THE CHARACTERISTICS AND EVOLUTION OF *MYCOBACTERIUM TUBERCULOSIS* DRUG-RESISTANCE AND CURRENT RESEARCH TOWARDS ENDING THE GLOBAL TUBERCULOSIS EPIDEMIC

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Abstract: Tuberculosis disease is one of the leading causes of death worldwide. It is caused by *Mycobacterium tuberculosis* infecting the lungs, where the bacteria interfere with the immune system in order to avoid eradication. Once inside the body, TB has the ability to exist in either a latent or active infection, with the latter presenting in a variety of respiratory symptoms. People who are immunocompromised or those that live in developing countries are most at risk for active TB. Currently, the Bacillus Calmette–Guérin (BCG) vaccine is the only preventative measure against the disease, however it has done little to control the epidemic. A variety of techniques, such as the TB skin test, are used to diagnose a TB infection. Once diagnosed, a rigorous antibiotic regimen is necessary to treat the disease. Unfortunately, TB has evolved in such a way that has made certain strains resistant to current drug therapy, exacerbating the problem. There are a variety of new diagnosis and treatment options currently in development, such as more accurate diagnostic tests, new vaccines, and shorter treatment schedules. However, drastic measures must be taken in order to target drug-resistant TB and end the global TB epidemic.

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CHAPTER I

INTRODUCTION

In 2017, over 1.3 million people died as a direct result of a tuberculosis (TB) infection while another 10 million people contracted the disease globally. According to the 2018 World Health Organization (WHO) global tuberculosis report, these statistics rank TB as the leading cause of death worldwide from a single infectious agent [15]. Despite TB co-existing with humans for thousands of years, we are no closer to eradicating the disease today than when it was first discovered. Due to a variety of factors, some modern strains of *Mycobacterium tuberculosis* have evolved in such a way that allows them to be resistant to current drug therapy. In 2017, the WHO reported that about 460,000 new TB cases were found to be resistant to multiple lines of treatment [15]. This thesis will explain how drug-resistant TB came to be such a widespread problem as well as detail existing and experimental treatment options to combat the growing problem of drug-resistant TB.

CHAPTER II

HISTORY OF *MYCOBACTERIUM TUBERCULOSIS*

In 2007, Kappelman et al. published an article in the *American Journal of Physical Anthropology* that detailed the discovery of an early Homo erectus skeleton in Western Turkey. Despite the incomplete nature of the remains, researchers were able to identify lesion patterns on the skull characteristic of a *Mycobacterium tuberculosis* infection. The remains were dated to be between 490,000 and 510,000 years old- making this the earliest known fossil record of a tuberculosis infection [5]. While documented cases of TB do not date back as far, the evidence provided by the skeleton points to a long, shared history between TB and human ancestors.

Hershberg and colleagues (2008) hypothesized that human tuberculosis originated in Africa and migrated outward along with the human race. This hypothesis is supported by the fact that Africa is the only continent in which all six of the main lineages of human tuberculosis can be found [4]. Traditionally, these lineages are each associated with a different part of the world. Figure 1 shows a .

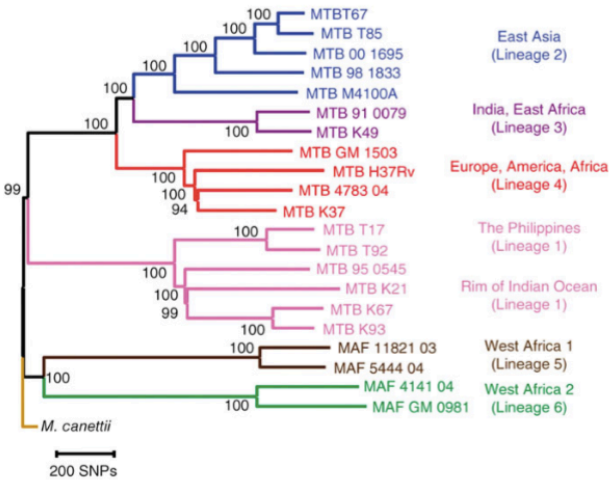

Figure 1: Phylogenetic breakdown of global Mycobacterium tuberculosis strains [17].

phylogenetic breakdown of this phenomenon. Ancient literature and modern DNA analysis of fossilized remains have provided researchers with a timeline, following the spread of the disease across the globe.

Ancient Egyptian art dating back some 5,000 years ago has been discovered depicting characteristic abnormalities associated with TB, including Pott's disease which will be detailed later in this paper. In 1997, the presence of TB in Ancient Egypt was confirmed when tissue samples from a mummy were analyzed via PCR-amplification. The amplified DNA was compared to the known *M. tuberculosis* genome and found to be homologous. The mummy was dated between 1550-1080 BC [7]. Some historians have even found references to the disease in biblical passages dating back to the same time period. In the books of Leviticus and Deuteronomy, people were threatened with a plague called "consumption" or "wasting disease." While TB was not specifically named in the ancient text, the original Hebrew word used to describe the wasting disease is used today for TB [3].

From Africa, TB quickly spread to the edges of Europe and Asia. TB was well known in ancient Greece. Hippocrates, the "father of medicine" himself, wrote about the disease. He called it "phthisis" and referenced both its ability to infect anyone, young or old, as well as its fatal nature, in his works. In 2003, Mays and Taylor found evidence that human TB had made its way to Britain prior to Roman invasion, as confirmed by skeletal remains found and dated to around 400-200 BC [6]. Skeletal remains dated around the first century AD, that exhibited classic TB lesions were also discovered in both Japan and South Korea [10].

In 2001 Rothschild and colleagues published an article detailing their discovery of *M. tuberculosis* complex in bison fossils found in Wyoming and carbon-dated to around 17,000 BP. The discovery was based on both molecular DNA testing and classic osteological indicators,

suggesting that TB prevalence in America may be two-fold. Human contraction could be traced to a zoonotic spread as well as the eventual human migration across the Bering Strait [8].

