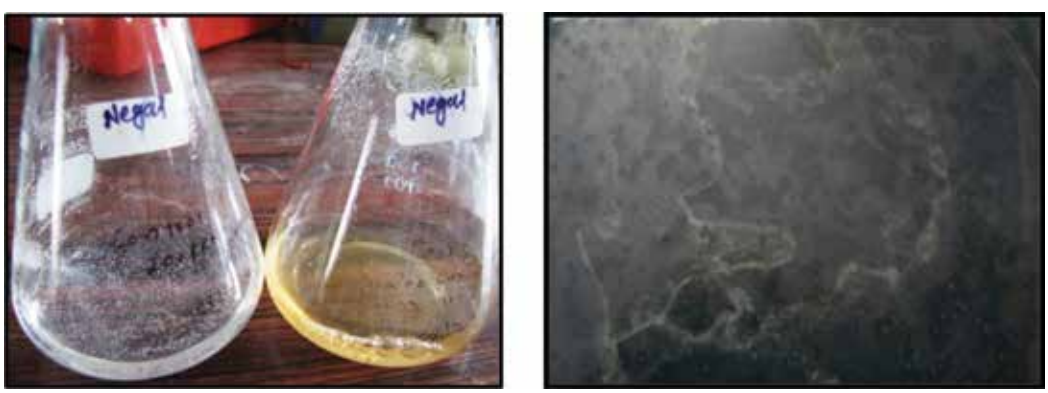

Figure 1. Fluoranthene degradation by *Mycobacterium litorale.*

genera are extensively used for removal of PAHs from contaminated sites by bioremediation techniques. It has been well established that *Mycobacteria* have exceptionally lipophilic surfaces which makes them a suitable candidate for the uptake of complex bound pollutants (i.e., PAHs) from the heavy contaminated soil particles. Thus, they have good catabolic properties toward the PAH molecule up to five benzene rings [17, 18]. Therefore, it indicates the PAH-degrading *Mycobacterium* strains are diversely distributed in the environmental soil.

The ability of the soil microbial community to degrade hydrocarbons depends on the number of microbes and its catabolic activity. *Mycobacteria* are metabolically versatile and are able to metabolize LMW and HMW PAHs. They have been reported to degrade HMW PAHs as pyrene, fluoranthene, and benzo[a]pyrene. Zeng et al. [19] demonstrated that *Mycobacterium* sp. NJ1 has an ability to degrade anthracene, pyrene, fluoranthene, and benzo[a]pyrene to various extents. Pagnout et al. [20] described *Mycobacterium* sp. SNP11 as possessing unique characteristics such as a cell wall rich with mycolic acids and the capacity to adhere strongly to hydrophobic compounds such as the HMW PAHs. This adhesion strongly facilitates the mass transfer of PAHs into the cells. Furthermore, Vila et al. [21] also reported that *Mycobacterium* sp. AP1 has the ability to degrade pyrene and produce intermediate metabolites.

3.2. Microcosm study

Soil microcosm is an approach to study microbial interactions with organic pollutants, in controlled and reproducible environmental conditions. Laboratory microcosms permit measuring of biodegradation and mineralization (CO_2 production) rates and can be used to study the effect of bioaugmentation and biostimulation on bioremediation process [22, 23]. Dave et al. [23], in our laboratory have constructed an efficient microcosm system for the enhancement of soil bioremediation process, which resulted in the improvement of HMW PAH degradation in simulated soil conditions (**Figure 2**). Addition of glucose, Triton X-100, and beta-cyclodextrin in presence of chrysene resulted in enhanced biodegradation of LMW and HMW PAHs up to six rings. In our previous study (unpublished work), we conducted a microcosm experiment in the laboratory using *M. litorale* as a bioaugmenting agent and addition of various biostimulating agents such as Triton X-100, agricultural compost, Bushnell Haas medium, and mixture of all agents, which exhibited significant biodegradation of PAHs (phenanthrene, anthracene, pyrene, fluoranthene, and chrysene) from PAH spiked soil. Actinomycetes are well known to grow under conditions ranging from obstructive to unfavorable environmental conditions for a long time. *Mycobacterium* AP1 is able to utilize pyrene, fluoranthene, and phenanthrene as a carbon source. *Mycobacterium* sp. AP1 plays a significant role in degradation of PAHs such as phenanthrene in soil microcosm conditions [22]. All over, bioaugmentation treatments showed better results than monitored natural attenuation treatments in remediating PAH-contaminated soils.

