Transepithelial migration of *Mycobacterium tuberculosis* strains and their affinity for glial cells *in vitro*

by

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Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science in the School of Laboratory Medicine and Medical Sciences, Department of Medical Microbiology, College of Health Sciences, University of KwaZulu-Natal, Durban

Supervisor: Prof. A. W. Sturm

02 October 2019

DECLARATION

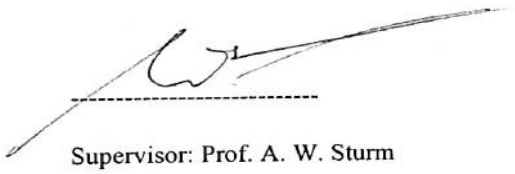
I, Taetso Nkwagatse (Student Number: 217050912), declare that this is my own unaided work. This work has not been submitted previously to the University of KwaZulu-Natal (UKZN) or any other University. Where I have used the work or ideas of others, the appropriate referencing conventions have been adhered to.

Student: Taetso Nkwagatse (217050912)

02/10/2019

Date

As the candidate's supervisor, I agree to the submission of this dissertation.



Supervisor: Prof. A. W. Sturm

2/10/19

Date

DEDICATION

For my mom, Seshegwane Wilheminah and son Tshephang Nkwagatse.

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LIST OF ABBREVIATIONS

Abbreviation	Abbreviated term
%	Percent
µL	Microlitre
°C	Degree Celcius
ANOVA	Oneway analysis of variance
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
BREC	Biomedical Research Ethics Committee
CFU	Colony forming unit
CNS	Central nervous system
CNS-TB	Central nervous system tuberculosis
CO_2	Carbon dioxide
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediamine tetra-acetic acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
HIV	Human immunodeficiency virus
IL	Interleukin
KZN	KwaZulu-Natal
MDR	Multidrug-resistant
MDR-TB	Multidrug-resistant tuberculosis

mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
nm	Nanometre
OADC	Oleic acid, albumin, dextrose, catalase
OD	Optical density
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SPSS	Statistical Package for the Social Sciences
TB	Tuberculosis
TEER	Transepithelial/ transendothelial electrical resistance
TER	Transmembrane electrical resistance
Th1	T helper 1
Th2	T helper 2
TNF	Tumour necrosis factor
UKZN	University of KwaZulu-Natal
USA	United States of America
WHO	World Health Organisation
XDR	Extensively drug-resistant
XDR-TB	Extensively drug-resistant tuberculosis

ABSTRACT

Background: Tuberculosis is an infectious disease caused by the bacillus *M. tuberculosis*. It is a disease that mainly infects the lungs, however, almost any other organ of the body can be infected. Central nervous system TB is a severe form of extrapulmonary TB representing 5 – 15 % of all cases of extrapulmonary TB and accounts for approximately 1 % of all TB cases. Microglia, the resident macrophages of the CNS, are located within the cerebral parenchyma by the BBB. These cells play a role as the first barrier against intra-cerebral infections such as TB. It is unknown whether the susceptibility characteristics of *M. tuberculosis* strains affects the rate at which the bacteria migrate through the alveolar epithelium to other sites of the body.

Materials and methods: M059K glial cells were cultured in a 24-well tissue culture plate then infected with different strains of *M. tuberculosis* (susceptible F1, MDR F28, susceptible and XDR Beijing, and susceptible, MDR and XDR F15/LAM4/KZN) at an MOI of 10. A549 alveolar epithelial cells and M059k glial cells were co-cultured in a 24-well transwell plate. A549 cells were cultured on the insert membranes and M059K glial cells in the wells below the inserts. The A549 cell monolayers were exposed to the different strains. The H37Rv laboratory strain was included as a reference strain.

Results: Invasion capacity, transmigration rate, adhesion and invasion rate following transmigration were established. The susceptible F15/LAM4/KZN and F11 strains showed a high invasion capacity for glial cells while the susceptible Beijing strain showed the lowest invasion capacity. The XDR strains displayed a greater transmigration rate over time. The MDR strains demonstrated lower transmigration rates compared to the susceptible strains. Only the susceptible Beijing strain did not show any transmigration ability. Following transmigration, the XDR strains demonstrated significantly high adhesion and invasion rates. The susceptible Beijing strain still did not show any dissemination ability.

Discussion: We found differences in invasion capacity and transmigration ability between the different strains of *M. tuberculosis*. The invasion capacity of *M. tuberculosis* into glial cells could differ from that of *M. tuberculosis* directly inoculated onto the glial cells. This could be a result of structural changes on the bacterial surface while migrating through the epithelium.

Conclusion: We conclude that *M. tuberculosis* isolates are able to pass through the epithelial lining of the alveoli to other body sites by transepithelial migration, and the XDR isolates pass A549 cells very effectively.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*). It is a disease that mainly infects the lungs, termed pulmonary TB, but almost any other organ of the body can be infected (Sarkar *et al*., 2016). Tuberculosis that occurs at any other organ of the body apart from the lungs is termed extrapulmonary TB (Click *et al*., 2012; Lee, 2015; Sarkar *et al*., 2016). In 2017, the World Health Organisation (WHO) reported 6.4 million incident cases of TB globally and extrapulmonary TB represented 14 % of those cases (World Health Organisation, 2018). Extrapulmonary TB develops as a secondary infection during the lymphatic or hematogenous spread of primary lung infection to other organs of the body (Sharma *et al.*, 2005; Golden & Vikram, 2005; Qin *et al*., 2015). The most common extrapulmonary sites affected include the central nervous system (CNS), bones and joints, lymph nodes, pleura, genitourinary tract, peritoneum and other abdominal organs (Golden & Vikram, 2005; Lee, 2015).

Central nervous system TB (CNS-TB) is a severe form of extrapulmonary TB representing 5 – 15 % of all cases of extrapulmonary TB (Qin *et al*., 2015) and accounts for approximately 1 % of all TB cases (Rock *et al*., 2004; Rock *et al*., 2005; Cherian & Thomas, 2011; Spanos *et al*., 2015). The infection manifests as tuberculous meningitis in most cases but can also manifest as intracranial tuberculoma or spinal arachnoiditis (Golden & Vikram, 2005; Tucker *et al*., 2016). Tuberculous meningitis most commonly affects children, people infected with the human immunodeficiency virus (HIV), and the elderly, causing high rates of morbidity and mortality in these population groups (Rock *et al*., 2004; Cherian & Thomas, 2011; Spanos *et al*., 2015; Tucker *et al*., 2016). Microglia are the local macrophages of the CNS (Cannas *et al*., 2011; Spanos *et al*., 2015). They are located within the cerebral parenchyma by the blood-brain barrier (BBB) functioning as the first barrier against intra-cerebral infections such as TB (Cannas *et al*., 2011; Spanos *et al*., 2015). Cells of the macrophage lineage are the host's primary target cells for *M. tuberculosis* and studies have demonstrated that they are efficiently invaded by this bacillus (Cannas *et al*., 2011; Qin *et al*., 2015; Tucker *et al*., 2016).

