THE ROLE OF EVOLUTION IN THE PATHOGENESIS AND VIRULENCE OF *MYCOBACTERIUM TUBERCULOSIS* AND THE IMPACT ON TUBERCULOSIS CONTROL

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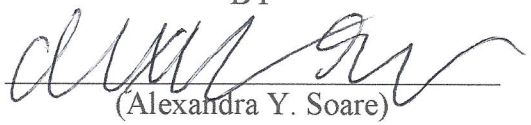

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AN ABSTRACT

Despite the development of a vaccine and several antibiotics, tuberculosis continues to be one of the leading causes in mortality in the world. The pathogenesis of the main causative agent, *Mycobacterium tuberculosis*, has puzzled many researchers for over a century. Research on the origin of *M. tuberculosis* can provide new knowledge on how the organism has evolved into the dangerous pathogen it is today. This thesis reviews recent literature on how the evolution of tuberculosis has contributed to the genetic diversity and positive control of select genes in the tuberculosis genome and how this can impact future development of therapeutic agents.

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Introduction

Tuberculosis is one of the leading causes of disease burden and death in the world, causing an estimated 1.7 million deaths each year (Lawn and Zumla 2011). After the development of the Bacille Calmette Guerin (BCG) vaccine in 1906 and the rise of antibiotics in the 1940s, tuberculosis was believed to be a disease of the past. But with the emergence of the HIV/AIDS pandemic in the 1980s, tuberculosis came back with a vengeance. Resistance to primary-line drugs, isoniazid and rifampicin, lead to development of multi-drug resistant tuberculosis (MDR-TB). In 1993, the World Health Organization (WHO) declared a global health emergency due to rapidly rising infection rates (Gagneux 2012). But even with new public health initiatives, tuberculosis has persisted and continues to acquire resistance to almost every antibiotic available (Calligaro and Dheda 2013). During this time, many studies began to show that BCD-vaccinated provided limited protection, with waning immunity and varied efficacy, leaving many vaccinated populations susceptible with few treatments available to combat drug-resistance (Colditz et al., 1994).

Despite increased awareness and funding, there is still much to be learned about *Mycobacterium tuberculosis,* the main causative agent of tuberculosis. Only within the past decade have scientists begun to examine and appreciate the origin of *M. tuberculosis* and the genetic variety within the strains. Understanding of the evolutionary tract of *M. tuberculosis* can give insight the biology of tuberculosis and how to ultimately eliminate it. This thesis study will review published literature on how *M. tuberculosis* has evolved over time and remained a serious threat to global health. First, I will start by characterizing *M. tuberculosis* and its role in human history. Then I will discuss the

bacterial origin and evolution over the time. Finally, this study will examine how this information impacts the development of therapeutic agents targeting tuberculosis.

Causative Agent of Tuberculosis

M. tuberculosis is a rod-shaped aerobic intracellular bacterium responsible for 80% of the tuberculosis cases around the world. It is not classified as Gram-positive or Gram-negative due to its unique waxy cell wall composition, which consists mainly of mycolic acids that make it impervious to Gram staining and many antibiotics (Smith 2003). Transmission of *M. tuberculosis* occurs through the inhalation of aerosol droplets containing the bacilli. Once inside a new host, *M. tuberculosis* migrates to the lung where they are taken up by alveolar macrophages. In most cases, uptake by macrophages would result in phagosomal maturation, with the macrophage delivering the pathogen to the lysosome where it is destroyed. However, in the case of tuberculosis infections, the macrophage is unable to mature due to bacterial interference with the phagosome endolytic pathway and overall maturation process (Jayachandan et al., 2013). Tuberculosis bacteria employ a number of mechanisms to prevent fusion with the lysosome, such as blocking the recruitment of the vesicular proton ATPase pump which prevents the macrophage from acidifying (Sturgill-Koszychki et al., 1994) or disrupting the phosphoinositide composition of the phagosome membrane to prevent acquisition of lysosomal constituents (Vergne et al., 2004).

Although the macrophage is unable to kill the bacteria, it does create an inflammatory response by recruiting cells involved in innate immune responses (i.e. natural killer cells, dendritic cells, T-cells, monocyte-derived macrophages, etc.) to the

lungs through secretion of chemokines. Once they arrive to the site of infection, the cells surround the infected macrophage and begin to form the granuloma, which create extreme environmental conditions (low oxygen, depletion of nutrients, acidic pH, etc.) to kill the pathogen (Ehlers and Schaaible 2013; Lugo-Villarino et al., 2013). However, *M. tuberculosis* has many virulence factors that allow it to survive in the harsh conditions of the granuloma. The DosR regulon has been identified as a set of genes within mycobacterium that allow for it to survive in anaerobic conditions (Bartek et al., Ehlers and Schaible 2013) and many studies have characterized the ability of mycobacteria to survive in highly acidic *in vitro* and *in vivo* conditions (Vandal et al., 2009).

