EX AFRICA SEMPER ALIQUID NOVI –
THE INTERTWINED GENEALOGIES OF
MYCOBACTERIA AND MAN

Prof Nicolaas Claudius Gey van Pittius VI
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BIOGRAPHY

Professor Nicolaas Claudius (Nico) Gey van Pittius VI was born in Pretoria, but grew up in Durban, South Africa. He is currently the Deputy Dean of Research in the Faculty of Medicine and Health Sciences, and a Full Professor of Molecular Biology and Human Genetics in the Department of Biomedical Sciences at Stellenbosch University. He is also a core member of the Department of Science and Technology (DST)/National Research Foundation (NRF) Centre of Excellence in Biomedical Tuberculosis Research (CBTBR) and the Medical Research Council Centre for Tuberculosis Research (CTR) hosted by Stellenbosch University.

After obtaining his BSc (cum laude), BScHons (cum laude) and MSc degrees from the University of Potchefstroom, and his PhD degree from Stellenbosch University, he spent some time as a postdoctoral research fellow, before being appointed Senior Researcher, and later Associate Professor, and now Full Professor in the Department of Biomedical Sciences.

He continued studying part time, obtaining a certificate in Intellectual Property Law (cum laude) and an LLB degree (cum laude) from the University of South Africa, and the degrees LLM and MBA from Stellenbosch University, and is currently registered for an MPhil degree in Science and Technology Studies at the same institution. He is an NRF B-rated researcher and was elected to membership of both the ‘Suid-Afrikaanse Akademie vir Wetenskap en Kuns’ (in 2006 at the young age of 33 years) and the Academy of Science of South Africa (ASSAf) (in 2010, at the age of 37 years). He is also a member of several other national and international scientific societies and committees, such as the American Society for Microbiology (ASM) and the International Union against Tuberculosis and Lung Disease (IUATLD).

Prof Gey van Pittius is a molecular mycobacteriologist who has worked on tuberculosis for the past 18 years, specifically focusing on the mechanisms of evolution of the mycobacteria and the development of mycobacterial pathogenicity and drug resistance. He has co-authored more than 80 papers and book chapters on various aspects of tuberculosis, with over 3 300 citations (and a current h-index of 31) and is a co-inventor of two granted and three provisional patents in the field. He has supervised numerous postgraduate students over the years and taught postgraduate modules in intellectual property law, bioinformatics, genomics and genome variation. His work has been presented in oral and poster form at more than 40 international and nearly 100 national conferences and meetings, and he has been invited to present lectures at numerous institutions worldwide.

To view a list of his publications, please scan:
I have had so many wonderful influencers in my life that I fear I may omit someone in a list of acknowledgements. However, I would like to recognise particularly the following people who contributed enormously to my personal and professional development over many years:

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_Deus vobiscum_

The ACKNOWLEDGEMENTS page of the document contains a list of individuals and groups who have contributed to the author's personal and professional development. The text is a heartfelt expression of gratitude and acknowledges the significant influence of various people over many years. The page also includes a dedication to the author's family and friends, and a final note expressing the author's thanks to all those who have supported them.
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There is a dread disease which so prepares the victim, as it were, for death; which so refines it of its grosser aspect, and throws around familiar looks, unearthly indications of the coming change – a dread disease, in which the struggle between soul and body is so gradual, quiet, and solemn, and the result so sure, that day by day, and grain by grain, the mortal part wastes and withers away, so that the spirit grows light and sanguine with its lightening load, and, feeling immortality at hand, deems it but a new term of mortal life; a disease in which death takes the glow and hue of life, and life the gaunt and grisly form of death; a disease which medicine never cured, wealth warded off, or poverty could boast exemption from; which sometimes moves in giant strides, and sometimes at a tardy pace; but, slow or quick, is ever sure and certain.

A description of tuberculosis taken from Nicholas Nickleby – Charles Dickens (1870)

INTRODUCTION

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), a disease which is a major threat to the health of millions of people worldwide and remains one of the world’s deadliest infectious diseases. Tuberculosis is regarded as the most lethal infectious disease in the history of humankind, being estimated to have killed over a billion people in the past 200 years alone (Paulson, 2013) (see Figure 1). It is further estimated that around a third of the world’s population (approximately 2 billion people) is currently infected with M. tuberculosis (WHO, 2014a). In 1993, the World Health Organization (WHO) took an unprecedented step and declared tuberculosis a global emergency, so great was the concern about the dangerously growing tuberculosis epidemic of the modern era (WHO, 1993). More than twenty years later we are not much better off, as in 2013 alone an estimated 9 million people developed TB and 1.5 million people died from the disease (360 000 of whom were HIV-positive) (WHO, 2014b). The BRICS countries (Brazil, the Russian Federation, India, China and South Africa) together account for almost 50% of reported TB cases worldwide, and 90% of TB cases occur in the developing world (WHO, 2014b). In Lesotho, South Africa and Swaziland, the best estimates suggest that approximately one person in every 100 (1 000 per 100 000 population) develops active TB each year, making this the region with the highest incidence of tuberculosis in the world (see Figure 2). This rate is similar to those recorded during the height of the tuberculosis epidemic in industrial Europe in the early 1800s before the advent of the antibiotic era (Daniel, 2006). Death rates in London, Stockholm and Hamburg approached 800 to 1 000 per 100 000 per year at that time (Daniel, 2006).