By the 19th century, TB was the cause of death in up to 1% of the population throughout Europe and North America. It had become such a common occurrence that references to TB can be found in classic literature, such as *Wuthering Heights* by Emily Brontë [2], and at the basis of folk lore. In New England specifically, consumption, as the disease was known, created American vampire stories. Due to the contagious nature of the disease, family members of those recently succumbed to TB would soon exhibit classic "wasting away" symptoms. According to the folk lore, this was the result of someone recently deceased (from TB), returning as a vampire and slowly draining the life from their relatives [9]. Interestingly, it was during this same time period that some of the biggest discoveries regarding the disease occurred. In 1865, Jean-Antoine Villemin demonstrated the transmissibility of TB by transferring the disease to a rabbit by injecting it with liquid from the lesion of an infected individual [2]. This served as evidence against the common belief that consumption was hereditary. Robert Koch expanded on this discovery and in 1882, he announced his isolation of the microorganism responsible. A year later, it was named *Mycobacterium tuberculosis* [1].

By the end of the 19th century, the prevalence of TB infections had drastically decreased in the developed world. Improved quality of life and the creation of better diagnostic and treatment techniques are thought to be the cause of the decline. By the early 1900s Koch's research had been expanded on and the tuberculin skin test was created. Not long after, the Bacille Calmette-Guérin (BCG) vaccine was accepted (both of these techniques will be detailed later in this paper). Unfortunately, places such as Sub-Saharan Africa did not have access to these new techniques and continued to see rampant TB [2].

The stark contrast in disease incidence between countries was exacerbated by the events of World War I. Despite efforts put in place by the allies, and Germany, to screen soldiers for TB, the disease still saw a resurgence during this time [2]. In response, the WHO's first major disease control project was started. Over the course of three years, 30 million people were tested for TB using the tuberculin skin test and 14 million people were given the BCG vaccine [11].

Unfortunately, the increased usage of TB vaccines gave rise to a new issue. TB strains resistant to drugs such as streptomycin were discovered as soon as 5 years after the treatment unveiling [12]. However, most researchers believed these resistant strains proved to be less virulent and therefore, were not a common threat [13]. A 1993 publication in the New England Journal of Medicine challenged this belief. The study involved collecting information from every patient in New York City who was positive for TB, and testing them for drug-resistant strains. Of the 466 patients tested, 33% were found to be carriers of TB strains that were resistant to one or more anti-tuberculosis medication (multi-drug-resistant; MDR). The article claimed that just 10 years earlier, only 10% of similar patients were infected by strains such as these [14].

In the last 20 years, new drug therapy has been unable to keep up with the emergence of drug-resistant TB strains. In 2006 the Centers for Disease Control adopted a new term to cover strains that are resistant to both first line and main classes of anti-tuberculosis drugs- extreme drug resistance (XDR) [13]. At the present time, not much headway has been made in combatting XDR-TB.

CHAPTER III

MYCOBACTERIUM TUBERCULOSIS EPIDEMIOLOGY

Worldwide TB infection has progressed to the point where, in 1997, the WHO began publishing yearly reports on the epidemic. The most recent report contains statistics from 2017, focusing heavily on the rate of TB in the HIV-positive population. Interestingly, the report noted that in 2017 57% of new TB patients were HIV-positive [15]. While HIV-positive patients possess a higher chance of contracting TB than healthy individuals, there are other lifestyle factors that play a role as well.

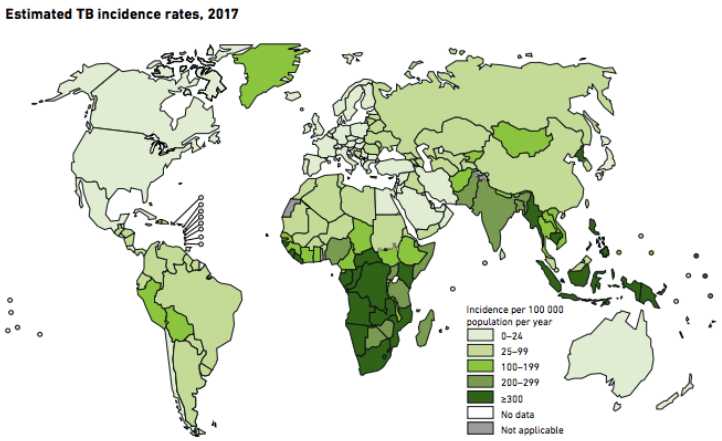

Figure 2: Map indicating global TB cases in 2017. Reprinted from Global Tuberculosis Report 2018 (p. 37), by the World Health Organization, 2018 [15].

TB most often presents itself in a form termed "respiratory tuberculosis" that spreads through aerosols expelled by the coughing and sneezing of an infected individual. When exposed to aerosols containing *M. tuberculosis,* one of three possible outcomes can arise; the infection can progress rapidly into an active TB infection, the bacteria may cause a latent infection, or the host's immune system will eradicate the organism [17]. Like most diseases,

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those who are immunocompromised are most at risk to the infection taking one of the first two paths- a characteristic best illustrated by the high rate of HIV- associated TB. TB is also closely related to poverty and its associated risk factors including malnutrition, diabetes, alcoholism, and tobacco use. With this in mind, it is not at all surprising that the WHO reported that South-East Asia and Africa collectively accounted for 69% of new TB cases and 82% of TB related deaths in 2017. Figure 2 compares the rate of TB cases in these areas to the rest of the world. Southern Asia and Africa are represented by some of the darkest colors on the map, indicating a higher TB rate than the rest of the world. These regions have a large percentage of the population living below the poverty line, and Africa, specifically, possesses the highest rate of HIV-associated TB worldwide [15].

TB cases attributable to selected risk factors, 2017

RISK FACTOR	RELATIVE RISK*	EXPOSED (MILLIONS IN 2017)	GLOBAL POPULATION ATTRIBUTABLE FRACTION (%)	ATTRIBUTABLE TB CASES (MILLIONS IN 2017)
Undernourishment	3.1–3.3	734	18	1.9
HIV infection	20	36	8.7	0.88
Smoking	1.6–2.5	1 047	7.9	0.83
Diabetes	2.3–4.3	460	7.5	0.79
Harmful use of alcohol	1.9–4.6	407	4.7	0.49

Figure 3: Prevalence and M. tuberculosis cases associated with common TB risk factors. Reprinted from Global Tuberculosis Report 201 (p. 147), by the World Health Organization, 2018 [15].