Since it is a highly aerobic pathogen, infection with *M. tuberculosis* results in pulmonary tuberculosis in 80-85% of cases, which presents with fever, bloody sputum, night sweats, chills, fatigue, and scarring of the lungs (Murray et al., 2013). The remaining 15-20% of cases results in the dissemination of *M. tuberculosis* to other organs. This manifestation is often seen in immunocompromised individuals when the granulomas are unable to encase the bacteria, allowing *M. tuberculosis* to escape the lungs and spread to other organs like the spine (Pott's disease), lymph nodes ("King's evil") or, as is the case of military tuberculosis, multiple organs at once (Sharma and Mohan 2012). Meningeal tuberculosis is an extra-pulmonary dissemination of tuberculosis, where the bacteria manage to escape the lung and enter the blood stream to spread to the meninges membrane that surrounds the central nervous system. Although it is not as common as pulmonary tuberculosis, meningeal tuberculosis is still very widespread and is often referred to as the most deadly dissemination of tuberculosis infection. Meningeal tuberculosis causes intracerebral inflammation that results in

clinical symptoms, including headache, fever, vomiting, convulsions, coma, and in many cases, death (Marais, et al., 2010).

There are a number of risk factors associated with *M.* tuberculosis infection. Areas that are endemic to *M. tuberculosis* are often endemic to other pathogens that could impact the host resistance. Human immunodeficiency virus (HIV) has a strong correlation with *M. tuberculosis* infection and increases the chance of reactivating latent disease (Pawlowski et al., 2012). Certain parasites have also been shown to have an effect on *M. tuberculosis* infection (Li and Zhaou 2013). Helminths (worms) infections induce Th2 responses, which impair host resistance to *M. tuberculosis* (Wasiulla et al, 2012; Rook 2007). Additionally, innate immunity to *Plasmodium berghei* (causative agent of malaria) can also induce a chronic tuberculosis infection (Mueller et al., 2012). Interestingly, infection with *Helicobacter pylori* is associated with protection against *M. tuberculosis* (Perry et al., 2010).

Areas that suffer from *M. tuberculosis* and other pathogenic infection also suffer from large amounts of malnutrition within the population. Low levels of Vitamin A and Vitamin D have been associated with higher risk to tuberculosis infections (Fox and Manzies 2013). Recently, Vitamin C has been shown to kill drug-resistant strains of *M. tuberculosis* when observed *in vitro* (Vilcheze et al., 2013). Other identified risk factors to *M. tuberculosis* include diabetes, alcoholism, chronic kidney disease and aging (Fox and Menzies 2013).

M. tuberculosis is part of the *Mycobacterium tuberculosis* complex (MTBC), a group of genetically related slow-growing mycobacteria responsible for causing tuberculosis in humans and animals. MTBC excludes mycobacteria that are pathogenic in

humans but do not cause tuberculosis, such as *M. leprae* or the *M. avium* complex (Murray et al., 2013). Pathogens included in the MTBC include animal-adapted strains such as *M. bovis* (cows), *M. microti* (voles), *M. pinnipedii* (seals), and *M. caprae* (goat). However, the animal-adapted strains of MTBC are opportunistic pathogens and rarely cause tuberculosis in humans (Smith et al., 2005). Areas without routine milk pasteurization is not routine see occasional cases of tuberculosis caused by *M. bovis*. However, the two main human-adapted strains of MTBC are *M. tuberculosis* and *Mycobacterium africanum* (Burgos 2013).

M. africanum is a phylogenetic variation of *M. tuberculosis* and is geographically exclusive to West Africa (Hershberg et al., 2008). First discovered in 1968 in Senegal, *M. africanum* was noted for its biochemical characteristics that distinguished it from *M. tuberculosis* (de Jong et al., 2010). Two subtypes of *M. africanum* have been identified by geographic and molecular characteristics: *M. africanum* type I, West African 1 (MAF1) is mainly found around the Gulf of Guinea while *M. africanum* type II, West African 2 (MAF2) is found in the far west of Africa. The genetic differences, as well as epidemiological occurrences, of *M. africanum* are extensively reviewed in de Jong et al., 2010. Although *M. africanum* is responsible for about 50% of tuberculosis cases in West Africa, there has been considerably less research done on it. For the purposes of this paper, MTBC will be used to describe *M. tuberculosis* and *M. africanum*.

History of Tuberculosis

Robert Koch was the first person to identify *M. tuberculosis* in 1882 when he managed to visualize *M. tuberculosis* in Bismarck Brown and then successfully infect

rabbits with *M. tuberculosis* colonies he grew from cattle-blood serum solid media. He presented his results to the Physiological Society of Berlin on March 24, which thereafter became celebrated as World Tuberculosis Day (Sakula 1983). Koch's findings came on the tail of experiments done by Jean-Antoine Villemin. In 1865, Villemin proved that an infectious agent caused tuberculosis when he inoculated rabbits with tissue from a cadaver that died from tuberculosis, thereby disproving previous theories that tuberculosis had a supernatural or hereditary cause (Smith 2013).

Koch's and Willemin's studies came after a large tuberculosis epidemic in Europe during the first half of the nineteenth century. The Industrial Revolution had caused a continental urbanization movement that had led to drastically increased population-density in many cities. It is estimated that one in every four Europeans died from tuberculosis during this time. Before the identification of *M. tuberculosis*, tuberculosis was referred to as the "White Plague" or "consumption", due to the dramatic weight loss often seen in infected individuals (Smith 2003).