There are some positive developments – full scale diagnosis and treatment of tuberculosis on a global scale has led to the worldwide TB death rate dropping by 45% between 1990 and 2013, and it is estimated that around 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment (WHO, 2014b). It is good news that TB rates seem to be gradually declining worldwide. However, this decline is extremely slow, and varies in different parts of the world; and the recent emergence of multidrug-resistant strains of M. tuberculosis poses a risk of reversing this trend. South Africa remains the country with both the highest incidence and prevalence of TB in the world, placing an immense burden on the health of the population (see Table 1). The additional pressures of HIV co-infection (61% HIV prevalence in TB cases in South Africa – Figure 3), multidrug resistance (MDR), and the emergence of extensive drug resistance (XDR)
and total drug resistance (TDR), for which there seem to be virtually no cure, are major causes for concern and ensure that the disease maintains its hold. TB is the leading cause of death among HIV-positive people, causing 25% of all HIV-related deaths. If not treated, around 90% of TB-infected HIV-positive people will die within months of contracting the disease (WHO, 2015). In 2013 the WHO stated that the rise of MDR-TB is an “urgent public health crisis” and “an imminent threat to global health”. Worldwide, an average of 3.6% of new cases of TB and 20% of those previously treated for TB have MDR-TB, with less than 50% of the MDR-TB cases identified in 2010 successfully treated, and nearly 10% of cases of MDR-TB being extensively drug-resistant (XDR-TB)(WHO, 2013). In 2011, treatment success reported for XDR-TB patients in South Africa was only 15%, and 40% of these patients died within a short time span (WHO, 2014b). Contracting XDR-TB is thus a proximate death sentence for many patients. The ineffectuality of the drugs available for treatment owing to the increase in resistance of M. tuberculosis is a major cause for concern worldwide, even more so since cases of totally drug-resistant TB (TDR-TB) are now beginning to emerge, also in South Africa (Klopper et al., 2013). Furthermore, despite more than a 100 years of research on M. tuberculosis, no vaccine currently exists which effectively protects adults against the most transmissible form of the disease, i.e. pulmonary tuberculosis (Brites and Gagneux, 2015). It is thus of utmost importance that novel drug and vaccine targets be identified, and new drugs and an effective vaccine against M. tuberculosis be developed, to enable us to successfully combat this disease.

In order to be able to develop the tools to successfully combat tuberculosis, we first have to understand the biology of the organism which causes the disease. My work over the past 18 years has focused on trying to elucidate the mechanisms of evolution of the mycobacteria and the development of mycobacterial pathogenicity and drug resistance. Mycobacterium tuberculosis has had a long and intertwined history with mankind and has co-evolved with us to become the most successful human pathogen known to date. For every defence we have come up with to halt the spread of this organism, it has developed a way to counter and outmanoeuvre our strategies. Discovering the biological basis for how mycobacteria became such successful pathogens gives us the opportunity potentially to interfere with this close relationship in a more targeted manner, and hopefully will help us finally to break the bond and eradicate this ancient disease.
Figure 2 – Estimated TB incidence rates across the world in 2013 (WHO, 2014b)

Figure 3 – Estimated HIV prevalence in new and relapse TB cases across the world in 2013 (WHO, 2014b)
THE HISTORY OF TUBERCULOSIS

The longer you can look back, the further you can look forward.
Winston Churchill (addressing the Royal College of Physicians – March 1944)

Tuberculosis (otherwise known as consumption, phthisis, the white plague or chronic wasting) has tormented humankind throughout its history and pre-history (Daniel, 2006), having been documented as early as 2 700 BC in ancient China, where the disease was described in a medical text by the Emperor Shen Nung as 2 700 BC in ancient China, where the disease was described in a medical text by the Emperor Shen Nung (2700 BC). A number of other ancient scholars, including Pliny the Younger, Aristotle, Galen, Vitruvius and Aretaeus, all described various aspects of the disease, some in great detail. It is clear that tuberculosis was a well-known disease to physicians and scholars even in these ancient times.

Mycobacterium tuberculosis has, however, been infecting human beings since much earlier in human history, as pathological signs of tubercular decay have been characterised and M. tuberculosis complex DNA has been detected in various archeological human specimens worldwide (Roberts and Buikstra, 2003). Tuberculosis can cause characteristic skeletal changes, such as collapse of the vertebrae (called Pott’s disease), periosteal reactive lesions, and osteomyelitis, enabling long-term sufferers from the disease to be identified through their skeletal remains because of the damage caused by the bacteria to their bones while alive (Ortner, 1981). A recent study in Turkey reported a 500 000-year-old fossil of *Homo erectus* which shows lesions characteristic of TB (Roberts et al., 2009). This finding is still somewhat controversial, but, if verified, it would mean that the evolution of *M. tuberculosis* pre-dates the origin of modern humans (Gagneux, 2012). The oldest undisputed evidence for tuberculosis infection of modern humans was discovered in human remains from the Neolithic era dating from 9 000 years ago in a submerged settlement called Atlit-Yam in the eastern Mediterranean Sea, off modern-day Israel (Hershkovitz, et al. 2008). Egyptian mummies with palaeopathological changes, reflecting Pott’s disease, have been a rich source for ancient tuberculosis evidence, from a 5 400-year-old skeleton from the Predynastic era (Crubézy et al. 1998), to various others from the Old, Middle and New Kingdoms of Egypt (Nerlich et al. 1997; Zink et al. 2003). One of the most recent examples is the so-called Granville mummy, from the necropolis of Thebes in ancient Egypt, being that of the lady Irytysenu of the 26th Dynasty, dated ca. 600 BC, who died of tuberculosis (Donoghue et al., 2010). Furthermore, tuberculous spondylitis was found in samples from Italy dating from the Neolithic period around 4 000 BC (Formicola et al., 1987; Canci et al., 1996), and others dating to the Bronze Age (Canci et al., 2001). *M. tuberculosis* DNA was also identified in Scandinavian Neolithic skeletons in Sweden dating to 3 200–2 300 BC (Nuorala et al., 2004). Tuberculosis was not only found to be widespread in the Old World, but has also been identified in skeletal remains from the New World (Roberts and Buikstra, 2003). For example, *M. tuberculosis* DNA was identified in the 1 000-year-old lung tissues of pre-Columbian mummies from Peru and northern Chile (Allison et al., 1973; Salo et al., 1994, Arriaza et al., 1995). A comprehensive account of the archeological evidence for tuberculosis from different sites across the Old and New World can be found in the manuscript by Roberts and Buikstra (2003). What is clear from the bioarchaeological evidence of tuberculosis is that the disease has its origins in ancient times and was established and found widespread in different parts of the world in human populations which were geographically isolated from each other for millennia, indicating an even earlier origin for the disease.