Even though HIV-positive individuals present a much higher risk of TB development than any other population, the additional risk factors mentioned are much more common and are therefore, more important to global TB epidemiology [17]. Figure 3 details the most common risk factors associated with TB. Interestingly, undernourishment is responsible for twice as many TB cases than HIV. The WHO notes that this is due to the high prevalence of malnourishment worldwide [15].

A 1996 study by Chan and colleagues looked specifically at TB prevalence in malnourished mice. They found that the malnourished mice exhibited a significantly depressed cell-mediated immune response when compared to the controls. They were able to pinpoint the

exact responses that were lacking in these deficient mice and found that the infection subsided to a less-fatal course when the the mice were fed a consistent, full diet [18].

Since TB is most commonly expressed as a pulmonary disease, it is no surprise that smoking leads to an increased chance of infection. Compounds found in cigarette smoke are known to be immunosuppressive. Specifically, studies have shown that chronic smoke inhalation leads to impaired pulmonary secretion production which hinders the immune system's ability to "flush out" bacteria, as well as decreased functioning of alveolar macrophages [17]. Alcoholism is also known to correlate with lowered immune function, and studies have shown that health issues associated with alcoholism can increase the rate of TB-related death. Lin and colleagues (2014) examined cases of TB-positive patients in a hospital in Taiwan. They aimed to determine the causes and risks associated with TB mortality. Ultimately, they determined that the presence of liver cirrhosis was a significant predictor for death in patients infected with TB, supporting the idea that alcoholism can worsen the course of TB infections [19]. Diabetes' association with TB is less well known. Several studies have found that diabetics are significantly more likely to contract TB than healthy individuals, but the mechanisms behind this difference are not clear at this time [17].

CHAPTER IV

PHYSICAL INDICATORS OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

a. Latent TB Infection

TB infection has the ability to exist in two forms: active and latent. According to the most recent statistics reported by the WHO, latent infection, the most common form, is thought to affect around 23% of the global population [32]. In this form, the person is infected but their immune system is able to keep the bacteria at bay. Those who are latently infected are unable to spread the disease [17]. There are also no clinical symptoms at this point. Because of this, carriers of latent TB are usually determined via a skin test that will be discussed later in this thesis. When an individual possesses a weakened immune system as a result of one of the risk factors previously listed, for example, the latent infection can quickly become active. In this form, the bacteria are able to replicate and spread throughout the body and potentially between individuals [16].

b. Active TB Infection

Once a TB infection becomes active, the infected individual can exhibit a variety of symptoms depending on the type of TB. Most infections will result from pulmonary TB in the lungs. Patients present with a persistent cough that is potentially coupled with hemoptysis, trouble breathing, weight loss that can progress to cachexia ("wasting away"), fever, and discomfort. It is worth noting that understanding the disease epidemiology is especially important because these

symptoms are characteristically non-specific to TB. For example, in a developed world, a patient presenting with trouble breathing and a chronic cough could be suffering from lung cancer or pneumonia. If this same patient were to live in impoverished Sub-Saharan Africa, however, pulmonary TB is a much more likely diagnosis [20]. In specific cases, long-term pulmonary TB results in more serious lung issues including, lung scarring leading to bronchiectasis, chronic pulmonary aspergillosis infection, and chronic obstructive pulmonary disease [21].

Additionally, people can present with a re-activated form of TB called "cavitary TB." This can occur in a very small portion of immunocompromised TB patients. When TB progresses to the point where host tissue death gets out of hand, open spaces, or cavities, are formed in the lungs. These cavities are connected to the airways, providing an oxygenated location for an abundance of *M. tuberculosis* bacilli to populate [52].

c. Extrapulmonary TB Infection

TB is also capable of infecting parts of the body aside from the lungs, causing an infection known as "extrapulmonary tuberculosis." Extrapulmonary TB infection is less common than, but often coupled with, pulmonary infection. Patients can exhibit pulmonary TB symptoms as well as obscure symptoms associated with the extrapulmonary organ involved. Infection can take place in virtually any organ, but is most common in the lymph nodes where it presents as glandular swelling of the neck [22].

Pott's Disease is a common name for spinal TB. This type of infection is characterized by the destruction of vertebrae leading to spinal deformities. The destruction leads to Pott's paraplegia in about 30% of spinal TB patients [22]. In section I, Pott's Disease was introduced as a common indicator of TB infection on ancient specimens. Remains have been found that exhibit the characteristic "humpback" that is often associated with Pott's Disease. Researchers have been

able to couple these physical findings with molecular tests to pinpoint ancient TB infections, such as those discussed in the first section of this paper [2].

CHAPTER V

HOW *MYCOBACTERIUM TUBERCULOSIS* UTILIZES THE IMMUNE SYSTEM

TB has survived within the human population for centuries. This has allowed the bacteria to learn how to manipulate the human immune system so that it can live and replicate comfortably without eradication. This section will use figure 4 as a template to explore the "life-cycle" of TB as it interacts with the immune system throughout infection, latency, and re-activation.