Figure 1: Paintings depicting tuberculosis in the 19th Century. "La Miseria" by Cristobal Rojas (top); "The Doctor" by Sir Luke Fildes (bottom)

Tuberculosis had already been plaguing the continent for several centuries, but by the 19th century, it had become so widespread that it became integrated into European culture, mainly through literature and art. The main characters in the operas *La Traviata* (1853) and *La Boehme* (1896) tragically die from tuberculosis in their lover's arms and

the paintings *La Miseria* by Cristobal Rojas (1886) and *The Doctor* by Sir Luke Fildes (1981) depict the subjects slowly dying from the disease (Figure 1). In *Les Misérables* (1862), Victor Hugo depicts the character of Fantine slowly succumbing to tuberculosis after she loses her jobs and falls into poverty (translation by Charles Wilbour):

> "Physical suffering had completed the work of moral suffering. This creature of twenty-five years had a wrinkled forehead, flabby cheeks, pinched nostrils, shriveled gums, a leaden complexion, a bony neck, protruding collar-bones, skinny limbs, an earthly skin, and her fair hair was mixed with grey."
> "Her conditions seemed to become worse from week to week. The handful of snow applied to the naked skin between shoulder blades, had caused as sudden check perspiration, in consequence of which the disease, which had been forming for some years, at last attacked her violently."
> "She had a strange brilliancy in her eyes and a constant pain in her shoulder near the top of her left shoulder blade. She coughed a great deal."

Documentation of tuberculosis cases has been written long before the 19th century. Hippocrates (400 B.C.) described the symptoms of tuberculosis, which was called phthisis, in *Of the Epidemics* as "the most considerable of diseases which then prevailed, and the only one proved fatal to many persons" which caused "fevers accompanied with rigors" and "sputa small, dense, concocted but brought up rarely and with difficulty" (translation by Frances Adams). The oldest documentations of tuberculosis-like-symptoms are found in the Rigveda scripts from ancient India (1500 B.C.) which details a charm used to drive a disease called "yaksma" away.

The development of new genomic techniques has allowed us to design PCR primers that recognize conserved polymorphisms and target sequences, such as insertion sequence IS*6110*, in the MTBC to confirm many of these cases in ancient DNA (aDNA) (Donoghue 2004). A study of 263 corpses found in a Hungarian sealed crypt used for middle-class families between 1731-1859 showed that 55% of the individuals were

positive for tuberculosis infection (Fletcher et al., 2008). However, skeletons found in Neolithic sites in Italy, Denmark, and the Middle-East dating back to 4,000 years ago show that tuberculosis has been around longer than historical documents have implied. In 2003, tissue samples from 85 ancient Egyptian mummies buried during the Middle Kingdom (2050-1650 B.C.) were analyzed for signs of tuberculosis. Of those 85 mummies, 25 had a positive signal for the amplification of the IS*6110* sequence. Further analysis by spoligotyping showed four of the skeletons were infected with *M. africanum* (Zink et al, 2003).

Some of these findings have been more open to interpretation than others. One study describes a 500,000-year-old fossil of *Homo erectus* with lesions characteristic of tuberculosis (Roberts et al., 2009). This study has been extremely controversial, as it suggests that *M. tuberculosis* is older than modern humans (Wilbur et al., 2009). Although Roberts's findings were ultimately inconclusive, they beg the question: how old is *M. tuberculosis* and where did it come from? How long has it been plaguing the earth and how did it become one of the most persistent diseases?

Origin and Evolution of Tuberculosis

The origin and age of a pathogen can give important insight into its pathogenesis and how to ultimately control it. Infectious diseases can be broadly classified into two categories: occurring before or after the Neolithic Demographic Transition (NDT). The NDT occurred almost 11,000 years ago when the development of agriculture lead from the switch of hunter-gatherer migratory patterns to human settlement and domestication of animals (Wolfe et al., 2007).

Post-NDT diseases are usually referred to as “crowd diseases”, due to their high virulence in populations of high numbers and density. To maximize transmission, these diseases usually manifest as an acute infection, which is easily passed to surrounding individuals. The host is either left with protective immunity or dies from the infection. The large and dense crowds needed to sustain the transmission of these pathogens did not exist until the NDT, when the development of agricultu re lead to rapid increases in population density. Therefore, the pathogens for most crowd diseases, such as smallpox, pertussis or measles, are believed to be no older than 11,000 years old (Wolfe et al., 2007; Comas et al., 2013).

In contrast, pre-NDT diseases are adapted to the low host population densities that resulted from nomadic patterns seen in small hunter-gatherer groups. These diseases, which include malaria, yellow fever, and Chagas’ disease, often result in chronic progression, which leaves the host contagious for months or years rather than mere weeks. While post-NDT diseases may present symptoms days after infection, pre-NDT diseases progress more slowly and do not present symptoms for several weeks. Additionally, the host is left with incomplete or non-last immunity, making them susceptible to future infections to the same pathogen (Wolfe et al., 2007).