Associations between *M. tuberculosis* strains and tropism for extrapulmonary sites have been reported in some studies (Caws *et al*., 2008; Be *et al*., 2011; Click *et al*., 2012; Sarkar *et al*., 2016). These studies have demonstrated that the site of extrapulmonary TB is not only host dependent but also dependent on bacterial characteristics. Understanding of the mechanisms used by *M. tuberculosis* to pass through epithelial barriers during infection was improved by studies using *in vitro* models of mucosal barriers (Birkness *et al*., 1999; Bermudez *et al*., 2002; Ryndak *et al*., 2016). These studies have demonstrated how *M. tuberculosis* exploits the intracellular environment of alveolar epithelial cells to enhance its infectivity and to traverse the alveolar barrier, gaining access into the bloodstream. This allows the bacilli to infect organs other than the lungs. Although these studies have established the association of *M. tuberculosis* strains and their tropism for extrapulmonary sites, the mechanisms by which these *M. tuberculosis* strains transmigrate from the lung to extrapulmonary sites remains understudied.

This is the first study to investigate the transmigration, from the lung to the brain, of endemic and globally relevant *M. tuberculosis* genotypes that spread in multidrug-resistant (MDR) and extensively drug-resistant (XDR) forms. The F15/LAM4/KZN (KZN) genotype is one of those genotypes. It was reported for the first time in KwaZulu-Natal Province, South Africa, in the early 1990s (Pillay & Sturm, 2007) and was responsible for the largest outbreak of XDR tuberculosis (XDR-TB) that occurred in Tugela Ferry, KwaZulu-Natal in 2005 (Gandhi *et al*., 2006). Other relevant genotypes include Beijing, F28, and F11. The Beijing genotype is globally disseminated and most prevalent in Asia (Bifani *et al*., 2002; Glynn *et al*., 2002; Hanekom *et al*., 2011). It is one of the most successfully spreading strains and is also associated with drug-resistant TB globally (Glynn *et al*., 2002; Hanekom *et al*., 2011; Chihota *et al*., 2012). The F28 and F11 genotypes have been reported in drug-resistant TB cases in the Western Cape Province, South Africa (Streicher *et al*., 2004; Nicol *et al*., 2005; Chihota *et al*., 2012). Although these strains have been reported in clinical cases of extrapulmonary TB, there is a lack of correlation between the relative transmigration levels and variation among these isolates in the pathogenesis of CNS-TB.

Extrapulmonary TB develops in a minority of cases, and it was previously not clear whether this was host or microbe dependent. However, studies suggest that both host and microbial factors play a role in the pathogenesis of extrapulmonary TB. A recent study in

our Department demonstrated that some strains of *M. tuberculosis* had a higher affinity for osteoblasts, a cell derived from mesenchymal stem cells present in bone, supporting previous findings that the site of extrapulmonary TB is also microbe dependent (Sarkar *et al*., 2016). We extended this study by comparing the transmigration of KZN, F28, F11 and Beijing strains, using an *in vitro* model comprised of human type II alveolar epithelial cells and human glial cells. Following transmigration, the bacteria were exposed to glial cells to evaluate the difference in affinity for glial cells among the strains to better understand which strain is more likely to cause tuberculosis in the CNS.

1.2. Literature review

1.2.1. *Epidemiology of tuberculosis*

According to WHO, an estimated 10 million people in the world developed TB in 2017. Amongst these people, 58 % were men, 32 % were women, and 10 % were children (World Health Organisation, 2018). Approximately 1.3 million people living without HIV have died from TB in 2017. Among all TB-related deaths, an additional 0.3 million deaths occurred among HIV-positive people (World Health Organisation, 2018). South Africa features on the three lists used by WHO to categorise countries with a high burden of TB, TB and HIV co-infection, and multidrug-resistant TB (MDR-TB). Approximately 227 224 cases of TB were reported in South Africa in 2017 (World Health Organisation, 2018) and of these 7 700 were infected with a drug-resistant strain. The rise in drug resistance, HIV co-infection and the lack of effective vaccines escalates the TB burden in the world.

Tuberculosis is the major cause of death among HIV-positive people (Gandhi *et al*., 2006). The weakened immune system in these patients makes it more challenging to fight the TB infection and therefore leads to the high TB-related mortality among them. The growing TB epidemic is associated with increased HIV infection in sub-Saharan Africa (UNAIDS, 2017; Mohammed *et al*., 2018). Among the 10 million new cases of TB reported in 2017, 9 % were among HIV-positive people, with 72 % of these resided in Africa (World Health Organisation, 2018). Majority of the TB and HIV co-infected cases were found in the southern Africa region (Figure 1.1) in some parts exceeding 50 %. This ranks Africa as the highest region of HIV and TB co-infection globally. South Africa alone constituted 39 % of the global burden of HIV- associated TB (World Health Organisation, 2018). The combined burden of the TB and HIV epidemics remain a crucial public health concern in South Africa (Karim *et al*., 2009; Middelkoop *et al*., 2014; Gouden *et al*., 2018) and KwaZulu-Natal province has the highest number of TB-HIV co-infection patients (Pietersen *et al*., 2014; Niehaus *et al*., 2015; Shah *et al*., 2017) with co-infection rates exceeding 70 % (Shah *et al*., 2017).

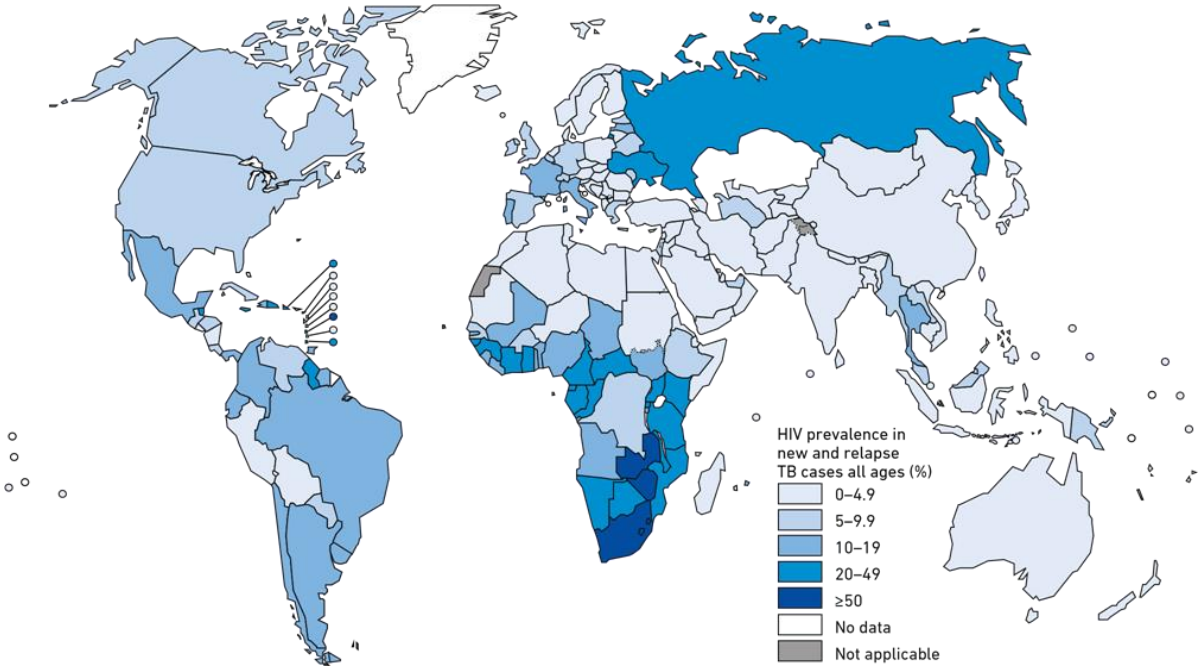

Figure 1.1: A worldwide estimate of the prevalence of HIV-TB co-infection in 2017 (World Health Organisation, 2018)