As mentioned previously, TB spreads via aerosols dispelled by an infected individual which are breathed in, making the lungs the initial site of infection. Once TB molecules are inhaled, our body works to activate an innate immune response against the invading microorganism. This first line of defense begins when toll-like receptors (TLR) on the surface of immune proteins recognize pathogen-associated molecular patterns (PAMPs)

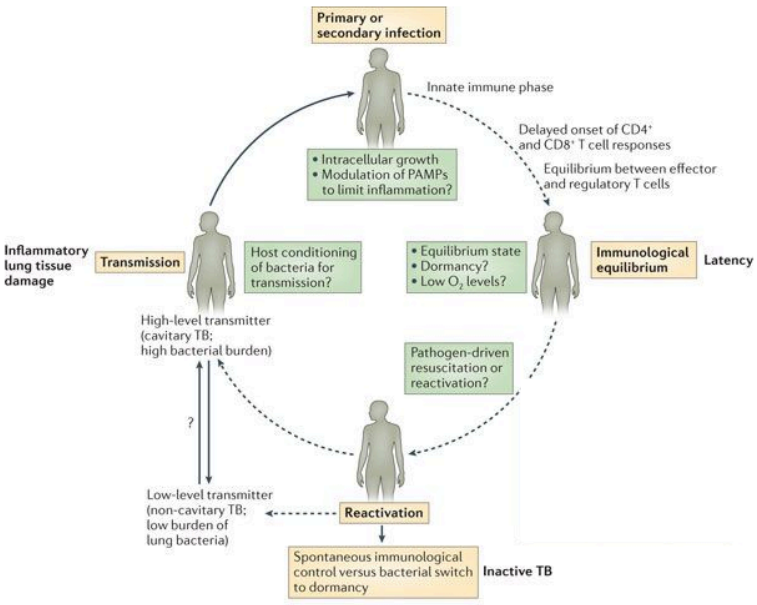

Figure 4: Immunological cycle of TB [41].

that are expressed on the pathogen. Studies show that TLR2 and TLR9, specifically, are key players in the detection of TB. TLR2 possesses the largest number of mycobacteria -specific agonists of any known TLRs and TLR9 detects mycobacterial DNA [41]. Following the detection of TB, cytokines such as TNF-α and INF-γ are recruited, creating an inflammatory response and signaling circulating phagocytes to ingest the bacteria into the phagosome [42]. During normal conditions, host phagocytes undergo a maturation process and phagosome-lysosome fusion. This creates an acidic environment inside the phagosome in which lysosomal enzymes can digest engulfed pathogens [43]. However, once inside the host phagosome, TB interferes to inhibit phagosome-lysosome fusion in order to avoid degradation [42]. Previously, it was believed that TB stayed within the phagosome after halting its maturation, giving TB a safe niche to replicate within [46]. However, more recent papers have reported that previous studies focused only on the initial stages of infection. After successfully avoiding degradation within the phagosome, TB then disrupts the phagosomal membrane and infiltrates into the cytosol of resident alveolar macrophages. Translocation to the cytosol was found to occur around 4 days following infection [46]. This is achieved through the use of a type VII secretion system.

Type VII secretion systems are a relatively new discovery, first observed in TB. This system allows TB to bypass its own complex cell envelope and secrete virulence factors which aid in its fitness within the host [47]. Specifically, TB secretes CFP-10 and ESAT-6 which help it to lyse the membrane of the phagosome. Studies have found that this secretion system is encoded for in a region of differentiation known as RD1. Interestingly, RD1 is the region responsible for *M. tuberculosis's* virulence. This gene region is deleted in the strain found in the BCG vaccine. Thus, cells infected with the BCG strain are non-virulent, and lack the secretion system, allowing for phagosome maturation and were therefore unable to translocate into the cytosol [46].

Access to the cytosol allows TB to migrate into interstitial tissue in the lungs. Once there, a storm of additional macrophages flock to the area creating an inflammatory response and

eventually, a granuloma [42]. Historically, granulomas were thought to be a host defense mechanism that helped to contain TB infections. However, a 2009 study provided evidence that TB has developed ways to manipulate the immune system and use granulomas to further its cell-to-cell spread within the host. It is worth noting that this study observed zebra fish that were infected with *Mycobacterium marinum* (Mm), which mimics early stages of TB infection. They concluded that following the initial macrophage infection, TB induces necrosis of the host cell [44]. Necrosis is an important aspect of TB's fight against the immune system. It causes the death of host macrophages while also ensuring the mycobacteria inside is released in such a way that it is still able to re-infect [48]. It also causes additional macrophages to be recruited to the area. TB then infects these new cells and the process repeats. This cell-to-cell spread did not occur in subjects infected with RD1 deficient strains, suggesting that TB's specialized secretion system may be responsible [44].

Nearly two weeks following the primary infection, infected dendritic cells (DC) migrate to lymph nodes to activate antigen-specific T cells. It then takes around a month for these T cells to proliferate, travel back to the lungs, and begin their attack. This process is significantly delayed. Comparatively, it only takes DC a few hours to present the influenza virus to lymph nodes [41]. At this time, researchers do not have a solid explanation for why this delay in immune response exists against TB. One likely explanation, is TB's ability to inhibit normal macrophage apoptosis [48]. Macrophage apoptosis is an important step in the immune system; it allows for DC to uptake the bacteria and transport the antigens to the lymph nodes [49]. By inhibiting apoptosis, TB is able to prolong the innate immunity phase, and thus, prolong its own replication [41].

Once adaptive immunity is initiated, TB replication is arrested and a latent infection progresses. During this time, the host granuloma is able to "cage in" infected cells. Even though the bacteria are still present in the body, latently infected individuals are asymptomatic and

incapable of spreading TB [41]. Additionally, a 2011 study of non-human primates found that TB was still able to replicate, mutate, and develop drug-resistance during latent infection [45]. Using macaques as their host, Ford and colleagues (2011) observed similar mutation rates in those infected with active TB and those infected with latent TB. While the true reasoning behind this is unknown, they suggest that prolonged time spent within host macrophages leads to extensive damage to bacterial DNA, and ultimately, mutations [50]. The above characteristics are best explained by considering that rather than being a state of bacterial inactivity, this latent period is most likely a state of equilibrium, with the immune system constantly battling to keep TB levels down [41].

When this state of equilibrium is thrown off, or when it is not reached to begin with, active infection progresses. In the case of an active infection, TB replicates to the point where it "spills out" from granulomas and spreads throughout the body [42]. Factors that can contribute to this re-activation include the lifestyle factors that were detailed in section II of this paper and anti-TNF treatment [41]. TNF suppression drugs are given to people to suppress immune inflammatory responses, such as with those who have arthritis or those who have received an organ transplant. Unfortunately, these drugs also suppress macrophage-mediated anti-mycobacterial activity and memory T cells, making these individuals susceptible to active infection [41]. Additionally, individuals who are latently infected can become re-infected if they come in contact with a new strain of TB [41].