MYCOBACTERIUM TUBERCULOSIS

*Mycobacterium tuberculosis* is an exceptionally slow-growing, rod-shaped bacterium which is remarkably well adapted to handle stressful conditions within the body, having a thick complex lipid-rich cell wall structure which is resistant to acidic conditions and impermeable to a number of compounds (Shinnick and Good, 1994). The bacterium is an intracellular pathogen of macrophages and is able to modify the maturation of the phagosome within which it resides, allowing the bacterium to bypass the microbicidal effector functions of the host cell (Clemens and Horwitz, 1995). It usually enters the body through inhalation of aerosol droplets...
caused by coughing. These droplets carry it into the lungs of an individual (Riley et al., 1959), after which disease progression involves phagocytosis of the organisms by alveolar macrophages, and subsequent survival and replication within these cells, followed by destruction of the lung tissue by the host's own immune response, in turn leading to the spread to new hosts (McKinney et al., 1998). Over 80% of tuberculosis cases present as pulmonary tuberculosis, which is also the most infectious form of the disease, and leads to coughing, chest pain, fever and night sweats, weight loss (wasting), loss of appetite, fatigue and the coughing up of blood (Hopewell, 1994).
The term ‘tuberculosis’ was first used by Franciscus de la Boé (Dr Silvius) in his Opera Medica of 1679 (Trail, 1970), and relates to the tubercles, tuberculous cavities and tuberculous lymph nodes that are associated with the disease. The first great step forward in the combat of tuberculosis came with the recognition by an English physician, Benjamin Marten, that tuberculosis may be caused by an airborne organism (McKinney et al., 1998). Before that, there was a huge debate raging between scholars and physicians on whether tuberculosis was an infectious or inherited disease. In his work, A New Theory of Consumption, published in 1722, Marten hypothesised that the etiological agent “may possibly be some certain Species of Animalculae or wonderfully minute living creatures that, by their peculiar Shape or disagreeable Parts are inimicable to our Nature; but, however, capable of subsisting in our Juices and Vessels …” (Dubos and Dubos, 1952). These “wonderfully minute creatures” were only isolated and described for the first time more than 100 years later, in the late 1800s, by the German physician Robert Koch (Koch, 1882). He revealed his discovery of the identification of M. tuberculosis as the causative agent of tuberculosis in a historical address to the Berlin Physiological Society on 24 March 1882 (McKinney et al., 1998). However, it was the cousin of M. tuberculosis, Mycobacterium leprae, the causative agent of leprosy in humans, that was the first mycobacterial species ever identified. Its discovery by Armauer Hansen was a breakthrough in medical history as the first ever convincing association of a microorganism with a human disease (Hansen, 1874, Hansen, 1880).

THE GENUS MYCOBACTERIUM

The genus Mycobacterium belongs to the class Actinobacteria, a group of guanine and cytocine (G+C)-rich Gram-positive bacteria generally found in soils, where they play an active role in the carbon cycle and other ecological processes (Jang et al., 2008). The genus currently consists of around 130 species and five subspecies (and an additional 34 species not validly published), 60% of which have only been identified in the last 15 to 20 years (see Figure 4). The genus Mycobacterium contains many species which have the ability to infect birds, fish, amphibians and mammals (including humans), and has evolved mechanisms by which it can invade and grow within host cells. The genus therefore contains a number of pathogenically important species causing human and animal diseases, such as tuberculosis (M. tuberculosis and M. bovis) (Michel et al., 2007; Espie et al., 2009); leprosy (M. leprae) (Gerber, 1994); Buruli ulcer (M. ulcerans) (Sizaire et al., 2006), pulmonary and nonpulmonary infections primarily in immunocompromised individuals (M. avium) (Havlir, 1994); and paratuberculosis or John’s disease (a chronic granulomatous enteritis of ruminants caused by M. paratuberculosis) (Chiodini et al., 1984).

It is hypothesised that the genus Mycobacterium originated more than 150 million years ago. This is based on the fact that M. ulcerans has very specific habitat requirements and a current geographic distribution in West Africa, Australia and South America that widely separates its endemic regions, making it virtually impossible to have reached these regions in the recent past owing to the extensive geographic barriers between them. These regions were last in contiguity as part of the Gondwanaland continental land mass that existed from approximately 510 to 180 million years ago during the Jurassic period (Daniel, 2006), indicating that the species must have developed before the break up of the continents when continental drift started.

Mycobacterial species are divided into two groups, (1) the rapidly growing species, which are able to form colonies on solid media in less than seven days, and (2) the slow-growing species, which are only able to form colonies on solid media after seven days or more (Shinnick and Good, 1994, Springer et al., 1996). Most of the slow-growing mycobacteria are pathogenic species, which seem to have evolved more recently and include the species causing tuberculosis, leprosy, paratuberculosis and other diseases. The fast-growing mycobacteria, on the other hand, seem to be more closely related to the progenitor of the mycobacteria and other closely related genera, including Corynebacterium, Nocardia, Rhodococcus and other actinomycete genera such as Streptomyces (Wayne and Kubica, 1986), which are predominantly non-pathogenic and found mostly in the environment as saprophytes (Shinnick and Good, 1994). It is therefore quite clear that at some stage in the evolution of the genus Mycobacterium, the slow-growing members of the genus have acquired the ability to survive and live in an intracellular environment, and have developed into both opportunistic pathogens (such as M. avium) and obligate pathogens (such as M. tuberculosis and M. leprae).

THE MYCOBACTERIUM TUBERCULOSIS COMPLEX

Mycobacterium tuberculosis belongs to a larger group of closely related slow-growing, tuberculosis-causing mycobacterial species within the genus Mycobacterium, the so-called Mycobacterium tuberculosis complex (MTBC) or tubercle bacilli. The M. tuberculosis complex comprises genotypically and phenotypically distinct
lineages which have evolved through clonal expansion from a common progenitor (Brites and Gagneux, 2015). The complex contains three different subgroups, based on its host adaptation profiles and phenotypes, namely (1) the smooth tubercle bacilli (including Mycobacterium canetti and other smooth tubercle bacilli found only in East Africa), the actual host range of which remain unknown (Gutierrez et al., 2005), (2) the human-adapted species (including M. tuberculosis and M. africanum) (Brites and Gagneux, 2015), and (3) the animal-adapted species, which includes the chimpanzee bacillus; M. mungi (the mongoose bacillus); the dassie bacillus; M. suricattae (the meerkat bacillus); M. microti (the vole bacillus); M. orygis (the oryx bacillus); M. caprae (the goat bacillus); and M. bovis (the bovine bacillus) (Alexander et al., 2010; Gey van Pittius et al., 2012a; Gey van Pittius et al., 2012b; Van Ingen et al., 2012; Coscolla et al., 2013; Parsons et al., 2013). These species are all able to cause tuberculosis disease in mammals, including humans, but are mostly restricted to ectotypes favouring disease in a specific host, e.g. the dassie bacillus is found mostly in the Cape hyrax (Procavia capensis), and M. pinnipedii in seals, etc. (Parsons et al., 2008).