Since *M. tuberculosis* has a unique dichotomy of characteristics found in both pre-NDT and post-NDT diseases, there has been a lot of deliberation to its origination. Its transmission by aerosol droplets allows tuberculosis to thrive in high-density populations. In 2010, it was projected that more than a third of worldwide tuberculosis cases would occur in India and China, the two most populous countries in the world (Dye and Williams 2010). However, *M. tuberculosis* is able to establish a latent infection that

won't progress into a disease until months later, which is often characteristic of pre-NDT diseases (Comas et al., 2013). Originally, it was believed that *M. tuberculosis* was a zoonotic crowd disease that derived from *M. bovis* after the domestication of cattle during the NDT (Donoghue et al., 2004; Smith et al., 2009). However, comparative genomic sequencing of *M. bovis* and *M. tuberculosis* has shown a smaller chromosome in *M. bovis* with clear genetic markers where genes were deleted from the *M. tuberculosis* genome. Therefore, it is far more likely that *M. bovis* is derived from *M. tuberculosis* rather than the other way around (Smith et al., 2009; Mostowy and Behr 2005; Gagneux 2012).

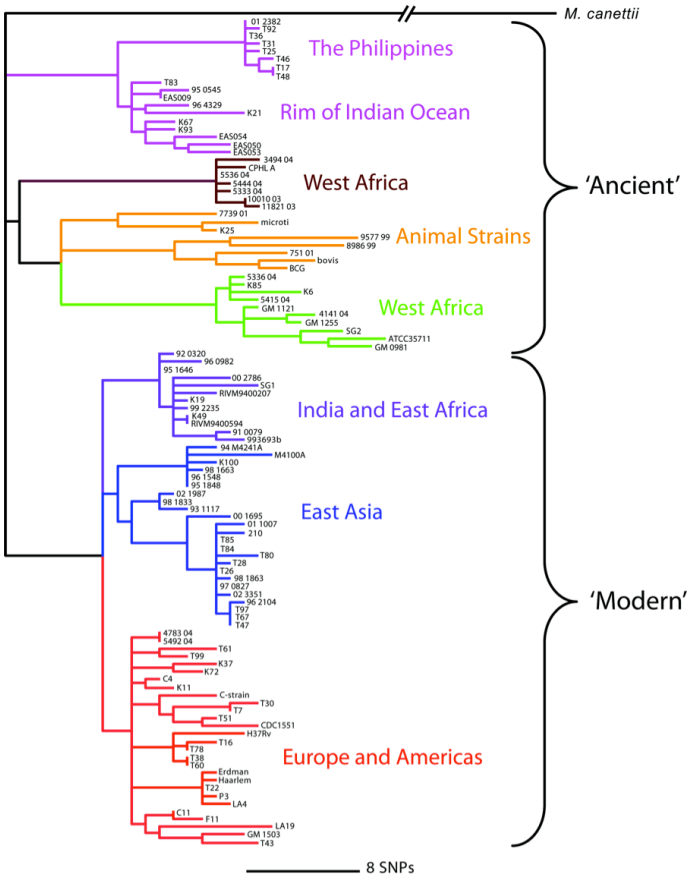

Figure 2: Maximum Parsimony Phylogeny of MTBC Using 89 Concatenated Gene Sequences in 108 Strains. Adapted from Hershman et al., 2008

Even after the *M. bovis* theory was disproven, the role of populations dynamics in tuberculosis infection lead many to believe that *M. tuberculosis* arose 10,000 years ago at the beginning of the NDT. However, a phylogenetic analysis of 108 MTBC strains introduced the "Out of Africa" theory where *M. tuberculosis* originated in Africa and was introduced to the rest of the world when the first anatomically modern humans migrated

out of Africa ~50,000 years ago (Hershberg et al., 2008). The migrations of modern humans out of Africa lead to the development of six distinct *M. tuberculosis* lineages (Table 1; Figure 2), which is extensively reviewed in Hershberg et al., 2008.

	Origin (Hershberg et al., 2006)	**Geographical association (Gagneux and Small 2007)**	**SNP marker (Gagneux and Small 2007)**
Indo-Oceanic (Lineage 1)	Ancient	East Africa, southeast Asia, south India	*OxyR* C371
East Asia (Lineage 2)	Modern	East Asia, Russia, South Africa	Rv3815c G81A
Central Asia (Lineage 3)	Modern	East Africa, north India, Pakistan	*RpoB* T2646G
Euro-American (Lineage 4)	Modern	Americas, Europe, north Africa, Middle-East	*KatG* T1388G *RpoB* C32431
West African 1 (Lineage 5)	Ancient	Ghana, Benin, Nigeria, Cameroon	N/A
West African 2 (Lineage 6)	Ancient	Senegal, Guinea-Bissau, The Gambia	N/A

Table 1: Lineages of Human Adapted MTBC

To summarize, three separate lineages became seeded with populations that migrated to Western Europe, Northern India, and East Asia (Figure 3b). Within the last millennia, civilizations in these areas experienced massive population growth and expansion due to increased travel and trade. The expansions of these three civilizations lead to the dispersion of three "modern" lineages of *M. tuberculosis* around the world (Figure 3c). The other three lineages remained more geographically isolated, leading to their classification as "ancient" lineages. The ancient lineages of *M. tuberculosis* include two subtypes of *M. africanum* that remained almost exclusive to West Africa and the Indo-Oceanic lineage, which corresponds with the earliest spread of humans out of Africa around 50,000 years ago. The populations associated with this lineage were believed to have settled in the Philippines where they remained isolated to the Indian Ocean (Hershberg et al., 2008).