In addition to the burden of TB-HIV co-infection, the rise in drug resistance poses a serious challenge to TB control programs in the world. Drug-resistant strains of *M. tuberculosis* include MDR and XDR strains. Multidrug-resistant strains are resistant to both isoniazid and rifampicin, which are the first-line drugs for anti-TB treatment, and XDR strains are resistant to rifampicin and isoniazid, plus resistance to any fluoroquinolone and any one of the three injectable drugs (amikacin, capreomycin and kanamycin) (World Health Organisation, 2018). Cases of infection with strains resistant to all the first-line and second-line anti-TB drugs available have been reported (Migliori *et al*., 2007; Velayati *et al*., 2009; Klopper *et al*., 2013). These strains are called totally drug-resistant strains. In 2017, an estimated caseload of 558 000 new cases of MDR-TB was reported globally, and this included 3.5 % of new cases and 18 % of previously treated cases (World Health Organisation, 2018). Fifty-four per cent of the total MDR-TB cases reported globally were notified in Russia, China and India (Figure 1.2). An average of 8.5 % of the estimated MDR-TB cases had XDR-TB. An absolute number of 10 800 cases of XDR-TB were reported, and a total of 230 000 deaths occurred among the drug-resistant TB cases. South Africa is the fourth amongst the five countries that

have reported the most significant number of XDR-TB cases with 747 cases (World Health Organisation, 2018). In South Africa, the highest burden of XDR-TB was reported in KwaZulu-Natal province with 1 027 cases diagnosed between 2011 and 2014 (Shah *et al*., 2017; Kapwata *et al*., 2017).

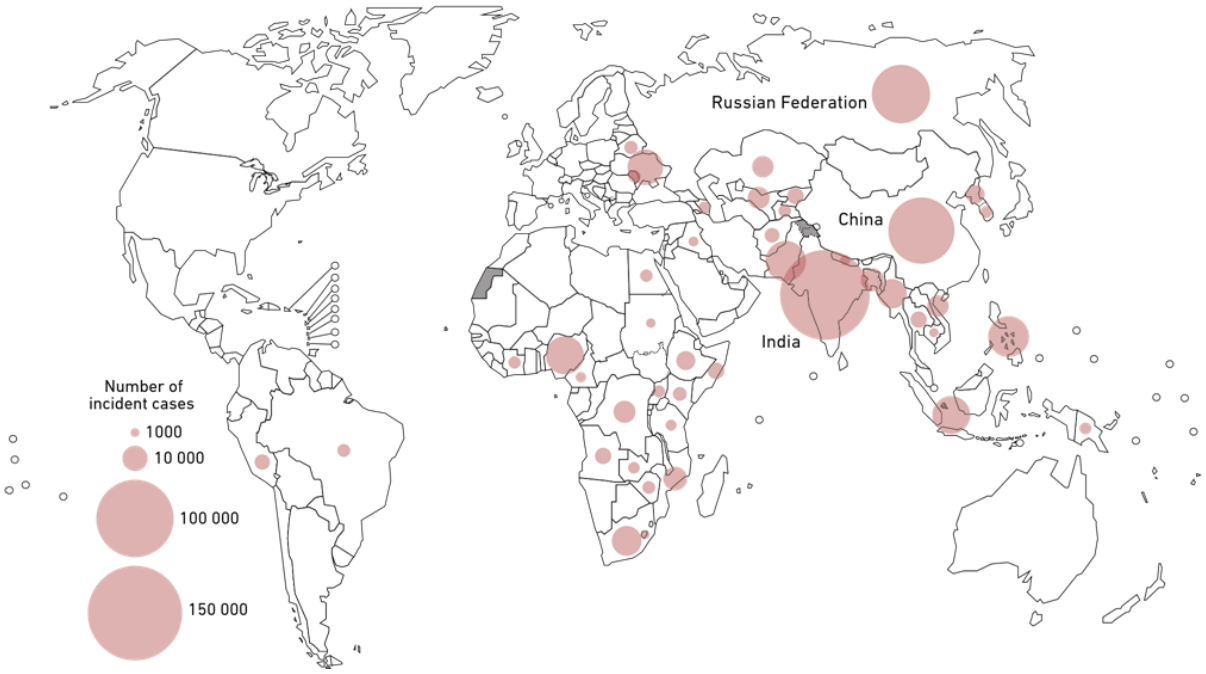

Figure 1.2: A worldwide estimate of the incidence of drug-resistant TB in 2017 (World Health Organisation, 2018)

Pulmonary TB is more prevalent than extrapulmonary TB, with the former being the most common among HIV-infected people (Gouden *et al*., 2018). A compromised immune system is one of the principal risk factors leading to the development of extrapulmonary TB (Naing *et al*., 2013; Gouden *et al*., 2018), making young children and HIV-infected individuals more susceptible to this form of the disease (Cherian & Thomas, 2011). Extrapulmonary TB constitutes 15 – 20 % of all cases of TB globally (Chitra *et al*., 2017). An estimate of 50 % of patients with extrapulmonary TB has still concurrent pulmonary involvement and HIV co-infection (Herath & Lewis, 2014; Lee, 2015; Chitra *et al*., 2017). According to WHO, the global incidence of extrapulmonary TB was estimated to be 14

% in 2017 (World Health Organisation, 2018). The highest incidence occurred in the Eastern Mediterranean (24 %) and the lowest incidence in the Western Pacific region (8 %) (Figure 1.3). Africa had the second highest incidence at 16 %, which is higher than the reported global incidence of 14 % (World Health Organisation, 2018).