MYCOBACTERIAL GENOMICS

A major breakthrough in the research of tuberculosis and the understanding of the mycobacteria came with the complete sequencing (in 1998) of the first whole genome sequence of the widely used M. tuberculosis reference laboratory strain H37Rv (Cole et al., 1998), as well as that of thousands of other strains and species within the genus Mycobacterium during subsequent years, including more than 600 strains of M. tuberculosis from our own laboratory at Stellenbosch University. The sequencing data of M. tuberculosis revealed that the genome is around 4.4 Mb, has a high G+C content of 67% and is made up of around 4 000 genes distributed evenly on both strands. Around 40% of the genes were identified to be of known function, 20% of hypothetical function, and 40% were totally unknown. Thus, the sequencing revealed a large number of previously unknown genes, all having the potential to be involved in the pathogenesis of the organism (Cole et al., 1998). Interestingly, approximately 52% of the genome originated as a result of gene duplication or domain shuffling events (Tekaiia et al., 1999). Another surprising observation was the fact that two unknown, novel gene families (representing areas on the genome with an exceptionally high G+C content of more than 80%) make up around 10% of the genome (Cole, 1999). These families were named the PE (99 members) and PPE (67 members) gene families, after the conserved proline (P) and glutamic acid (E) motifs found in the N-termini of the encoded proteins. The sequencing of the genomes of thousands of mycobacterial strains and species over the past 18 years has presented an extraordinary opportunity to discover important genes through the newly evolved science of comparative genomics (Cole, 1998), through which the genomes and gene sequences of mycobacterial species and strains are compared and the differences analysed in detail.

Mycobacterial genomics opened the door to understanding how mycobacterial species and strains are related to each other, and what changes took place during their evolution, causing the varying phenotypes that we see today to arise. The addition of subsequent technologies such as comparative proteomics, comparative transcriptomics and comparative metabolomics has vastly increased the wealth of information available to us and has led to the birth of the new field named comparative mycobacterial ‘omics’, or ‘mycobactomics’ for short. The results of these analyses provided an unprecedented view of the genomic and proteomic variation of the mycobacteria, and provided insight into the evolution of fitness, virulence and drug resistance. Mycobactomics also provided new tools to identify chromosomal regions, genes, proteins as well as metabolic compounds of interest which can be used in the development and application of methods of detecting pathogenic and non-pathogenic strains and species, in other words for use in the development of novel mycobacterial diagnostics.

EVOLUTION OF VIRULENCE IN THE MYCOBACTERIA

Most mycobacteria are environmental organisms and are found in soil and water. They cause disease only occasionally when they encounter a susceptible, usually immune-compromised, human or animal host. However, a few slow-growing species, such as the members of the M. tuberculosis complex, have acquired the ability to survive in host macrophages during the course of evolution and have become major pathogens of humans and animals (Jang et al., 2008). As mentioned above, the availability of whole genome sequence data for a large number of mycobacterial species has greatly facilitated comparative genomics research aimed at addressing genetic variability within and between mycobacterial species. One such study identified 48 M. tuberculosis chromosomal regions with atypical characteristics, signifying acquisition through horizontal gene transfer events (Becq et al., 2007). These specific regions
account for 4.5% of the genome (199 kb) and include a large number of genes orthologous to those from other bacterial species present in soil, in particular members of the proteobacteria and actinobacteria (Becq et al., 2007). Building upon this work, Veyrier et al. (2009) later identified 137 genes in M. tuberculosis which were likely acquired through the process of horizontal gene transfer in a step-wise manner during the divergence of the slow-growing mycobacteria from the fast growers, 60% of these genes originating from other actinobacteria found in the same environment as the saprophytic mycobacteria. Among others, there has been a specific acquisition of genes encoding for proteins involved in metabolic functions related to adaptation to anaerobic conditions (Veyrier et al., 2009), thereby preparing the organism for an intracellular lifestyle.

These early episodes of horizontal transfer of genomic islands from surrounding environmental species may have contributed to the evolution towards the virulence of M. tuberculosis, specifically by helping the bacilli firstly to persist in protozoa found within their natural environment and, subsequently, in mammalian phagocytes. It has been shown in many studies that pathogenic mycobacterial species have acquired the ability to survive in protozoa. M. fortuitum, M. xenopi, M. avium, M. marinum and M. bovis all resist being killed and multiply within Acanthamoeba sp. (Taylor et al., 2003), indications even being that their virulence increases with subsequent passages in this amoeba (Cirillo et al. 1997). It is hypothesised that this intra-protozoal lifestyle and the bacterium’s increased capacity to live intracellularly and resist being killed by the host cell prepared the progenitor of the M. tuberculosis complex for an intracellular lifestyle.

The ancestors of the M. tuberculosis complex, having acquired the ability to survive intracellularly, subsequently had to evolve strategies to overcome the immune response of the host, to transmit effectively between hosts, and to avoid becoming extinct in small human populations by tempering its own virulence (Brites and Gagneux, 2015). At some stage during their evolution, the members of the human and animal-adapted lineages of the M. tuberculosis complex split off from the smooth tubercle bacilli and seemed to have lost their ability to undergo horizontal gene transfer. This happened during an evolutionary bottleneck or selective sweep which occurred around 35 000–40 000 years ago, probably linked to their change in lifestyle status from an opportunistic pathogen to that of an obligate pathogen (Gutierrez et al., 2005). Divergence of the smooth tubercle bacilli (including M. canetti) seems immediately to predate this bottleneck or selective sweep, while all other members of the complex are the result of the clonal expansion of only a small number of surviving bacteria (McEvoy et al., 2012). Comparative genomics analyses show that M. tuberculosis has undergone a biphasic evolutionary process involving both genome expansion (through gene acquisition and duplication) and reductive evolution (through gene deletions) (Veyrier et al., 2011). Gene duplication has specifically been shown to have played an enormous role in the divergence and adaptation of the species, and subsequent increase in virulence of M. tuberculosis, with around 52% of the organism’s proteome having been shown to be derived from gene duplication events (Tekai et al., 1999). These duplication events include the five copies of the immunopathologically important ESAT-6 (ESX) gene clusters (Gey van Pittius et al., 2001), as well as the large multi-protein PE and PPE families (Gey van Pittius et al., 2006), both of which are hypothesised to play a key role in the survival and virulence of the pathogenic mycobacteria.