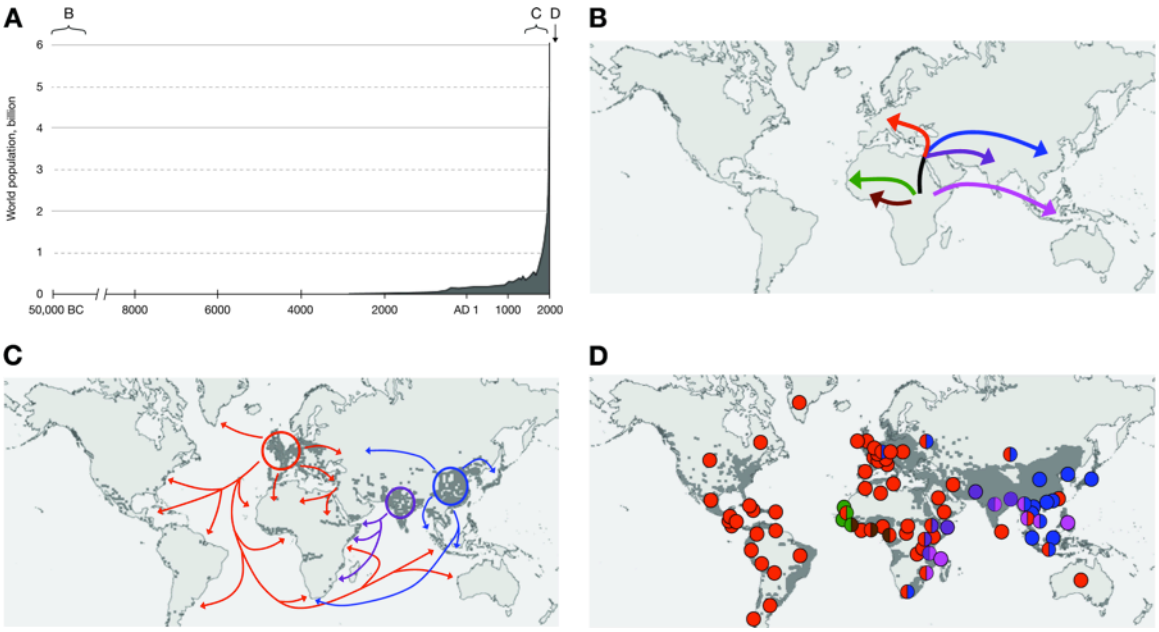

Figure 3: "Out Of-And-Back-To-Africa" Scenario for the Evolutionary History of Human-Adapted MTBC. Adapted from Hershberg et al., 2008 (A) Global human population size during the last 50,000 years. The letters above the graph indicate the time periods corresponding to (B), (C), and (D), respectively (B) Hypothesized migration out of Africa of ancient lineages of MTBC. (C) Recent increase of global human population. Each dark grey dot corresponds to 1 million people. These three geographic regions are each associated with one of the three modern MTBC lineages (red, purple, and blue). (D) The human population has reached 6 billion. The distribution of the six main human-adapted MTBC lineages we observe today is shown.

Other human migratory patterns can explain the presence of different lineages around the world. The presence of the Euro-American lineage in regions of Middle East, Africa and Asia correspond with European colonization in the 16th century. Additionally, the presence of the East-Asian lineage in South Africa can be traced to the import of slaves from Southeast Asia by Dutch colonists in the 17th and 18th century as well as the influx of almost 50,000 Chinese workers at the beginning of the 20th century to work in South African gold mines (Mokrousov et al., 2005).

The "Out of Africa" theory was more or less proven when an international team analyzed 34,167 single nucleotide polymorphic sites (SNPs) across 259 MTBC strains to

give more insight into the genetic diversity and evolutionary history of *M. tuberculosis*. Their findings not only supported Hershberg et al's theory but suggested that MTBC emerged about 70,000 years ago, implying that *M. tuberculosis* has been co-evolving with modern humans and has been readily able to adapt to numerous demographic changes (Comas et al., 2013).

Genetic Diversity in *M. tuberculosis* Strains

In addition to offering insight into the age and role of population dynamics in the spread of *M. tuberculosis*, Hershberg et al. provided convincing proof that *M. tuberculosis* was more genetically diverse than previously implied. In the past, it was believed that all strains of *M. tuberculosis*, were genetically similar and the few differences that distinguished the strains from each other had no effect on disease dissemination (Sreevastan et al., 1997; Musser et al., 2000). However, in Hershberg's study, the human-adapted strains of MTBC (*M. tuberculosis* and *M. africanum*) were shown to be as genetically diverse as the animal-adapted strains, even though the animal-adapted strains were adapted to four separate ecotypes (Hersheberg et al., 2008). Over the years, more studies have documented distinct genetic differences in different strains of *M. tuberculosis* and on occasion, *M. africanum* (Coscolla and Gagneux 2010).

One of the most notable variances is the difference in virulence between ancient and modern strains. Human monocyte-derived macrophages (MDMs) infected with 28 different *M. tuberculosis* strains produced distinctly different inflammatory responses to ancient and modern linages. Modern strains produced significantly less pro-inflammatory cytokines and chemokines, such as interleukin-6 (IL-6) and interferon-α (IFN-α),

compared to ancient strains. Furthermore, these results were reproducible across eight different human donors (Portevin et al., 2011). A previous study in Gambia showed similar results when strains of modern origin had a significantly higher chance of rapidly progressing to active disease than those of ancient lineages (de Jong et al., 2008). The population expansion experienced by the modern strains through travel and expansion allowed them to adapt to higher virulence and shorter latency periods (Burgos 2013). The lower virulence of *M. africanum* in comparison to *M. tuberculosis* can explain why there is no *M. africanum* in the Americas despite the large number of West Africans that migrated to the Americas through slave trade. It is possible that the Euro-American lineage of *M. tuberculosis* outcompeted *M. africanum*, which prevented *M. africanum* from being established in the United States (de Jong et al., 2010).