3.3. Bacterial enzymatic routes

In the aerobic degradation, cytochrome P-450 monooxygenases are complex multicomponent systems present generally in fungi and are like the bacterial aromatic ring dioxygenases. These enzymes are generally membrane bound and have broad substrate specificities. PAH

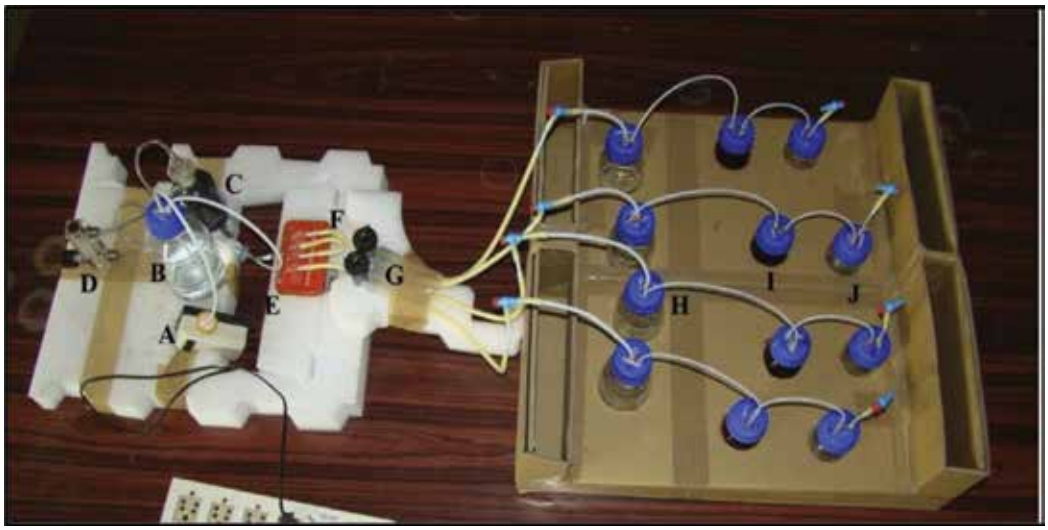

Figure 2. Microcosm system constructed in the laboratory [23]. Air pump (A), 2 M NaOH (B), activated charcoal (C), rotameter (D), 0.2 μ cellulose acetate filter (E), glass manifold (F), air regulator (G), sterile MilliQ water bottle to maintain humidity (H), microcosm flask (I) and CO2 trap (J).

is converted into arene oxide by addition by one atom of molecular oxygen by the monooxygenase (**Figure 3**), while the other atom is reduced to water.

The bacterial aerobic degradation of PAHs is generally initiated by the action of multicomponent dioxygenases that can catalyze the incorporation of both atoms of oxygen and two electrons from NADH to form *cis*-dihydrodiol. These multicomponent dioxygenases usually consist of reductase, a ferredoxin, and a third component consisting of two proteins, large and small iron-sulfur proteins [24]. Subsequent dehydrogenation by dehydrogenase forms dihydroxylated intermediates, which can further be degraded through *ortho*- or *meta*- (intradiol or extradiol) ring cleavage pathway which then eventually enters the TCA cycle (**Figure 3**). Dioxygenases have a number of applications such as in various clean-up technologies for wastewater treatments, biodegradation/bioremediation of PAHs, and other organic compounds in various contaminated niches.

Majority of dioxygenase enzymes were studied with Gram-negative bacteria but certain reports are also on gram-positive bacteria, specifically actinobacteria [25]. Silva et al. [26] reported that *M. fortuitum* has an ability to degrade anthracene maximally and increase their metabolic activity by changing various physical conditions, that is, pH and temperature. The other route of PAH degradation is accomplished by the action of monooxygenases. Initial oxidation by monooxygenases in bacteria forms trans-dihydrodiols; this activity is slower than dioxygenases. The cytochrome P-450 monooxygenase is a complex multi-enzyme protein of fungal origin that shares similarities to its bacterial counterparts.

Figure 3 represents the major routes for the degradation of PAHs by various enzyme systems. Among these, degradation of PAHs by dioxygenase-dehydrogenase enzyme system