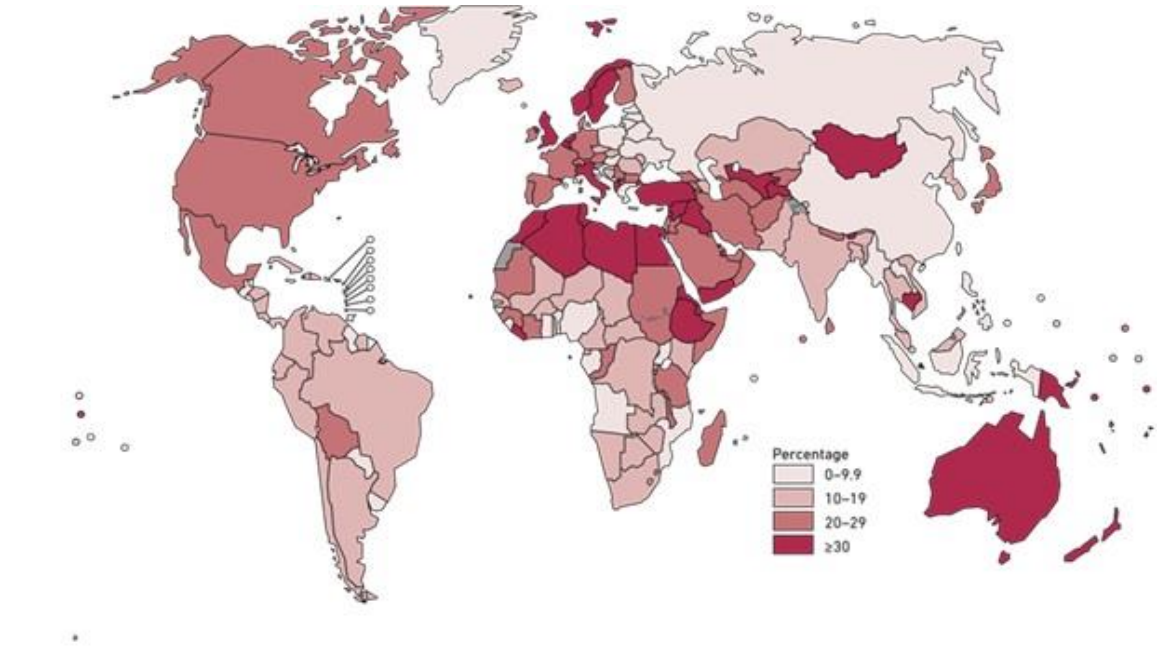

Figure 1.3: The global incidence of extrapulmonary TB in 2017 (World Health Organisation, 2018)

1.2.2. *Predominant Mycobacterium tuberculosis strain families in South Africa*

Molecular epidemiological studies of TB have enhanced our understanding of the diversity of the *M. tuberculosis* genotypes (Bos *et al*., 2014; Coscolla & Gagneux, 2014). Seven lineages of *M. tuberculosis* have been identified and linked with different geographic areas (Bos *et al*., 2014; Coscolla & Gagneux, 2014). Among these seven lineages, lineage 2 (East Asian) and 4 (Euro-American) are the most globally distributed and most virulent lineages when compared to the others (Coscolla & Gagneux, 2014). Figure 1.4 shows that lineage 2 (which includes the Beijing strain family) is predominant

in the Eastern Asia region, but also circulates in the Russia and Southern Africa regions. In South Africa, it is well established that there is a high level of diversity among *M. tuberculosis* strains. The Beijing, F15/LAM4/KZN (KZN), F28 and F11 genotypes were found to drive the burden of TB disease in the country (Streicher *et al*., 2004; Gandhi *et al*., 2006; Mlambo *et al*., 2008; Chihota *et al*., 2012; Kamudumuli *et al*., 2015).

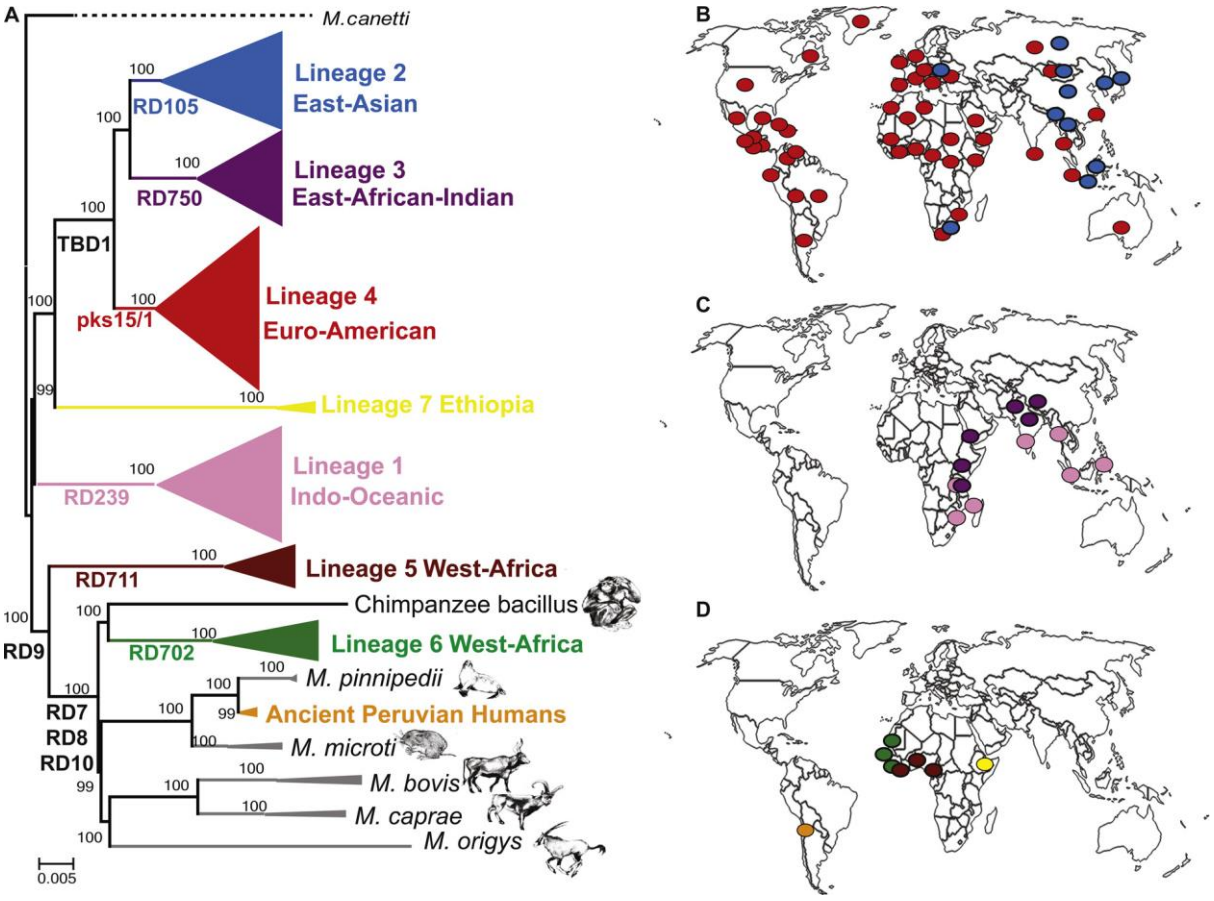

Figure 1.4: (A) Lineages of *M. tuberculosis* represented in colour, (B) the most geographically distributed lineages, (C) lineages with intermediate distribution, and (D) lineages with the least geographical distribution (Coscolla & Gagneux, 2014)

The Beijing strain genotype is one of the main lineages identified to be distributed globally (Gagneux & Small, 2007). This genotype was described for the first time in 1995 and was the first spreading *M. tuberculosis* strain to be found (van Soolingen *et al*., 1995).

It was described as the predominant genotype in Beijing, China, and was found to be distributed similarly in neighbouring countries, including Mongolia, Thailand and South Korea (van Soolingen *et al*., 1995). The lineage has been reported all over the world and also in cases associated with drug resistance in various settings (Frieden *et al*., 1993; Bifani *et al*., 1996; Bifani *et al*., 2002; Glynn *et al*., 2002; Brown *et al*., 2010), including in South Africa (Johnson *et al*., 2010).