Several studies have documented the variety in clinical presentation and immune response of animal models infected with *M. tuberculosis*. Strains isolated from British patients with pulmonary tuberculosis were more virulent when injected into guinea pigs than strains isolated from Indian patients (Mitchison et al., 1960). The same results were shown when the guinea pigs were infected through the respiratory route by aerosol (Williams et al., 2005). Rabbits infected with East Asian strains suffered from more severe meningitis and higher bacterial load in their system than rabbits infected with Euro-American strains (Tsenova et al., 2005). A review published by Coscolla and Gagneux reviews 67 studies that report phenotypic differences seen *in vitro* or in animal models between different MTBC strains (Coscolla and Gagneux 2010).

Coscolla and Gagneux also identified 33 studies that have described clinical differences seen in different strains of MTBC. In a study testing 187 Vietnamese adults

with meningeal tuberculosis and 237 Vietnamese adults with pulmonary tuberculosis, the Euro-American linage was shown to have caused significantly less cases of meningeal tuberculosis than the Indo-Oceanic or East-Asia lineages as well as lower mortality from meningeal tuberculosis (Caws et al., 2008). Another study in Tuscany, Italy found a significant association with extrapulmonary tuberculosis and infection from the Central Asia lineage and *M. bovis* (Lary et al., 2009).

Due to its virulence, most studies on tuberculosis pathogenesis have been centered on the East-Asia lineage. Out of the 33 studies reviewed by Coscolla and Gagneux, 22 of the studies focused on the East-Asia lineage (Coscolla and Gagneux 2010). The W genotype, a particularly virulent strain of the East-Asia lineage, is responsible for the outbreaks among HIV-infected individuals in New York City during the 1990s (Bifani et al., 1999). The persistence of the East Asia can be traced back to a phenolic glycolipid (PGL) produced by *pks 15/1* gene. PGL is believed to inhibit innate immunity by increasing production of macrophage deactivating cytokines, such as interleukin-11 (IL-11) and interleukin-13 (IL-13), while suppressing proinflammatory cytokines such as tumor necrosis factor- α (TNF-α) and the p40 subunit of interleukin 12 (IL-12p40). The reduced expression of TNF-α is important for the formation of the granuloma, which keeps the *M. tuberculosis* at bay and prevents the infection from becoming systemic (Reed et al., 2004; Caws et al., 2008).). The ability of the East Asia genotype to inhibit granuloma formation explains its association with extrapulmonary tuberculosis and meningeal tuberculosis (Kong et al., 2007; Thwaites et al, 2008). The virulence of the Beijing genotype family, a subgroup within the East Asia lineage, are extensively reviewed in Parwati et al., 2010.

Despite causing 50% of tuberculosis in West Africa, less research has been done on the immunogenic variations of *M. africanum*. The genetic distance between MAF1 and MAF2 suggest that there are phenotypic differences between the two lineages (Figure 2). However, there have been very few studies that have examined genetic differences between strains of *M. africanum*. However, phenotypic differences between *M. tuberculosis* have been investigated, particularly with MAF2. One study in Gambia showed that the prevalence of HIV co-infection is higher for MAF2 than *M. tuberculosis* or MAF1. However, in Ghana, there was no difference in HIV co-infection or disease severity between *M. tuberculosis* and *M. africanum* (de Jong et al., 2010). As mentioned, these studies are preliminary and lack the robust analysis needed to properly characterize the immunogenic profile of *M. africanum*.

In order to decipher the cause behind the differences in innate immune responses, one study focused on the contents of the cell wall to see if the lipid content was responsible for the different immune responses. Stimulation of lipids isolated from the cell wall of different *M. tuberculosis* lineages with murine bone-marrow-derived macrophages showed significant differences in cytokine expression. Specifically, East Asian and Indo-Oceanic strains induced higher concentrations of TNF-α than the Euro-American strains. When the lipids were fractioned and compared through thin layer chromatography, distinct differences in lipid-profiles were observed. Euro-American strains were characterized by large amounts of phthiocerol dimycocerosate (DIM A) and small amounts of phthiodiolone dimycococerosate (DIM B) and Indo-Oceanic strains were characterized by a novel lipid that has been named “Lipid Y” (Krishnan et al., 2011). Along with PGL, East Asian strains were characterized by phthiotriol

dimycocerosate, an important virulence factor in preventing phagocytosis and protecting *M. tuberculosis* from reactive nitrogen intermediates in macrophages (Rousseau et al., 2004; Astarie-Dequeker et al., 2009). Evading phagocytosis allows the bacteria to escape the lung and disseminate into the blood stream, which can explain the association between the East Asia lineage and meningeal tuberculosis (Caws et al., 2008).