It is also worth noting that not all active infections harbor the same level of transmissibility from infected to non-infected. Section III described characteristics of an extreme type of infection called "cavitary TB." People suffering from cavitary TB present a very high person-to-person transmission risk due to the increased number of infectious bacilli present in the cavities [41]. Interestingly, individuals co-infected with HIV are less likely to transmit TB than non-HIV positive individuals [51]. This is because those co-infected with HIV/TB are less likely

to be smear positive, meaning they harbor a lower number of bacilli than normal infected individuals. This is based on a sputum smear test that will be detailed, along with other testing techniques, in the next section.

CHAPTER VI

CURRENT DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS*

a. Tuberculin Skin Test

In 1909, Clemens Freiherr von Pirquet, a pediatrician from Austria, published an article detailing his use of tuberculin as an indicator of latent TB infection. Tuberculin had previously been cited by Robert Koch as a treatment for TB, however, he experienced an adverse reaction when he injected himself with the substance. Extensive research eventually led to the version of tuberculin that is still used in TB testing today [2].

The tuberculin skin test is one of two main diagnostic tests used today. It is sometimes referred to as the Mantoux test, after the scientist who helped develop the technique in the early 1900's. Its execution is fairly straight-forward. According to the CDC, two visits to a health-care provider are necessary to appropriately carry out the test and determine a result. During the first visit, a syringe is used to inject a small amount of tuberculin into the skin of the forearm. A follow-up visit is required 2-3 days later. At this point, the health-care provider will measure the diameter of the inflamed area surrounding the initial site of injection. The size of a positive result varies based on a patient's history. For example, immunosuppressed individuals or people who have recently been in contact with TB will present with a reaction of at least 5mm when they are positive for TB, while healthy individuals with no known ties to TB will present with a reaction site of 15mm when they are infected [24].

The tuberculin skin test works by activating pre-existing mycobacteria-specific T lymphocytes. When a person becomes infected with TB, their immune system creates and circulates T cells specific to the bacteria. By injecting tuberculin under the skin, these T cells proliferate and create a delayed hypersensitivity response that is characterized by redness at the site of injection. T cells recruit additional immune cells to the site causing vasodilation and edema during a reaction that peaks 48 hours after injection, thus rendering a second appointment necessary to read the results [25]. While this is the most common test used to determine TB infection, it is not the most reliable. A variety of factors can invalidate the results including reader bias and false positives. If a patient is infected with a non-tuberculosis *Mycobacteria,* their body may still produce a reaction that could be interpreted as a positive result [25]. It is also important to note that false negative results can arise if a patient is recently infected, was infected many years prior to testing, or if their immune system is overwhelmed by the infection [24].

b. Interferon Gamma Release Assays

False positive skin tests can also arise when a patient has previously been exposed to the BCG vaccine. When a health-care provider is aware of this, they will oftentimes order a TB blood test (interferon gamma release assay) instead. The mechanisms behind this technique are similar to the TB skin test. Blood is collected and exposed to TB antigens, and if this exposure causes the patient's white blood cells to release interferon gamma, it could signify TB infection. There are two types of TB blood tests: QuantiFERON®-TB Gold In-Tube test (GFT-GIT) and T-Spot. These tests take anywhere from 8-30 hours and different TB antigens are used in each one. When put through the GFT-GIT test, any blood that is positive for interferon gamma is considered potentially TB positive. A positive result during the T-Spot test is indicated by the presence of interferon gamma producing cells in the blood [26].

c. Additional Diagnostic Tests

Sputum smear testing and chest x-rays are also used to diagnose TB infection. Low-income countries depend on sputum testing for the majority of their TB diagnostic testing. With this technique, sputum is smeared and stained with a Zeihl-Neelson acid-fast stain. This test allows for the staining and detection of acid-fast organisms such as *Mycobacterium*. Even though this type of TB testing is so prevalent in countries most affected by the disease, it is worth noting that the test requires at least 10,000 organisms per mL of sputum, often giving a false-negative result in patients that are co-infected with HIV [27]. Chest x-rays are often used as a supportive test, especially in cases where false negatives are suspected. According to the Mayo Clinic, clinicians look for white spots or drastic changes within the lungs [28]. Chest x-rays are less conclusive in areas prone to HIV. There is a high rate of other pulmonary diseases in HIV patients, each of which can cause chest x-ray abnormalities [26].

In 2010, the WHO approved a new type of TB diagnostic testing, the Xpert MTB/RIF assay. This test is recommended for HIV patients and those who may be exposed to drug-resistant TB. It works by exposing a sputum sample to a sample reagent containing sodium hydroxide and isopropanol. This concoction reduces the possible pathogenicity of the sample, making the testing process safer for the technicians. The sample is then loaded into the GeneXpert device where the gene of interest is amplified via RT-PCR, after which 5 probes are used to detect different segments of this gene. Only 2 of the 5 probes need to give a positive signal in order for TB to be detected [29].

This test focuses on the *rpoB* gene found in TB. This gene was chosen because virtually all Rifampicin- resistant TB strains carry a mutation on this gene. Interestingly, this region is flanked by *M. tuberculosis* specific DNA sequences. This close proximity allows for Xpert MTB/RIF to easily test sputum for TB while simultaneously testing for drug resistance. The test is also more sensitive, providing a better diagnostic technique for HIV patients [29].