The first outbreak of MDR-TB caused by the Beijing strain occurred in the 1900s, in New York, in a population that had a high prevalence of HIV (Frieden *et al*., 1993; Bifani *et al*., 1996). In South Africa, MDR-TB was identified for the first time in the Western Cape Province in 1985, but information about the genotype from these isolates is not available (Streicher *et al*., 2004). Since then, MDR-TB and XDR-TB cases associated with the Beijing strain have been reported, predominantly in the Eastern and Western Cape provinces (Brown *et al*., 2010; Chihota *et al*., 2012; Klopper *et al*., 2013). This strain has been classified as the most successful *M. tuberculosis* strain family due to its worldwide distribution, together with its emergence of drug resistance (Hanekom *et al*., 2011). An association between the Beijing strain and extrapulmonary TB has been reported in past studies (Dale *et al*., 2005; Nicol *et al*., 2005; Maree *et al*., 2007; Caws *et al*., 2008; Click *et al*., 2012). However, these findings are contradictory as positive, negative, and no association between the Beijing strain and extrapulmonary TB has also been reported.

The F15/LAM4/KZN (KZN) strain is the primary strain driving the epidemic of resistant TB in the KwaZulu-Natal province, in South Africa (Gandhi *et al*., 2014). Like the KZN strain, F15 is a strain family that is part of the Latino-American and Mediterranean (LAM) family, and its spoligotype pattern has similarities with the LAM4 sub-lineage (Filliol *et al*., 2002; Streicher *et al*., 2007). The KZN strain has been in existence in KwaZulu-Natal province as early as 1994, the same year in which the first MDR isolate of the strain was identified (Pillay & Sturm, 2007). The first XDR-TB case associated with the KZN strain was notified in 2001 in one patient (Pillay & Sturm, 2007), then followed by the largest XDR-TB outbreak that occurred in Tugela Ferry in 2005 (Gandhi *et al*., 2006). During this outbreak, 98 % of HIV co-infected patients died within an average of 16 days. The F15/LAM4/KZN genotype has developed into a family of closely related strains accompanied by progression from drug-susceptible to MDR then to XDR (Pillay & Sturm, 2007). The rapid spread of the KZN strain and the high mortality rate that occurred

during the 2005 Tugela Ferry outbreak implies increased virulence. The association between the KZN strain and affinity for extrapulmonary sites was reported in one study using osteoblasts (Sarkar *et al*., 2016). The study reported that the invasion capacity of strains increased with resistance level, with the KZN-XDR variant having the highest number of intracellular organisms when compared to other variants (Sarkar *et al*., 2016).

The F28 and F11 strain families are two of the four strains that were identified in more than 70 % of drug-resistant TB cases in the Western Cape Province, South Africa (Streicher *et al*., 2004). The spoligotype pattern of the F28 family corresponds with the S sub-lineage (Filliol *et al*., 2002; Nguyen *et al*., 2003; Brudey *et al*., 2006; Streicher *et al*., 2007) and that of the F11 family corresponds with the LAM3 sub-lineage (Filliol *et al*., 2002; Streicher *et al*., 2007). It has been reported that the F28 strain is responsible for 9.7 % of TB cases among patients from the urban communities in Cape Town (Richardson *et al*., 2002). Although the F28 genotype appears to be common in the Western Cape Province, comparison of the spoligotypes and IS6110-RFLP types in the international National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands database suggests that this strain family may be more worldwide distributed than previously thought (Sarkar *et al*., 2016). This strain family was never reported from other parts of South Africa and other regions of Africa. According to Sarkar *et al*., (2016), the MDR F28 strain has a decreased affinity for osteoblasts (representing an extrapulmonary site) when compared to the MDR- and XDR-KZN strains, and the drug-susceptible F11 strain (Sarkar *et al*., 2016). In the Western Cape Province, the F11/LAM3 strain family is present in 21.4 % of all TB patients, suggesting that it is as much successful as the Beijing strain family which is responsible for 16.5 % cases (Streicher *et al*., 2004; Victor *et al*., 2004). Although the F11/LAM3 strain is predominant in the Western Cape, the strain has also been reported from other parts of South Africa (Mlambo *et al*., 2008; Chihota *et al*., 2012; Kamudumuli *et al*., 2015) and was also reported to be globally distributed (Victor *et al*., 2004). Findings from Sarkar *et al*., (2016) demonstrated the F11/LAM3 strain as the most invasive strain in osteoblasts (Sarkar *et al*., 2016), implying increased virulence in this extrapulmonary site.

1.2.3. *Pathogenesis of tuberculosis*

Tuberculosis infection occurs when droplet nuclei containing *M. tuberculosis* bacilli, are expelled into the air from the lungs of a person infected with TB and inhaled by a healthy person (Torrelles & Schlesinger, 2017; Koul *et al*., 2011). In the classic pathogenesis model, after inhalation of the bacilli, they settle in the alveoli of the lung, where alveolar macrophages then phagocytose them. Recent findings indicate that the bacilli also invade alveolar epithelial cells, but not bronchiolar cells (Ashiru *et al*., 2010). The infection can either be eliminated by innate and adaptive immune responses or become an asymptomatic infection known as latent TB infection or develop into active disease. Innate and adaptive immune responses are initiated, following cytokine secretion and immune cell recruitment induced by pathogen recognition processes, to clear the pathogen from the infected site (Cantone *et al*., 2017). During latent TB infection, bacilli are surrounded but not completely eliminated, and infected individuals are asymptomatic upon initial infection (Kumar *et al*., 2011). This latent infection can last a lifetime, but a compromised immune system increases the risk of disease activation (Kumar *et al*., 2011). Active TB develops in only 5 - 10 % of people with latent TB. This is referred to as reactivation disease, a term which refers to the reactivation of dormant bacilli, not to the second episode of disease. Active TB is characterised as an infection with signs of clinical disease. It develops when the host fails to develop an immune response that can control the initial infection (Gengenbacher & Kaufmann, 2012).

Although the lung is the primary site of infection, *M. tuberculosis* can disseminate and also infect other parts of the body. *Mycobacterium tuberculosis* can initiate extrapulmonary TB by lymphatic or hematogenous dissemination of the bacilli from the lung to other organs of the body (Sharma *et al.*, 2005; Golden & Vikram, 2005; Qin *et al*., 2015), including the CNS (meninges), lymph nodes, bones and joints, pleura, genitourinary tract, peritoneum and other abdominal organs (Golden & Vikram, 2005; Lee, 2015). The CNS is the most serious location where infection may result in meningitis or a brain tuberculoma, which is fatal in most cases (Golden & Vikram, 2005). Tuberculous meningitis is an essential manifestation of the disease in both infants and immunocompromised individuals. Central nervous system TB is a secondary infection resulting from the dissemination of infection from the lungs to the meninges and brain

parenchyma via the circulation (Donald *et al.*, 2005; Rock *et al.*, 2008; Be *et al.*, 2009). Despite the protective properties of the BBB, it has been suggested that *M. tuberculosis* can pass through the BBB and invade the CNS as free bacilli. These findings are supported by studies that have illustrated the upregulation of a specific gene of *M. tuberculosis* associated with traversal of the BBB (Jain *et al.*, 2006). However, the mechanisms by which *M. tuberculosis* evades the protective properties of the BBB remain understudied. Infection of the meninges, the formation of localised foci (Rich foci), followed by the release of bacilli into the subarachnoid space are first events characterising CNS-TB (Donald *et al.*, 2005). When the bacilli are deposited in large numbers, tuberculous meningitis or formation of tuberculomas may occur, especially in infants or young children (Be *et al*., 2009). However, deposited bacilli may not induce an immune response in older children and adults. This may cause latent CNS disease, and when the bacilli are later recognised by the immune system or reactivated, the formation of tuberculomas in the CNS ensues (Be *et al*., 2009). These tuberculomas may then rupture into the cerebrospinal fluid, leading to severe inflammation and tuberculous meningitis. Tuberculous meningitis typically presents an insidious subacute course, and for most untreated patients, death ensues within five to eight weeks of the onset of illness (Ramirez-Lapausa *et al*., 2015). Figure 1.5 below illustrates the dissemination of pulmonary TB to extrapulmonary TB in the CNS.