The ESAT-6 (or ESX) gene cluster regions express proteins which form a dedicated, multi-component, binding protein-dependent active transport system encoding a novel type VII secretion system, responsible for the sec-independent secretion of, inter alia, the family of the T-cell antigens ESAT-6 and CFP-10 (Gey van Pittius et al., 2001, Abdallah et al., 2007, Bitter et al., 2009). The five different ESX systems in the genus Mycobacterium evolved through gene duplication, in the order ESX-4, ESX-1, ESX-3, ESX-2 and most recently ESX-5 (Gey van Pittius et al., 2001). Although they are all involved in the transport of small proteins across the cell membrane, these immunologically important secretion systems have evolved to perform different functions (a well-known consequence of gene duplication and the subsequent redundancy that arises) and do not appear to be able to complement each other when knocked out. ESX-4 was shown to be the most ancestral region, being the smallest ESX locus (containing only the minimal set of genes required for transport), orthologs of which are also present in bacteria outside of the genus Mycobacterium, and play no apparent role in virulence (Gey van Pittius et al., 2001; Sassetti et al., 2003). ESX-1 was the first ESX system to be discovered, and has been shown to be directly involved in pathogenicity and phagosomal escape of the organism, but is not essential for in vitro growth (Simeone et al., 2012). ESX-3, on the other hand, seems to be involved in iron and zinc homeostasis and is essential for in vitro growth of this organism (Serafini et al., 2013). Virulence of ESX-
I mutants is severely attenuated in macrophage cell lines and in in vivo infection models, owing to the fact that they are unable to escape the phagolysosome of macrophages (Houben et al., 2012, Simeone et al., 2012). Deletion of a large part of the ESX-1 genetic locus is also the major cause of the attenuation of the vaccine strain M. bovis BCG (Pym et al., 2002) and other non-virulent mycobacterial species. Interestingly, the last duplication (of ESX-5) coincided with the emergence of the slow-growing mycobacterial species, being absent in the genomes of all fast-growing species studied thus far (Gey van Pittius et al., 2006, Abdallah et al., 2009). This system is responsible for the secretion of many proteins, which are found only in the slow-growers and are absent in the genomes of all fast-growing species studied thus far (Gey van Pittius et al., 2006, Abdallah et al., 2009). Since most pathogenic mycobacteria are slow-growing, the presence of ESX-5 and the expansion of the PE and PPE protein families in only the slow-growers seem to indicate a link to an adaptation towards an intracellular and virulent lifestyle. A possible role has been identified for ESX-5 as having evolved into a nutrient import system in lieu of the porin systems in the fast-growers (which have been shown to be detrimental to the intracellular survival of fast-growing mycobacteria and are absent in the slow growers), ensuring adequate nutrient acquisition while maintaining the impermeability of the outer membrane required for in vivo persistence (Ates et al., 2015). Duplication of the original gene cluster encoding for the ESAT-6 secretion system, and the linked proteins of the PE (and specifically PE_PGRS), PPE and ESAT-6-like protein families, therefore enabled functional diversification into a number of novel pathogenic mechanisms.

ORIGINAL SELECTIVE FORCES – HOST ADAPTATION AND CO- EVOLUTION

The intertwined genealogies of mycobacteria and man commenced the moment that the first progenitor of the M. tuberculosis complex gained the ability to stably infect a member of Homo sapiens. Since that moment a relationship of interplay developed between host and bacterium which lasts to this day. The conversion from an opportunistic pathogenic lifestyle to that of an obligate pathogen placed immense pressure on the bacterium to ensure that it could survive in the host and spread to new hosts (through the mechanism of active disease), but not in a manner which would wipe out all available hosts and thereby destroy its own ability to subsist. The bacterium’s very survival therefore became intricately linked to the existence of the host (and influenced by the host’s immune system), and any changes in the internal and external environment of the host posed the potential threat also deeply to affect the survival of the bacterium. An iterative process of host adaptation and co-evolution thus took place over millennia (and still takes place today), whereby evolutionary changes in the pathogen that increase infectivity and survival are countered by evolutionary changes in the host that increase resistance to, and clearing of, the infection (Brites and Gagneux, 2015). This process of reciprocal, antagonistic effects on the fitness of both mycobacterium and man have led to one of the key features of tuberculosis as we know it, namely latency, a condition in which the bacterium is able to latently and persistently infect the host without causing disease or being able to spread, held in check by the host’s immune system, until such time as conditions become favourable (e.g. old age or immune suppression) to cause active disease and spread to a new host (Brites and Gagneux, 2012). Mycobacterium tuberculosis has adapted to this lifestyle in such a unique and exceptionally effective way that it is estimated that around a third of the world’s human population is latently infected by the organism (WHO, 2015). During the millennia that followed the first infection of humans by the ancestor of M. tuberculosis, the two main original selective forces which primarily acted on the adaptation of the organism have been the host immune response and changes in human demography (Brites and Gagneux, 2012).