Role of Evolution in the Granuloma Formation

It has been established that there is a large amount of genetic diversity within the MTBC with large implications for phenotypic differences, but has not been well studied. However, host genetic factors, such as low β-defensins production or production of $Foxp3^{+}CD4^{+}$ cells, have been identified as having an impact on disease outcome as well (Hernandex-Pando et al., 2009; Paula et al., 2011). One cohort study in South Africa performed whole-blood microarray gene expression analysis on tuberculosis patients, healthy donors with a latent *M. tuberculosis* infection and non-infected donors to determine gene expressions associated with susceptibility and resistance in tuberculosis infections (Martzdorf et al., 2011).

In a study comparing individuals suffering from pulmonary tuberculosis, patients with similar demographics and disease phenotype exhibited different inflammatory profiles that are linked to an individual's ethnic background (Coussens et al., 2013). Furthermore, certain genetic polymorphisms associated with those inflammatory profiles make them more susceptible to certain lineages of *M. tuberculosis* over others. Patients in Ghana with autophage gene variant *IRGM*-261T were able to confer protection against the Euro-American lineage of *M. tuberculosis* but not the *M. africanum* lineages

(Intermann et al., 2009). In the previously mentioned study that examined the association between the East-Asia lineage and meningeal tuberculosis, individuals with a certain polymorphism in the Toll-like receptor-2 (*TLR-2*) gene were more likely to have tuberculosis caused by the East-Asian lineage (Caws et al., 2008).

The influence of host and bacterial genotype on the manifestation of tuberculosis and the adaption of different lineages to different populations suggests that over the past 70,000 years, MTBC and humans have engaged in an "evolutionary arms-race" where the pathogen and host try to outwit each other through immune pressure by the host and immune evasion by the pathogen. This phenomenon is indicative of a host-pathogen coevolution, which is defined as "the process of reciprocal, adaptive genetic change in two or more species" (Woolhouse et al., 2002).

Many pathogens have undergone selection for genes encoding antigens as method to evade host immunity in this "evolutionary arms-race". To determine if co-evolution between humans and MTBC has led antigenic variation in tuberculosis infections, an international team compared the genome of 21 strains that were selected to best represent the global diversity of MTBC. The authors divided the genes into "essential genes", "nonessential genes" and "antigens". Before the study, the authors hypothesized that they would find evidence of antigenic variation in MTBC by measuring the ratio of rates of non-synonymous and synonymous changes (dN/dS), of epitope regions that interact with T-cells. If the antigenic regions were genetically diverse, there would be a high dN/dS, signifying a decrease in purifying selection across *M. tuberculosis* strains. To the surprise of the authors, the dN/dS of epitope regions was measured to be 0.5, which is lower than the dN/dS of non-essential genes (0.65) and even lower than the dN/dS than essential

genes (0.53). Furthermore, out of the 491 epitopes tested, 95% showed no amino acid change across the strains and only five epitopes had more than one variable position. When those variable antigens were excluded from the analysis, the dN/dS of the epitopes decreased down to 0.25. Contrary to the author's original hypothesis, the T-cell epitopes of MBTC are evolutionarily hyperconserved (Comas et al., 2010).

This new revelation raised several questions about the pathogenesis of MBTC, particularly in the formation of the granuloma. It was always assumed that the creation of the granuloma after *M. tuberculosis* infection was for host protection. Findings of "healed fibrotic and calcified tuberculosis granulomas in healthy individuals" suggest that without the granuloma, the pathogen would continue to replicate and cause a more severe systemic disease such as TBM (Ramakrishnan 2012). However, this study by Comas and his team suggests that bacterial recognition by macrophages and the resulting formation of the granuloma might be part of the tuberculosis pathogenesis. By allowing MBTC strains to be recognized when it infects the host, the bacteria can create cavities in the lung to increase transmission and cause tissue damage (Comas et al., 2010).

Other studies have hinted that T cell immunity may play dual role of protection and pathogenic in tuberculosis infections. HIV-positive tuberculosis patients with low CD4 T cell counts are less likely to present with cavities than HIV-positive patients with higher CD4 T cell counts (Kwan and Ernst 2011). In a zebrafish model infected with *M. marinum*, 6 kDa early secretory antigenic target (ESAT-6), one of the hyperconserved antigens in Comas' study, was shown to induce matrix metalloprotease 9 (MM9), an enzyme which recruits more macrophages to the site of infection to help with granulomas

maturation. When MM9 production was disrupted, bacterial growth and granuloma formation was stalled (Volkman et al., 2010).

Although the study disproved the authors' original hypothesis, it still offers proof of coevolution between humans and MTBC through more dualistic role of the granuloma during tuberculosis infections. Although the granuloma prevents the dissemination of the bacteria to other parts of the body, unresolved granulomas can eventually develop into lung cavities, which causes necrosis of lung tissue similar to those found in cancer cases (Hunter 2011). Additionally, the bacteria continue to grow in these cavities, making them a breeding ground of highly infectious organisms. Eventually, the center necrotic area can burst open and release high levels of bacteria into the lung and create an active infection in a previously unaffected patient (Krishman et al., 2010). Comas' study is fairly recent and more research needs to be done to further validate his findings. However, if we are to follow the coevolution trajectory between MTBC and humans, it is possible that these epitope regions became conserved after the NDT, when higher rates of population density allowed for increased transmission of MBTC. The recognition of T-cells and the formation of the granuloma may have been MBTC's method of evolving with humans after the NDT.