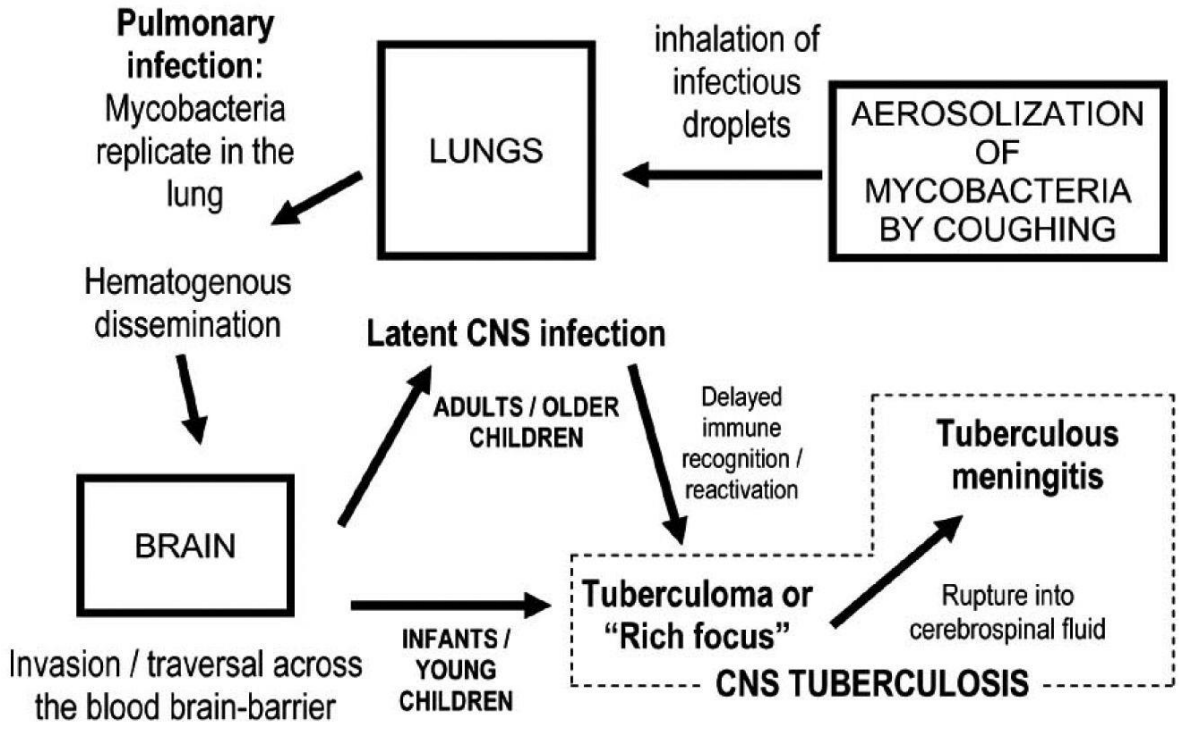

Figure 1.5: Dissemination of TB from the lungs to the CNS (Be *et al*., 2009)

1.2.3.1. *Immune response to tuberculosis*

The human immune response consists of the innate and adaptive immune responses. Cooperation of both immune responses is required for the host to efficiently eliminate invading microbial pathogens (Smith, 2003; Dannenberg, 2006; Kawai & Akira, 2010). An innate immune response is a form of natural immunity whereby immune components, mainly complement, neutrophils and NK-cells, provide the first line of protection against an invading pathogen that the host has not encountered previously. This natural immunity is facilitated by macrophages and dendritic cells (DCs) resident at the site of infection following the recognition of *M. tuberculosis* bacilli (O'Garra *et al*., 2013).

Pathogen recognition elicits chemokine and cytokine secretion resulting in immune cells recruitment and activation, initiating an immune response to clear the pathogen from the infected site (Roitt *et al*., 2002). Once the pathogen is recognised, the mycobacteria are engulfed by alveolar macrophages. These alveolar macrophages are considered to be the

first cellular element of the immune response (Chen *et al*., 2018). The ability of these cells to destroy ingested pathogens depends on their innate microbicidal capacity (Chen *et al*., 2008; O'Garra *et al*., 2013). Dendritic cells and neutrophils, as well as the pulmonary epithelial cells lining the alveoli also internalise the bacilli (Bermudez & Goodman, 1996; O'Garra *et al*., 2013). Studies report that *M. tuberculosis* is more likely to encounter alveolar macrophages than alveolar epithelial cells after inhalation of the bacilli (Cohen *et al*., 2018; Huang *et al*., 2018). However, the number of alveolar epithelial cells present in the alveolar space is high compared to the number of macrophages (Bermudez & Goodman, 1996; Chuquimia *et al*., 2012; Randall *et al*., 2015). Therefore, the likelihood of the bacilli encountering an alveolar epithelial cell instead of a macrophage is higher (Bermudez & Goodman, 1996; Bermudez *et al*., 2002; Ashiru *et al*., 2010; Chuquimia *et al*., 2012; Randall *et al*., 2015). This initial interaction of *M. tuberculosis* with epithelial cells might result in chemokine secretion (Cantone *et al*., 2017), which recruit macrophages and neutrophils to the primary site of infection (Chuquimia *et al*., 2012). When strong enough, the innate immune response can eliminate the pathogen. However, if the bacilli survive via its mechanisms acquired to allow intracellular survival and proliferation, they continue to replicate actively in the macrophages and subsequently spread to adjacent cells and tissues (Wolf *et al*., 2008). The intracellular proliferation of the bacilli induces a robust inflammatory response in the macrophages, inducing production of cytokines like interleukin (IL)-1, IL-8, IL-12, interferon (INF)-γ and tumour necrosis factor (TNF)-α (Silva *et al*., 2012). An inflammatory response is essential for the host to control the infection (Smith, 2003). The inflammatory response results in the activation and recruitment of more macrophages from the circulation and other phagocytic cells (Torrelles & Schlesinger, 2017). Simultaneously, resident DCs in the lung engulf the bacilli or products derived from its intracellular proliferation, and the ongoing inflammatory signals promote DC migration from the lung to the draining lymph node, activating T cells of the adaptive immune system (Tian *et al*., 2005; Wolf *et al*., 2008).