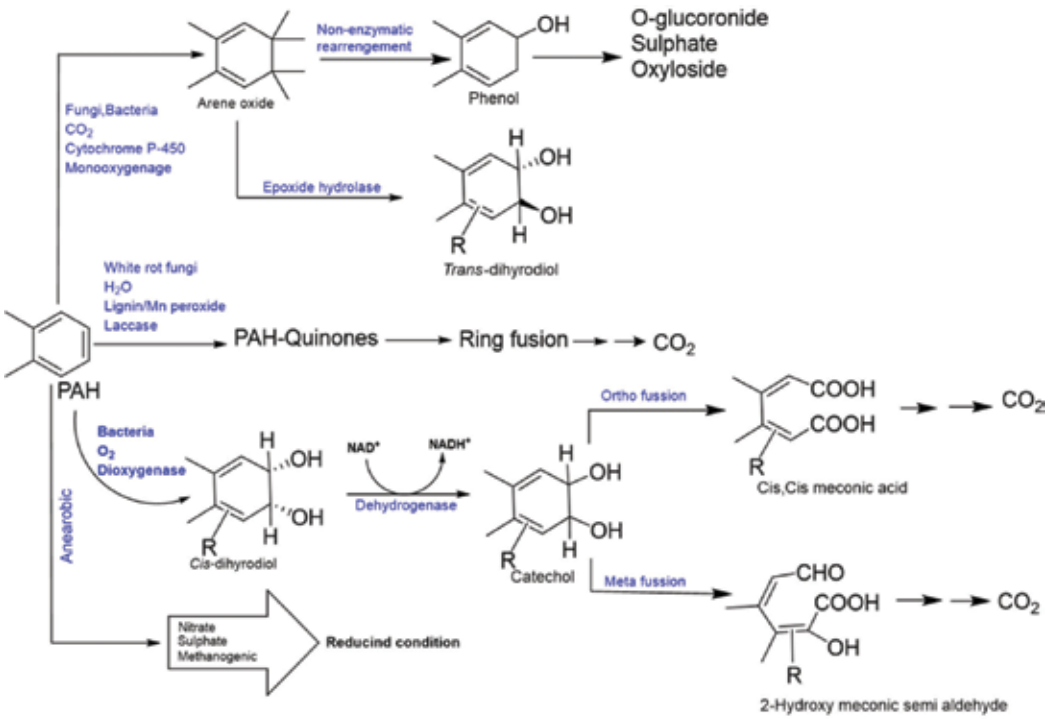

Figure 3. Microbial metabolisms of PAHs by various routes [17].

is commonly used by bacteria. Bacterial genera, capable of degrading PAHs commonly, include species of *Rhodococcus*, *Nocardia,* and *Mycobacterium*. This is a relatively small range of genera considering the prevalence of PAHs in the environment. Gram-positive actinobacteria as *Mycobacterium* spp. have been reported for the degradation of PAHs containing four or more fused aromatic rings at various extents. This is probably due to the hydrophobic cell surface which allows their adhesion to hydrophobic PAHs, thus facilitating mass transfer of the substrates inside the cells [27].

3.4. Biotransformation by *Mycobacterium* species

Many *Mycobacterial* species as *M. vanbaalenii* PYR-1 have been elucidated for the degradation of naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene, which produces key intermediate metabolites during degradation [28]. These results are significant because they have expanded our understanding of the enzymatic capabilities of bacteria to biodegrade HMW PAHs.

Mycobacterium strains have ability to degrade PAHs via either monooxygenase or dioxygenase enzymatic mechanisms, which form *trans*- and *cis*-dihydrodiol as an intermediate metabolite. Dean-Ross [29] recognized biodegradation of fluoranthene via fortuitous metabolism by an *M. flavescens* strain through *meta*-cleavage.

M. holderi was isolated from PAH-contaminated soil and was reported to grow on fluoranthene and co-oxidize pyrene in the presence of fluoranthene. It produced 29 metabolites during fluoranthene biodegradation [30]. Therefore, generated intermediate metabolite by *Mycobacterium* sp. showed a significant reduction of genotoxic potential after biodegradation of pyrene, fluoranthene, and phenanthrene [20, 31, 32].

M. vanbaalenii PYR-1 has been studied in detail with respect to enzymatic functions of various genes involved in PAH degradation [33–36]. Gene-encoding PAHs ring-hydroxylating oxygenases as *nidA, nidB*, and *nidD* are involved in PAH biodegradation [33]. These genes are expressed in *Mycobacterium* cells, which actively participated in phenanthrene, pyrene, and fluoranthene degradation. Guo et al. [37] also described that the dioxygenase *nidA* genes are involved in biodegradation of PAHs such as phenanthrene, pyrene, and fluoranthene.

4. Conclusion

Organic pollutants such as PAHs, PCB, and pesticides are resistant to degradation and are predominantly present in the environment; thus, they cause severe toxicological effects on humans as well as marine biota. Therefore, there has been growing interest in mycobacterial strains as potential bioremediation agents and as important components of indigenous PAH and other xenobiotic compound degradation. Various researchers reported the use of *Mycobacterium* for PAH degradation in different environmental conditions. Mycobacterium possesses peculiar characteristics for degradation of HMW PAHs due to their potential enzymatic mechanisms, which encoded the PAH ring-hydroxylating oxygenases genes, participating in PAH biodegradation. A member of the genus *Mycobacterium* is responsible for HMW PAH removal and their catabolic enzyme like monooxygenase/dioxygenase, which is converted into less harmful and simpler end products. Thus, *mycobacteria*, isolated from different habitats in the environment, can be exploited for their potential to remediate contaminated sediment/soil. Based on the study, interpretations will aid notable information to the scientific community for future research on bioremediation of recalcitrant high molecular weight (HMW) PAHs. Based on the previous study *Mycobacterium* has tremendous capability to remediate the contaminated sites and transform them to less toxic end products. Biodegradation is considered as the best approach to restore PAH-contaminated soils. Therefore, bioremediation is a feasible option for cleaning up PAHs because it is simple, applicable over large areas, cost-effective, and eco-friendly green approach.

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This book arose from the combination of diverse areas of knowledge, experience, research, and points of view that try to demonstrate that mycobacteria are a complex science and very relevant to scientific studies that affect the human being in the world. Sophisticated techniques for improving human health do not guarantee that the "battle" against mycobacteria has been won, since tuberculosis, mycobacteriosis, and leprosy are a daily challenge in the world.

The book includes contributions made by prestigious experts and research groups in different areas of mycobacteria, and they have contributed new perspectives of their area giving a comprehensive, important, and fascinating emphasis of this field that continues to offer challenges that lead various disciplines to understand their biology and pathogenicity. It is hoped that these chapters will be very useful for learning and discussion.

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