EX AFRICA SEMPER ALIQUID NOVI – OUT OF AFRICA ALWAYS SOMETHING NEW

Comparative genomics analyses revealed that, although the members of the M. tuberculosis complex differ remarkably in their phenotypes, pathogenicity and primary host range (which ranges from chimpanzees to humans, voles, goats, oryx, dassies, cattle, meerkat, mongoose and seals), they are genetically very close and share 99.9% identity between their DNA sequences (Brosch et al., 2002). Members of the M. tuberculosis complex have lost the ability to undergo horizontal gene transfer, and as such, the only genetic changes that can still take place in these organisms are deletions, duplications, genetic rearrangements, transposon insertions and single nucleotide polymorphisms (Karboul et al., 2006; McEvoy et al., 2007; Karboul et al., 2008; McEvoy et al., 2009a; McEvoy et al., 2009b;
Weiner et al., 2012). *M. tuberculosis* made (and still makes) use of all these mechanisms to ensure that it counters any attacks by its host on its survival (see for example below for a description of the evolution of drug resistance through single nucleotide polymorphisms). The evolution of these different species and strains of the *M. tuberculosis* complex is thought to have occurred from a common progenitor which existed more than 70 000 years ago, when the first mycobacterium is thought to have infected a member of *Homo sapiens* living in East Africa (Comas et al., 2013). Up to about a decade ago, it was thought that *M. tuberculosis* evolved from *M. bovis*, spreading to humans only after the domestication of cattle (its current maintenance host) around 10 000 years ago (Stead, 1997). However, genomics analyses have proved this wrong, as human-adapted lineages of *M. tuberculosis* are phylogenetically more ancestral than those of the animal-adapted species (see Figure 5) and *M. bovis* has lost several genes still present in *M. tuberculosis* and other animal-adapted species (Brosch et al., 2002). In fact, several studies have shown that animal tuberculosis already occurred in the late Pleistocene (between 20 000–11 000 years ago), long before the domestication of cattle (Rothschild and Martin, 2006). This is confirmed by the presence of characteristic tubercular lesions in fossils of bighorn sheep (*Ovis canadensis catclawensis*), extinct musk ox (*Botherium bombifrons*) and mastodons (*Mammut americanum*) in North America (Martin and Rothschild, 1989; Rothschild and Laub, 2006), and through lesions and the presence of ancient *M. tuberculosis* complex DNA in the metacarpal of an extinct North American long-horned bison that was radiocarbon dated at more than 17 800 years old (Rothschild et al., 2001). Mastodons lived in North America starting about 2 million years ago and thrived there until around 11 000 years ago (around the time humans arrived on the continent) when they and many other large North American mammals became extinct, leading to the theory that the introduction of ancestral tuberculosis through human contact with these large mammals might have contributed to their extinction (Rothschild and Laub, 2006).

Notwithstanding the high clonality of the species belonging to the *M. tuberculosis* complex, different lineages, sublineages and strains have evolved over the last 70 000 years (through the mechanisms described above), including the progenitor of the animal-adapted species, which have lost a region of the genome called RD9, a region which is thus absent in all extant animal-adapted species but still present in the human-adapted members of the complex (Brosch et al., 2002). Although *M. tuberculosis* and *M. africanum* have been shown to be able to infect other mammalian species (see for example Parsons et al., 2012, and others), they are only able to stably infect and spread in humans. It is therefore possible that this deletion of RD9 opened the way for

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**Figure 5** – The family tree of the members of the Mycobacterium tuberculosis complex (adapted from Alexander et al., 2010)
differentiation and spread of the organism into other mammalian host species, the earliest of which originate from the African continent (chimpanzees, hyrax, meerkat and mongoose), supporting the view that the complex originated in Africa and that diversification took place on this continent before dispersal to the rest of the world. Evidence exists for animal tuberculosis occurring in Eurasia long before being present in North America, and being brought to North America by immigrant bovids, supporting the theory of the spread from Africa through Eurasia to America (Rothschild and Martin, 2006). The different animal-adapted species all underwent their own specific genetic adaptations (mostly deletions) subsequent to RD9, in order to adapt to the new host niches which they ventured into, to such an extent that these specific deletions can today be used as a tool to differentiate genetically among all of the different members of the *M. tuberculosis* complex (Warren et al., 2006).

Comparative genomics and phylogeographic analyses have shown that *M. tuberculosis* evolved as a human pathogen in Africa and subsequently spread to the rest of the world along with modern humans as they migrated out of Africa over the last 70 000 to 40 000 years (Gutierrez et al., 2005; Hershberg et al., 2008) (see Figure 6). As human populations diversified, these ancient *M. tuberculosis* strains also underwent early diversification based on the changing human demography and genotypes in order to ensure that the optimal host-pathogen relationship was maintained (Brites and Gagneux, 2015). Host-specific adaptation in isolated human populations meant that several main lineages of *M. tuberculosis* with distinct features developed in isolation over thousands of years. The species *M. tuberculosis* that we see today therefore consists of a large number of different strains (currently there are more than 1 000 known strains) falling into more than 40 different larger strain families (many of these presenting with different pathogenic characteristics, fitness and host adaptations) and all located within seven large known lineages (Gagneux et al., 2006; Coscolla and Gagneux, 2014). These seven lineages have a strong phylogeographical structure, having differentiated and adapted to different human populations in different parts of the world. They are separated into the ancient lineages and the modern lineages, based on the deletion of a region named TbD1 (Brosch et al., 2002). The ancient lineages are more closely related to the progenitor and to the animal-adapted species and consist of lineage 5 (*M. africanum* West-African 1) and lineage 6 (*M. africanum* West-African 2), which are both restricted geographically to the western African countries, as well as the recently described lineage 7.

![Figure 6. Out-of-Africa and Neolithic expansion of the human-adapted species of the Mycobacterium tuberculosis complex (M. tuberculosis and M. africanum). Major splits are annotated with the median value (in thousands of years) of the dating of the relevant node (taken from Comas et al., 2013).](image-url)
Cape's climate for curing tuberculosis, thus attracting thousands of travellers from both East Asia and Europe (especially Britain) and India came to South Africa on account of the sunny, temperate climate which was thought to be beneficial in terms of the East Indian and Asian people who were willingly in terms of the Europeans and unwillingly in terms of the European and East Asian origin as the dominant strain types in this country. As mentioned above, different strains of \( M. \) \( \text{tuberculosis} \) have acquired different phenotypic properties through evolution, resulting in strains which are, for example, hyper- or hypovirulent in certain populations (Aguilar et al., 2010). We also observe specific strain families which seem to outcompete others in a certain setting (Mardassi et al., 2005, Hanekom et al., 2007). The incidence of cases with strains from the Beijing genotype (originally from East Asia), for example, has been shown to be increasing exponentially over time within our host population in the Western Cape, South Africa, with an estimated doubling time of only 3.8 years (Johnson et al., 2010, Hanekom et al., 2011). The Beijing strain family has been shown to grow faster than other strains, to be more likely to transmit, and to have a greater propensity to cause disease (López et al., 2003). The F11 LAM genotype (originally from Europe), on the other hand, is also currently one of the largest strain families in the Western Cape of South Africa and is believed to be the cause of approximately 15% of tuberculosis cases worldwide (Gibson et al., 2008). Between Beijing and F11, these two families, originating from vastly different parts of the world, account for close on 45% of the tuberculosis cases in this setting (Victor et al., 2004). In other South African study settings, such as KwaZulu-Natal, the KZN strain family (also part of the larger LAM genotype and a relative of the F11 LAM strain family prevalent in the Western Cape) is a highly virulent strain endemic and predominant in this region, which has recently experienced an outbreak of extensively drug-resistant tuberculosis (Pillay and Sturm, 2007). In Zimbabwe and Zambia, the F9 LAM11 strain was found to be predominant, infecting approximately half of the patients in this region (Gibson et al., 2008). Outside Africa, the RD\(^{\text{Ko}}\) \( M. \) \( \text{tuberculosis} \) family is a LAM sublineage that is found worldwide, but is the predominant cause of TB in South America, representing 38% of the total strains and 70.4% of the LAM strains found in this setting (Gibson et al., 2008). Similar to the Beijing family, strains belonging to the LAM family show a fitness advantage when comparing their rate of growth with that of non-LAM strains. The advent of globalisation has significantly altered the relationship between mycobacteria and man. What seemed to have been a relatively stable relationship shaped by millennia of co-evolution and reciprocal antagonistic pressures has been disturbed by