The adaptive immune system is extremely specific, as it is influenced by the immune system's previous interaction with a pathogen, or its immunogenic components (antigens) (Vankayalapati & Barnes, 2009). It is mediated by T and B lymphocytes, facilitated by specific antigen receptors that are expressed on the surface of these cells, and its

immunological memory makes the adaptive response long-lasting. Following the recognition of the infected macrophages and migration of DCs to the draining lymph nodes, T cell activation and expansion occurs (Shi & Sugawara, 2013). T cells can be grouped into two subsets, T helper (Th) 1 and Th2, based on the cytokines they produce. T helper 1 cells play a significant role in defence against tuberculosis, and they suppress Th2 cells during tuberculosis (Shi & Sugawara, 2013). Th1 cells produce significant amounts of TNF-α and IFN-γ, which increases the microbicidal capacity of the infected macrophages, which eventually result in the intracellular killing of *M. tuberculosis* bacilli (O'Garra *et al*., 2013). The Th1 response, however, does not lead to complete elimination of the bacilli but the formation of granuloma. The granuloma attempts to "wall off" and restrict the growth of the bacilli by surrounding it with immune cells (Russell, 2007; Russell *et al*., 2009; Ramakrishnan, 2012; Mahamed *et al*., 2017). Live *M. tuberculosis* bacilli remain confined inside the granuloma, protected from the host immune response, often in a non-metabolically active state for a long time or most often for a lifetime. This state is called latent infection (Silva *et al*., 2012). Although granulomas contain the infection, they do not always succeed. They may progress to central necrosis and cavitation, promoting multiplication and dissemination of the pathogen into the airways, release of the *M. tuberculosis* into the environment and transmission of the infection to susceptible hosts (Russell, 2007; Barry *et al*., 2009; Russell *et al*., 2009; Ramakrishnan, 2012; Mahamed *et al*., 2017). Failure of the granuloma to contain infection and reactivation of infection may result from events that weaken the host immune defences such as HIV infection (Kumar *et al*., 2011; Silva *et al*., 2012).

Within the CNS, microglial cells are located in the brain parenchyma by the BBB and are the first line of defence against *M. tuberculosis* (Spanos *et al*., 2015). Following infection with *M. tuberculosis*, activated microglia release proinflammatory cytokines that contribute towards defence against the pathogen (Rock *et al*., 2005; Yang *et al*., 2007; Hernandez Pando *et al*., 2010; Be *et al*., 2011; Cannas *et al*., 2011). Some of the chemokines and cytokines reported in *in vitro* studies include TNF-α, IL-6 and IL-1β (Rock *et al*., 2005; Cannas *et al*., 2011). Similar cytokines were reported in *in vivo* studies (Hernandez Pando *et al*., 2010; Be *et al*., 2011; Francisco *et al*., 2015).

1.2.4. *Interaction between epithelial cells and Mycobacterium tuberculosis*

An important component of the mucosal barrier and innate immune response of the lung that protects it against respiratory pathogens is the alveolar epithelium. This epithelium is made up of type I and II pneumocytes, providing a barrier function that detects pathogens through pattern recognition receptors, and secretion of surfactant proteins, chemotactic factors, cytokines and antimicrobial molecules (Lin *et al*., 1998; Chuquimia *et al*., 2013). The focus of most studies is to investigate the role of alveolar macrophages and other professional phagocytes such as neutrophils, dendritic cells and monocytes in the development of *M. tuberculosis* infection. However, increasing evidence implicates the type II alveolar epithelial cells in both host cell defence and bacterial pathogenicity (Chuquimia *et al*., 2012; Ryndak *et al*., 2015). The logic for studying the interaction between mycobacteria and epithelial cells in addition to the ability of *M. tuberculosis* to infect these cell types is that the alveoli of the lung are made up of over 95 % epithelial cells (Bermudez & Goodman, 1996; Chuquimia *et al*., 2012). Therefore, it is more likely that *M. tuberculosis* will interact with epithelial cell first before encountering any other cell (Ashiru *et al*., 2010; Chuquimia *et al*., 2012). The interaction between *M. tuberculosis* and epithelial cells may result in the activation of macrophages and could facilitate their migration to the site of infection.

The first study of *M. tuberculosis*-epithelial cell interactions established the use of the immortalised human alveolar epithelial cell line A549 (McDonough & Kress, 1995). This model proved to be suitable for examining the interaction of *M. tuberculosis* with the alveolar epithelium. This study revealed two significant findings. First, *M. tuberculosis* can adhere to and invade respiratory epithelial cells with virulent strains noted to be more cytotoxic and second, the intracellular passage of *M. tuberculosis* within macrophages enhances the association between *M. tuberculosis* and epithelial cells (McDonough & Kress, 1995). Subsequent to this study, Bermudez and Goodman (1996) reported on findings that demonstrate the intracellular replication of *M. tuberculosis* within A549 cells (Bermudez & Goodman, 1996). It was observed that efficient invasion and intracellular replication in alveolar epithelial cells is possible (Bermudez & Goodman, 1996). Other studies have also supported this theory by comparing the intracellular multiplication of *M. tuberculosis* in cultures of macrophages and bronchial cells (Mehta

et al., 1995; Ashiru *et al*., 2010). These comparison studies demonstrated that the rate of intracellular replication of *M. tuberculosis* was significantly higher in alveolar epithelial cells than in the other cell types (Mehta *et al*., 1995; Ashiru *et al*., 2010).

During the progression of TB, *M. tuberculosis* destructs and disrupts the alveolar epithelium to allow the bacilli to gain passage back into the airways to enable dissemination to a new host. Based on this, several studies were initiated to demonstrate whether the A549 model could be used to reproduce this phenomenon *in vitro*. Cytotoxicity of epithelial cells is a result from infection with virulent *M. tuberculosis* strains (McDonough & Kress, 1995) and this cytotoxicity is caused by cellular necrosis (Dobos *et al*., 2000; Ashiru & Sturm, 2015). Dobos *et al*., (2000) reported that the cellular necrosis in A549 monolayers did not occur due to intracellular or extracellular growth but as a result of A549 cell membrane permeation (Dobos *et al*., 2000). This necrosis required infection with viable *M. tuberculosis*, suggesting that the observed cell toxicity was due to a bacterial factor(s) (Dobos *et al*., 2000). It was further demonstrated that cytotoxicity levels in alveolar epithelial cells are significantly higher in virulent strains grown under anaerobic conditions (Ashiru & Sturm, 2015).

Studies have enriched our understanding of mechanisms used by *M. tuberculosis* to pass through epithelial barriers during infection using *in vitro* models of mucosal barriers (Birkness *et al*., 1999; Bermudez *et al*., 2002; Ryndak *et al*., 2016). Birkness *et al*., (1999) devised the first *in vitro* model of an alveolar epithelium (Birkness *et al*., 1999). A polarised bilayer of epithelial and endothelial cells was assembled and used to measure the ability of *M. tuberculosis* to cross the bilayer. Using this model, it was observed that large numbers of bacteria adhered to and invaded the epithelial cells and that a small percentage of the inoculum translocated the bilayer (Birkness *et al*., 1999). Furthermore, the migration of peripheral blood mononuclear cells through the bilayer to the upper chamber was observed when these cells were added to the lower chamber. These cells were seen associated with *M. tuberculosis* on the epithelial cells (Birkness *et al*., 1999). Similarly, Bermudez *et al*., (2002) confirmed the previously observed translocation of *M. tuberculosis* across the bilayer (Bermudez *et al*., 2002). In this study, bacteria recovered from infected A549 cells or macrophages was increased, again demonstrating that an invasive phenotype emerges following intracellular passage (Bermudez *et al*., 2002).