CHAPTER VII

CURRENT TREATMENT OPTIONS FOR *MYCOBACTERIUM TUBERCULOSIS*

a. The Bacille Calmette-Guérin Vaccine

Not long after the implementation of the tuberculin skin test, Albert Calmette and Camille Guérin were able to attenuate *Mycobacterium bovis* and develop the TB vaccine Bacille Calmette-Guérin (BCG) [2]. This vaccine is missing a key TB genomic region known as RD1. This region encodes for virulence factors, such as a type VII secretion system. Since these genes are missing, the vaccine is highly unlikely to cause TB and is therefore safe for all ages [42]. Initially the vaccine was only given to children who tested negative to TB. However, eventually research conducted by the WHO showed no ill-effects presented in TB-positive vaccinated children. This led to millions of people being vaccinated and no need for costly diagnostic tests [30]. The WHO still recommends that children living in high-risk countries receive BCG along with their usual childhood vaccines [15]. Unfortunately, despite its massive acceptance, when it comes to preventing adult TB, the vaccine is highly variable. So much so, BCG protection ranges from 0-80% with high-risk countries experiencing less protection [30]. With the only available TB vaccine being so inefficient, there is a definite need for new techniques.

b. Antibiotics

Aside from the BCG vaccine, a variety of drugs are currently used to treat both latent and active TB. The first-line drugs are used during an intense round of treatment in individuals who are not known to be infected with a drug-resistant strain. These drugs include: isoniazid (INH),

rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) [31]. Each drug uses a different mechanism to attack TB. For example, INH is activated by the presence of the *katG* mycobacterial enzyme and works to disable to production of a cell wall in TB, while RIF binds to the β-subunit of bacilli RNA polymerase to block mRNA elongation, therefore blocking protein synthesis [37].

According to the CDC the initial "intensive phase" of treatment consists of attacking TB with all four first-line drugs for a total of 8 weeks. This is followed by an 18-week "continuation phase" where the individual takes only INH and RIF. The situation surrounding the infected individual (are they HIV positive, is this their first TB infection, etc.) affects how often the drugs are taken throughout treatment; with some taking antibiotics every day for the entire 26-weeks and others taking the drugs only twice weekly [31]. This type of combination therapy is necessary to properly combat basic TB infections. By combining treatments that have different modes of action, there is a higher probability that the strain causing the infection will be responsive to at least one of the drugs used. However, there are instances where the first-line drugs are not enough. When drug-resistant TB strains are suspected additional treatment protocols must be implemented.

Group A. Fluoroquinolones[b]		Levofloxacin	Lfx
		Moxifloxacin	Mfx
		Gatifloxacin	Gfx
Group B. Second-line injectable agents		Amikacin	Am
		Capreomycin	Cm
		Kanamycin	Km
		(Streptomycin)[c]	(S)
Group C. Other core second-line agents[b]		Ethionamide / prothionamide	Eto / Pto
		Cycloserine / terizidone	Cs / Trd
		Linezolid	Lzd
		Clofazimine	Cfz
Group D. Add-on agents (not part of the core MDR-TB regimen)	**D1**	Pyrazinamide	Z
		Ethambutol	E
		High-dose isoniazid	H[h]
	D2	Bedaquiline	Bdq
		Delamanid	Dlm
	D3	*p*-aminosalicylic acid	PAS
		Imipenem–cilastatin[d]	Ipm
		Meropenem[d]	Mpm
		Amoxicillin-clavulanate[d]	Amx-Clv
		(Thioacetazone)[e]	(T)

Figure 5: Treatment recommendations for drug-resistant TB, according to the 2016 WHO treatment guidelines [38].

According to the WHO treatment

guidelines for drug-resistant TB, secondary TB drugs are classified into the 4 groups shown in figure 5 [38]. Group A consists of fluoroquinolones, a group of antibiotics that inhibit type II topoisomerase in TB, ultimately blocking DNA synthesis [37]. Group B, injectable antibiotics, work in a variety of ways to inhibit protein synthesis within TB [37]. Group C consists of ethionamide, which works to inhibit mycolic acid biosynthesis, interfering with TB's cell wall production [39]; cycloserine, which inhibits cell wall synthesis by blocking peptidoglycan biosynthesis [40]; linezolid, which blocks TB's ability to undergo protein synthesis [40]; and clofazimine, which attacks TB by disrupting its metabolism [40]. Group D consists of a variety of additional attack agents that disable TB by using many of the mechanisms already mentioned. The WHO recommends that these additional drugs be used in combination to combat drug-resistant strains of TB.

c. Directly Observed Therapy

In addition to conventional drug therapy, the DOT strategy has also been put into place all over the world. DOT stands for "directly observed therapy" [36]. This therapy was created as an answer to the ever-growing problem of patients not abiding by drug therapy schedules. As mentioned previously, TB treatment is a long process that often lasts months and failure to complete treatment can lead to a variety of problems, the most serious of these being increased drug-resistance or death. With the help of DOT, patients take their medication under direct observation and are thus more likely to complete the entire course of treatment. While the observation can be conducted by a family member or health official, the records are reported back to the health system, adding an additional layer of accountability [36].

CHAPTER VIII

DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

In addition to the 1993 MDR-TB study that was mentioned in section I, there have been a number of drug-resistant TB outbreaks across the globe in more recent years. In 2012, a manuscript published by Samuel Loewenberg in *The Lancet,* described an outbreak of (what the researchers deemed) totally-drug-resistant TB cases in Mumbai. While TB mutations are a known cause of modern drug resistance, this article places the blame on inadequate treatment. Residents of Mumbai are no stranger to TB. With much of the city plagued by overcrowding and poverty, it has created the perfect environment for the disease to easily spread. Government facilities are often overcrowded, leading to a high rate of spread in hospitals as well as diseased individuals being turned away due to lack of beds. As a result, people have started to turn to private doctors for treatment. Unfortunately, some of these doctors lack proper medical training and many of them provide counterfeit drugs or incomplete treatment plans. This inadequate treatment often leads to TB not being completely eradicated in a patient, leading to increased drug-resistance. All of the factors mentioned above have snowballed and led to India having one of the highest rates of drug-resistant TB worldwide [33].

Russia joins India as one of the countries with the highest prevalence of drug-resistant TB. In fact, along with China they account for 47% of the global total, according to the 2018 WHO tuberculosis report [15]. Interestingly, the WHO noted that their national TB epidemiological review found that while overall TB rates in Russia are decreasing (due to tight

regulations on regular TB screening for all adult residents), MDR-TB rates are increasing. Unfortunately, the WHO was unable to provide an explanation for this counterintuitive rise in MDR-TB cases [15].