\( \text{OUT OF AFRICA \ldots AND BACK \ldots} \)

Southern Africa was located in a geographically central position along the historical trade routes between the East and West for many hundreds of years, and tens of thousands of travellers from both East Asia and Europe travelled through this region and settled here (mostly willingly in terms of the Europeans and unwillingly in terms of the East Indian and Asian people who were brought here as slaves). It is also well known that tuberculosis sufferers from Europe (especially Britain) and India came to South Africa on account of the sunny, temperate climate which was thought to be beneficial and could lead to cure. Advertisements even appeared in the \textit{Illustrated London News} extolling the virtues of the Cape's climate for curing tuberculosis, thus attracting large numbers of fresh cases of tuberculosis in the 1870s and 1880s to the region, the most famous of which probably being Cecil John Rhodes (Packard, 1989). We therefore today observe a miscellany of \( M. \) \( \text{tuberculosis} \) strains from both European and East Asian origin as the dominant strain types in this country. As mentioned above, different strains of \( M. \) \( \text{tuberculosis} \) have acquired different phenotypic properties through evolution, resulting in strains which are, for example, hyper- or hypovirulent in certain populations (Aguilar et al., 2010). We also observe specific strain families which seem to outcompete others in a certain setting (Mardassi et al., 2005, Hanekom et al., 2007). The incidence of cases with strains from the Beijing genotype (originally from East Asia), for example, has been shown to be increasing exponentially over time within our host population in the Western Cape, South Africa, with an estimated doubling time of only 3.8 years (Johnson et al., 2010, Hanekom et al., 2011). The Beijing strain family has been shown to grow faster than other strains, to be more likely to transmit, and to have a greater propensity to cause disease (López et al., 2003). The F11 LAM genotype (originally from Europe), on the other hand, is also currently one of the largest strain families in the Western Cape of South Africa and is believed to be the cause of approximately 15% of tuberculosis cases worldwide (Gibson et al., 2008). Between Beijing and F11, these two families, originating from vastly different parts of the world, account for close on 45% of the tuberculosis cases in this setting (Victor et al., 2004). In other South African study settings, such as KwaZulu-Natal, the KZN strain family (also part of the larger LAM genotype and a relative of the F11 LAM strain family prevalent in the Western Cape) is a highly virulent strain endemic and predominant in this region, which has recently experienced an outbreak of extensively drug-resistant tuberculosis (Pillay and Sturm, 2007). In Zimbabwe and Zambia, the F9 LAM11 strain was found to be predominant, infecting approximately half of the patients in this region (Gibson et al., 2008). Outside Africa, the RD\(^{\text{Ko}}\) \( M. \) \( \text{tuberculosis} \) family is a LAM sublineage that is found worldwide, but is the predominant cause of TB in South America, representing 38% of the total strains and 70.4% of the LAM strains found in this setting (Gibson et al., 2008). Similar to the Beijing family, strains belonging to the LAM family show a fitness advantage when comparing their rate of growth with that of non-LAM strains. The advent of globalisation has significantly altered the relationship between mycobacteria and man. What seemed to have been a relatively stable relationship shaped by millennia of co-evolution and reciprocal antagonistic pressures has been disturbed by
the breaking down of traditional geographical barriers, leading to mycobacterium encountering novel genotypes of man, and man encountering novel genotypes of mycobacterium, and which gave rise to a new conflict between the two ancient adversaries raging worldwide. This new situation has been further complicated by two recent events in the long and intertwined history of mycobacteria and man, namely the introduction of anti-microbials and the advent of the HIV/AIDS epidemic. These new selective forces have impacted substantially on the relationship and will shape the epidemic for years to come.

MODERN SELECTIVE FORCES – HIV AND ANTI-MICROBIALS

The discovery of anti-microbials have revolutionised medicine in the 20th century, and humankind has never before in its history had such a strong array of defences against microbial diseases as we have today. In conjunction with vaccination, many frightful diseases have been nearly eradicated through anti-microbial therapy. However, overuse, ineffective prescription and non-compliance have led to a new scourage of bacterial resistance. The rise in bacterial resistance to antibiotics in the last few decades has prompted the World Health Organization to classify anti-microbial resistance as a “serious threat [that] is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country” (WHO, 2014c).