Implications for Product Development

Information on the genetic diversity in *M. tuberculosis* strains as well as the conservation of T cell epitopes has many implications for developing new diagnostic tools, antibiotics and vaccine for tuberculosis. Comas's study has great potential for developing a more accurate diagnostic test for tuberculosis. The slow growing nature of

M. tuberculosis makes it difficult to grow in culture and the efficacy of the tuberculin skin test has drawn criticism due to false positives from individuals who have received the BCG vaccine (Gagneux and Small 2008). The creation of a more precise test that could identify the lineage of the causative agent would significantly improve treatment protocols and decrease the spread of drug-resistant strains. Different strains of *M. tuberculosis* are intrinsically more resistant to antibiotics than others or are more prone to conserve certain mutations that lead to acquired resistance. In isoniazid-resistant *M. tuberculosis* strains, the Euro-American lineage is more likely to conserve the *katG* (*Rv1908c*) S315 mutation, while the Indo-Oceanic linage naturally selects the *inhA* (*Rv1484*) mutation (Gagneux et al., 2006). Although both mutations lead to isoniazid resistance, each gene has a different function that, when lost, leads to the resistance and thus a different "level" of resistance. The *katG* mutation corresponds with a higher level of resistance than *inhA*, which further supports the higher virulence seen in modern lineages than ancient lineages (Fenner et al., 2012). As well as its increased virulence, the Beijing subgroup has been heavily associated with increased rates of drug resistant tuberculosis (McGrath et al., 2014). Several studies have investigated the association between MBTC genotype and drug resistant tuberculosis (Warner and Mizrahi 2013; Borrell and Gagneux 2011; Smith et al., 2013).

The creation of a diagnostic tool that would be able to differentiate between different linages of MBTC as well as drug-resistant strains is daunting but foreseeable. But the most daunting task in light of the genetic diversity within MBTC is the creation of a new vaccine for a pathogen that has created many setbacks in vaccinology. This should come as no surprise as there have been extremely varied results in the efficacy of

BCG, despite its global use (Zwerling et al., 2011). A study comparing the efficacy of BCG between populations in Malawi and populations in the UK showed that IFN-γ responses to *M. tuberculosis* purified protein derivative (PPD) were ten times higher in the UK than in Malawi (Black et al., 2002). BCG has also been shown to induce less protection in the more virulent strains of *M. tuberculosis*, especially against the progression of TBM (Tsenova et al., 2007)

In light of the new results on hyperconserved antigens across the MBTC genome, the antigen in any vaccine would have to be carefully considered to make sure we are not merely inducing the reaction that *M. tuberculosis* wants to increase transmission (Comas et al., 2010; Achkar and Casadvall 2013). One method of bypassing this is to mimic vaccine development for *Neisseria meningitides* and create a multivalent vaccine to protect against as many strains as possible or to create a vaccine targeted to certain areas where the molecular epidemiology of *M. tuberculosis* is well defined. However, these are extremely expensive methods and would require copious amounts of funding.

Most vaccines that have been developed against tuberculosis (and are continuing to be tested to this day) have been focused on creating cell-mediated immunity and harnessing the right cytokines to elicit protection (Cooper and Khader 2008). However, given the cytokine responses for different strains, it may be necessary to consider other forms of immunity in vaccine development for *M. tuberculosis*. Since *M. tuberculosis* and other members of the MBTC are intracellular pathogens, vaccines that induce antibody-mediated immunity have not been extensively explored or even considered (Nunes-Alves et al., 2014).

However, many researchers are calling for antibody-mediated immunity to be included in vaccine development for tuberculosis due to research showing evidence of protective antibodies against mycobacteria (Achkar and Casadevall 2012). Since *M. tuberculosis* is able to survive in a host that can mount a disease-preventing immunity, the immune response elicited by a vaccine against tuberculosis must be stronger than one created during natural infection. While cell-mediated immunity is necessary to combat tuberculosis, humoral immunity can help mount a stronger response needed to combat *M. tuberculosis* (Kozakiewicz et al., 2013)

Another way to prevent a pathological outcome from the innate immune response to *M. tuberculosis* is to create a vaccine that induces mucosal immunity, especially since *M. tuberculosis* infects the host through mucosal tissue in the respiratory tract. This could prevent the bacteria from entering the lungs in the first place and completely bypass the formation of the granuloma (Li et al., 2013). Immunization by the respiratory tract has already been shown to be highly effective in several animal models including mice, guinea pigs, cattle, and primates (Beverley et al., 2014). However, there has not been a robust or conclusive analysis on the mucosal response to different strains of *M. tuberculosis*.

Conclusion

Despite advances in molecular epidemiology and genome sequencing, there is still much to be learned regarding the complexity of *M. tuberculosis*. Examination of the evolution of *M. tuberculosis* can lead to important discoveries, such as differing levels of virulence within lineages or hyperconservation of T-cell epitopes, which can have a large

influence on the development of products to combat tuberculosis. Due to advances in genome sequencing, groundbreaking research in this area has only occurred within the past seven years. Naturally, some of this research is still speculative. However, it is clear that further examination into the evolution of *M. tuberculosis* should be done to fully understand this dangerous pathogen that continues to be a leading cause of morbidity and morality after 70,000 years.

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