While the previously mentioned articles provide a prime example of how developing countries are struggling to combat the growing problem of drug-resistant TB, the issue is seen worldwide, as shown in Figure 6. Many studies note that a possible reason for the global prevalence of drug-resistant TB is simply migration or travel of infected individuals from developing countries.

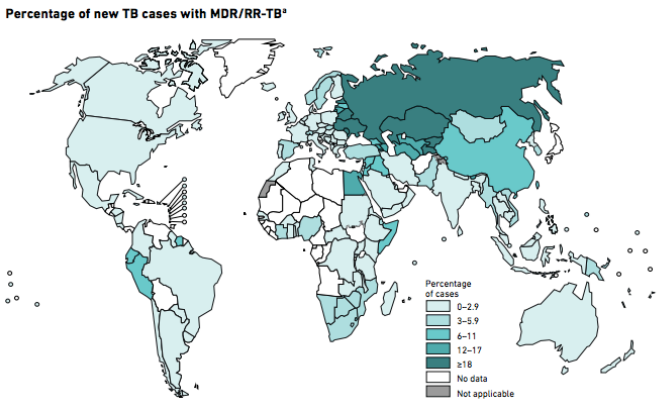

Figure 6: Global MDR-TB incidence. Reprinted from Global Tuberculosis Report 2018 (p. 51), by the World Health Organization, 2018 [15].

Modern treatment of TB is becoming increasingly difficult due to the high occurrence of drug resistant TB strains. The WHO recognizes 5 types of drug-resistant TB: mono-resistance, or resistance to only one first-line anti-TB drug; poly-resistance, or resistance to more than one first-line anti-TB drug (not including INH and RIF); multidrug resistance (MDR-TB), or strains resistant to both INH and RIF; extensive drug-resistance (XDR-TB), or strains resistant to any fluoroquinolone, and at least one of three second-line injectable drugs (capreomycin, kanamycin and amikacin), in addition to multidrug resistance; and finally, RIF resistance (RR-TB), or strains found to be resistant to RIF using phenotypic or genotypic methods of detection, with or without resistance to other anti-TB drugs [35]. Some studies have begun to use the term "totally-drug-resistant-tuberculosis" to describe strains which are invulnerable to all known TB therapies, however, the WHO does not recognize this classification. Koch and colleagues (2018) note that it is especially important to consider these levels of drug-

resistance when formulating a treatment plan. Errors in treatment or using a standard "blanket" treatment plan for each case is only contributing to the drug-resistant TB epidemic [41].

Inadequate treatment is not the only cause of drug-resistant TB. A variety of spontaneous mutations have been observed in drug-resistant TB strains that render current treatments ineffective. Each type of treatment works by targeting distinct TB gene sequences in order to execute its specific method of attack. However, TB is prone to single-nucleotide polymorphisms (SNPs) which lead to genetic variation. When these SNPs happen along a gene used in drug interactions, resistance is possible [40]. For example, section VI listed RIF as one of the primary drugs used to combat TB, but strains resistant to this primary defense are common (RR-TB). These strains are the result of a mutation on the *rpoB* gene that codes for the β-subunit of RNA polymerase that RIF targets. This mutation leads to conformational changes to the subunit which decreases drug affinity and therefore decreases drug effectiveness [37]. It is also important to note that most strains that are RIF-resistant are also resistant to other drugs, usually INH. INH resistance can be caused by a variety of mutations, most notably in the gene responsible for its activation, *katG*. Mutations seen in this gene cause a deficient form of isoniazid to be activated, hindering its antimicrobial abilities [37].

Heteroresistance is also possible within an infected individual, further complicating treatment. Heteroresistance occurs when both resistant and non-resistant strains are present within a patient. This can happen by co-infection as well as by TB mutating during replication within the body [41]. This phenomenon further stresses the importance of resistant strain detection as well the need for combined therapy.

The need to find additional treatment methods to combat drug-resistant TB strains is becoming a race against the clock. In July of 2017, Sharma and colleagues published an article in *The Lancet* detailing incident projections for 2040. Doctors at the CDC used a series of

mathematical models to conclude that by 2040, person-to-person spread of drug-resistant TB strains will lead to one third of TB cases in Russia being drug-resistant and about 12% of Indian cases, with nearly 9% of these cases being XDR [34].

CHAPTER IX

THE FUTURE OF *MYCOBACTERIUM TUBERCULOSIS* TREATMENT

The 2018 WHO Global Tuberculosis Report lists 3 distinct pillars of action that are necessary in the fight to end TB: integrated, patient centered care and prevention; bold policies and supportive systems; and intensified research and innovation [15]. The WHO's main goal is to develop universal protocols that will provide treatment access to anyone who is, or could become, infected. This report notes that it is important to distinguish drug-resistant strains during the diagnosis stage and to develop new vaccines and less-intense treatment regimens. The "End TB Strategy" created by the WHO aims to end the global TB epidemic by 2030 [15]. This section will expand on the aspects of this strategy, specifically focusing on new advances in TB treatment.

a. Current Research in new TB Diagnostic Techniques

Section V touched on the currently accepted diagnostic techniques used to detect TB infections and the limitations of each technique. Efficient diagnostic systems are a key first step in combatting the epidemic. New methods must not only take into account several external factors in order to determine a definitive diagnosis, but also factor in possible drug-resistance of the strain. The WHO reports that while there are many diagnostic techniques being researched at this time that could potentially meet these requirements, not much significant progress has been made recently [15]. One major downfall of many of the methods is affordability. The areas of the

world that are most in need of new diagnostic techniques are also some of the poorest. Most techniques are best executed in a laboratory setting that is not feasible in real, third-world situations [42].