Selman A Waksman (1964), the discoverer of the antibiotic Streptomycin, stated the following in his book The Conquest of Tuberculosis:

But most important, the ancient foe of man, known as consumption, the great white plague, tuberculosis, or by whatever other name, is on the way to being reduced to a minor ailment of man. The future appears bright indeed, and the complete eradication of the disease is in sight…

Waksman could not have been more wrong. Humankind has forgotten that for every action there is a reaction, and actions affecting its intertwined relationship with the mycobacteria are no different. The use of antibiotics against M. tuberculosis initially seemed very successful, and rates of tuberculosis started to decline – so much so that all development of new anti-mycobacterial drugs were stopped for a number of decades after the discovery and introduction into clinical practice of the first successfully active compounds against M. tuberculosis between the 1940s and 1960s. But indiscriminate use of these drugs has led to the emergence of drug resistance, multidrug resistance (MDR), extensive drug resistance (XDR) and even total drug resistance (TDR) (Müller et al., 2013) and the utterance of these abbreviations is now invariably accompanied by feelings of fear in tuberculosis conversations around the world. Humans mounted a new assault against the mycobacterium, namely antibiotics, by this means trying to disturb the age-old host-pathogen relationship in their favour, and celebrated battles won, but forgot that the war was not yet over. The mycobacteria responded in their own way to defend themselves through mechanisms such as the selection for resistance through single nucleotide polymorphisms in drug target genes and reducing susceptibility through efflux of the drugs (Louw et al., 2009; Louw et al., 2011; Müller et al., 2011; Sirgel et al., 2012). The modern selective pressure of anti-microbial therapy has even benefited some mycobacterial strains, which were in a better position to respond to the new environment they encountered through the sudden presence of antibiotics (Van Rie et al., 2005). We therefore observe much higher levels of drug resistance in the Beijing family in the Western Cape compared to other strains families such as the LAM family. This is a significant observation, as the LAM strains currently have a nearly equal representation in the population of Cape Town compared to the Beijing strains, and indicates that there are strain-specific drug-resistance acquisition and adaptation mechanisms within the Beijing family. Our epidemiological data indicate that there is an exponential increase in Beijing strains in this setting compared to other strains, which have stayed relatively constant or even decreased in number over the past 13 years (Van der Spuy et al., 2009). We thus observe changing M. tuberculosis population patterns, indicating clade-specific pathogenic and adaptive characteristics. In other study settings, such as the Eastern Cape, drug-resistant atypical Beijing strains are of extreme importance and a large outbreak of these strains is currently occurring in this setting (Klopper et al., 2013). Drug resistance and multi-drug resistance is being acquired and transmitted on a large scale (loeger et al., 2010) and is on the increase worldwide, and the emergence of extensively and totally drug-resistant mycobacteria, resistant to both first line and second line treatment, is cause for extreme alarm (Hoek et al., 2009; Chihota et al., 2012; Streicher et al., 2012). We urgently need to find new ways to replace traditional methods of genotyping (Hanekom et al., 2008, Warren et al., 2009b) and new tools to shorten time to diagnosis and look for resistance (Hoek et al., 2008, Warren et al., 2009a, Parsons et al., 2011, Barnard et al., 2012a, Barnard et al., 2012b). Drug resistance is a
selective pressure which is favouring the mycobacterium, and an inadequate response from the host will have a detrimental effect on the survival of mankind in the battle between mycobacterium and man.

The other recent event that had a major impact on the relationship between mycobacteria and man was the advent of the HIV/AIDS epidemic (Brites and Gagneux, 2012). HIV disease has been pandemic for only the last 30 years, but has had a huge influence on the health of humans, and on the intertwined relationship of mycobacteria and man. Currently there are around 35 million people worldwide living with HIV, and around 2.3 million new infections annually (Lucas and Nelson, 2015). Seventy per cent of HIV-positive people live in sub-Saharan Africa. HIV has the ability to cause systemic T-cell destruction and reduced cell-mediated immunity that leads to a wide range of opportunistic infections and cancers (Lucas and Nelson, 2015). Chief among these is infection with *M. tuberculosis*, an organism which lives in close interplay with the host’s immune system, and which therefore thrives under conditions where the immune system is compromised. The modern selective force of HIV co-infection has ensured that strains of *M. tuberculosis* are now experiencing favourable host conditions that they have never encountered before in their long relationship with mankind. These favourable immunocompromised conditions created by HIV have allowed *M. tuberculosis* to find new niches for infection, leading to more frequent extra-pulmonary disease, and resulting in atypical clinical presentations (Wasserman and Meintjes, 2014). TB-HIV co-infection is the most important cause of the high rates of infectious morbidity and mortality in South African adults (Wasserman and Meintjes, 2014). The risk of contracting TB is up to 40-fold higher in HIV-positive patients than in HIV-negative patients (Getahun et al., 2010), and TB is the leading cause of death among HIV-positive people, causing 25% of all HIV-related deaths. If not treated, around 90% of TB-infected HIV-positive patients will die within months of contracting the disease (WHO, 2015). An HIV-negative person latently infected with *M. tuberculosis* has a lifetime risk of 10% of progressing to TB disease, while in an HIV-positive co-infected person that risk rises to 10% per year (McShane, 2005).

It is clear that the modern selective pressures of the introduction of anti-microbials and the advent of the HIV/AIDS epidemic have played a significant role in the recent events surrounding the intertwined genealogies of mycobacteria and man. Although man won a number of battles with the introduction of antibiotics, he lost many more through his indiscriminate use thereof. The war has taken a turn in favour of the bacterium not only through the mechanisms of multidrug resistance, but also with the added complications of a new disease, namely HIV/AIDS, which compromises immunity, thereby neutralizing the host’s first level of defence against the bacterium.

CONCLUSION

So it is said that if you know your enemies and know yourself, you can win a hundred battles without a single loss. If you only know yourself, but not your opponent, you may win or may lose. If you know neither yourself nor your enemy, you will always endanger yourself.

Sun Tzu, The Art of War, Chapter 3 (6th century BC)

Echoing the words of Sun Tzu, in order to be able to conquer the disease, it is essential to understand not only ourselves, but also the organism causing the disease. My work over the past two decades has specifically focused on trying to understand how mycobacteria have evolved to be such successful human pathogens. This has led me on a fascinating voyage of discovery through time, and has allowed me to get a glimpse of the extraordinary, intertwined genealogies of mycobacteria and man, a relationship which began many thousands of years ago on the continent of Africa, a continent on which one of the largest battles in this age-old war is currently being fought. Who will win only time will tell... The war is far from over, with both sides trying to outmanoeuvre the other, and the intertwined history of mycobacterium and man is still in the making...

“We have made substantial progress toward TB elimination in this country, but TB remains a formidable opponent with thousands of cases still diagnosed each year. TB can be fatal and treatment remains long and difficult. This ancient bacterium has demonstrated its ability to evade our attacks many times before.”

Dr. Jonathan Mermin, Director, USA Centers for Disease Control and Prevention’s National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention (2014)

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