One major contender is an updated version of the Xpert MTB/RIF assay. The Xpert MTB/RIF Ultra has been shown to have increased sensitivity that allows for more accurate detection in populations of low bacilli numbers, such as those with HIV. The WHO plans to refine this technique and it could be used as early as 2019 [15]. Research groups are also working on developing assay cartridges that are able to detect resistance to other drugs such as INH and fluoroquinolones. Additionally, research is being done to refine one of the more basic diagnostic techniques, the chest X-ray. This research aims to eliminate the reader bias and chance of human error by using a computer program to detect hallmarks of TB in X-ray images. The system is currently being calibrated using historic images of the disease and the WHO will evaluate the system further this coming year [15].

b. New Vaccines on the Pipeline

As mentioned previously, the only current vaccine used against TB is the BCG vaccine. This treatment has proven to be invaluable in the treatment of young children, less than 5 years of age, however its efficacy varies greatly throughout the older population. Since transmissibility of the disease is much more likely in the adolescent and adult age range, it is important to find a vaccine with a greater success rate. Additionally, with the growing emergence of drug-resistant strains, it is also important that any new treatments be at least equally effective against both non-resistant and resistant strains [42]. It has been proposed that a key component of new vaccines includes accounting for the virulence factors TB utilizes during infection. Specifically, since TB is now known to leave the phagosome, new vaccines should be effective in the cytosol, unlike BCG [47].

The 2018 WHO Global Tuberculosis Report lists a remarkable 12 vaccines that are currently in various stages of clinical trials. The main goals of a successful vaccine include those that are able to not only lower the risk of initial infection, but also limit the activation of the disease in those who are already latently infected. It is important to note that following laboratory testing, treatments go through 3 phases of clinical trials in which they are administered to humans. Phase I includes giving the trial vaccine to a small test group of healthy individuals to test the safety of the drug. Phase II includes expanding the test group and administering the vaccine to a population of people who are similar to the target population in order to test the vaccine's ability to produce the desired effect. And eventually, phase III tests both safety and efficacy of the vaccine on a large scale in order to determine licensure [53].

Of these 12 vaccines undergoing development, the majority are still in phase I or early phase II, however there are two that are in phase III of development. The Vaccae vaccine has completed phase III of testing and its results are currently being analyzed. The study of this vaccine has lasted a decade and included 10,000 people- illustrating just how extensive drug research is [15]. Vaccae includes a heat-inactivated form of the nonpathogenic mycobacterial species of the same name. This vaccine is believed to boost the host immune system against mycobacterium, specifically, it boosts lymphocyte activity to kill infected macrophages. The vaccine is being tested in combination with drug therapy in latently infected individuals and those infected with MDR-TB strains [54]. While this vaccine has been approved in China, further analysis is necessary for global approval [15].

VPM1002 is the second vaccine in phase III of development. It is a live recombinant BCG vaccine in which the urease C gene is replaced by a lysteriolysin O (LLO) gene from *Listeria monocytogenes.* The urease C gene is believed to be a key player in TB's ability to neutralize the phagosome and hinder its maturation, allowing for the survival of TB within the macrophage. However, by replacing this gene with LLO the phagosome is able to acidify and

progress into phagosome-lysosome fusion. LLO also creates a pore, allowing antigens and bacterial DNA to escape into the cytosol, activating immune responses outside of the macrophage as well. The majority of testing of this vaccine has been done on neonates, however it is also being tested as a vaccine used to prevent reoccurrence in previously infected adults. In 2017 a phase III study was implemented on 2,000 adults who had previously undergone treatment for TB [55].

c. The Future of Chemotherapy to be Used Against TB

A variety of drugs are also being tested to aid or even replace current chemotherapy. One of the more promising contenders is the drug delamanid. It is currently in phase III of testing and the WHO has conditionally approved it for combination therapy in individuals with suspected MDR-TB [15]. Delamanid works by inhibiting the synthesis of mycobacterial cell wall components [57]. Research has shown that delamanid is ideal for combination therapy due to its low chance of drug-drug interaction. Interestingly, therapy including delamanid could also inhibit the development of drug resistance during treatment, specifically to fluoroquinolones [56].

Research is also looking at shorter treatment regimens. With current drug therapy lasting anywhere from 6-24 months, a lot of patients find it difficult to keep up with the strict treatment schedule. Developing a shorter treatment plan could aid in lessening the emergence of drug-resistant strains as well as prove to be a more realistic plan for those who struggle to access treatment. For example, the Nix-TB regimen combines the oral-drugs bedaquiline and linezolid with a new drug called pretomanid to treat XDR-TB infected individuals in just 6 months [15]. It is currently in phase III, with participants in the study being bumped to a 9-month plan if they did not show improvement by month 4 [56].

d. Monitoring TB Throughout Treatment

This need for close monitoring of treatment progression is why it is also important to develop detection techniques that can provide an assessment of TB levels within the host. A university in Scotland is working on developing The Molecular Bacterial Load Assay (MBLA) to quantify the TB bacilli load throughout treatment using RT-qPCR. To date, MBLA is still in the lab-based testing phase [15].

Unfortunately, there is still a substantial amount of research needed to fully understand TB and create effective testing and treatments against it. This requires a great amount of funding. The WHO reports that current calculations fall short of the estimated $2 billion necessary for adequate research [15]. With TB being most prevalent in the poorest communities, private pharmaceutical companies are hesitant to invest in research [42]. In an effort to accelerate current responses the United Nations organized a high-level meeting in September of this year. This meeting was the first of its kind, and while details discussed in the meeting have not been released, it is clear that funding was the main topic of discussion. Leaders discussed the importance of funding research and developing affordable diagnostic and treatment plans [23].

CHAPTER X

CONCLUSION

M. tuberculosis infection is one of the leading causes of death worldwide. Despite this bacteria being a source of infection in the human population for centuries, today's technological and medical advancements have brought us no closer to its eradication. This is largely due to its ability to mutate, rendering certain strains resistant to our current chemotherapy options. While there are many new diagnostic and treatment techniques currently in varying stages of testing, lack of funding has been a major drawback to TB research. Going forward, there is still much to learn about TB, and its drug-resistant strains, before there is any hope of its eradication.

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Master of Science

Thesis: THE CHARACTERISTICS AND EVOLUTION OF *MYCOBACTERIUM TUBERCULOSIS* DRUG-RESISTANCE AND CURRENT RESEARCH TOWARDS ENDING THE GLOBAL TUBERCULOSIS EPIDEMIC

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