Furthermore, the addition of infected and uninfected monocytes to the bilayer demonstrated efficiency in the translocation of infected monocytes than the uninfected ones (Bermudez *et al*., 2002). Additionally, infection of the A549 cells in the bilayer with *M. tuberculosis* also increased monocyte translocation (Bermudez *et al*., 2002). Although these studies have demonstrated that monocytes serve as a transport vehiculum for the dissemination of *M. tuberculosis* through the epithelial-endothelial bilayer, other studies have proven that the bacilli are also able to migrate independently (Pethe *et al*., 2001; Ryndak *et al*., 2016). These results strongly suggest that *M. tuberculosis* could exploit epithelial cells as protection against immune responses in which they also expand and acquire a phenotype that enables rapid dissemination during primary infection. This ability to pass and damage the epithelial barrier may, therefore, contribute to the dissemination of bacilli from the lungs to other organs.

1.2.5. *Interaction between glial cells and Mycobacterium tuberculosis*

Glial cells, along with neurons, form the CNS. Glial cells support and nourish neurons, and isolate and shield the nervous tissues against injuries from foreign bodies (Cannas *et al*., 2011). Microglia, one of the glial cells, are the inhabitant macrophages of the brain parenchyma, and they share numerous, if not all, characteristics of macrophages in other tissues. Such characteristics include the production of different cytokines and chemokines induced by several stimuli (Curto *et al*., 2004; Cannas *et al*., 2011) such as *M. tuberculosis* infection. For *M. tuberculosis* to reach the CNS, it must first cross the BBB. Mechanisms involved in this process are not well studied. A study by Jain *et al*., (2006) developed an *in vitro* model of the BBB which was used to identify microbial components of *M. tuberculosis* associated with invasion and traversal of the BBB during CNS-TB pathogenesis (Jain *et al*., 2006). In this study, virulent *M. tuberculosis* strains were able to invade and traverse the endothelial cell monolayer more efficiently than non-pathogenic strains. This *in vitro* model used human brain microvascular endothelial cells, which makes up the BBB but did not include cells found in the brain side of the BBB. Therefore, the role played by these cells in defence against entry into the brain parenchyma is not well studied.

In vitro studies have established microglial cells as the principal host cells of *M. tuberculosis*. Invasion of these cells results in the activation and release of proinflammatory cytokines such as TNF-α and IL-6 (Rock *et al*., 2005; Cannas *et al*., 2011). Following infection of human microglia and astrocytes with *M. tuberculosis in vitro*, Rock *et al*., (2005) observed an invasion of 76 % and 15 % respectively (Rock *et al*., 2005). Furthermore, microglia released robust amounts of numerous cytokines and chemokines than astrocytes (Rock *et al*., 2005), implying that microglia play an essential role in the pathogenesis of CNS-TB. In another study, it was demonstrated that non-invasive lysates of *M. tuberculosis* induce a similar inflammatory response and reactive oxygen species (ROS) generation as in infection with live *M. tuberculosis* in microglial cell lines (Yang *et al*., 2007). In addition, primary mixed glial cells (astrocyte-enriched cultures) induced less robust cytokine production and ROS generation compared to the microglial cell lines (Yang *et al*., 2007). These findings support previous findings that *M. tuberculosis* selectively infects human microglia rather than astrocytes (Rock *et al*., 2005). These studies have established that the activation of microglial cells by *M. tuberculosis* occurs through cytokine and chemokine responses, and protein and gene expression studies support these findings (Qin *et al*., 2015). Similar as *in vitro* studies, animal models have also demonstrated that activated microglia are recruited to TB lesions and are noted to have intracellular bacilli (Hernandez Pando *et al*., 2010; Be *et al*., 2011; Francisco *et al*., 2015; Tucker *et al*., 2016). These *in vitro* and *in vivo* studies have sufficiently explored host-microbe interactions by targeting crucial inflammatory pathways involved in CNS-TB pathogenesis.

Past studies have indicated that the differences in *M. tuberculosis* genotypes can contribute to variations in host immune response, virulence and clinical outcome of disease (Caws *et al*., 2008; Click *et al*., 2012; Sarkar *et al*., 2016). Additionally, it has been demonstrated that dissemination and pathology due to strain specificity is possible, suggesting that virulence factors might enable invasion and survival of *M. tuberculosis* in the CNS (Hernandez Pando *et al*., 2010; Be *et al*., 2011). When bacterial and host genotypes, including their interaction, were compared across two large groups of Vietnamese adults with meningeal or pulmonary TB, Caws *et al*., (2008) demonstrated an association between *M. tuberculosis* genotype and disease outcome (Caws *et al*., 2008). Disease caused by the Euro-American lineage was less likely to be meningeal than

pulmonary, suggesting that this lineage may be less capable of extrapulmonary dissemination in this Vietnamese study population (Caws *et al*., 2008). Data from this study also suggest that specific genes found in hosts are associated with meningeal TB caused by the East Asian (Beijing) genotype (Caws *et al*., 2008). In contrast, when the East Asian/Beijing lineage was compared with three other lineages (East-African Indian, Euro-American and Indo-Oceanic lineages), the East Asian lineage was relatively less associated with extrapulmonary TB rather than pulmonary TB (Click *et al*., 2012). Previous studies have also reported a positive association between the East Asian lineage with pulmonary TB (Dale *et al*., 2005). Although the above findings prove that there is an association between *M. tuberculosis* genotype and disease outcome, studies from the Western Cape Province in South Africa found no association between *M. tuberculosis* genotype and clinical presentation or disease outcome (Nicol *et al*., 2005; Maree *et al*., 2007).

Microglia phagocytose virulent *M. tuberculosis* more rapidly and efficiently than less virulent strains (Curto *et al*., 2004). It is in these cells where *M. tuberculosis* bacilli retain a potential to proliferate, therefore providing a conducive environment for persistence and mechanism for subsequent reactivation should a state of immunosuppression be acquired (Curto *et al*., 2004; Cannas *et al*., 2011). Based on findings that the invasion capacity of the KZN strain increases with the level of resistance when compared to the F28 (MDR) and F11 (susceptible) strains (Sarkar *et al*., 2016), and that *M. tuberculosis* strains are able to migrate across *in vitro* alveolar barriers (Ryndak *et al*., 2016), it is of importance to determine how these strains compare in terms of their ability to cross the alveolar barrier and subsequently invade extrapulmonary sites like the CNS.

1.3. Significance of this study

Studies have adequately explored the association between *M. tuberculosis* strains and site of extrapulmonary TB. However, these studies investigated mostly the H37Rv laboratory strain and the CDC 1551 clinical strain, suggesting a lack of strain diversity when investigating host-microbe interaction in the development of extrapulmonary TB. Additionally, most of the *in vivo* studies have used intra-cerebral injection models which

do not imitate the natural CNS disease occurrence which is caused by hematogenous dissemination of *M. tuberculosis* from the lung, followed by the invasion of the CNS. A previous study in our Department has reported an association between different *M. tuberculosis* strains and tropism for osteoblasts. Therefore, we extended this study and compared the ability of the same strains to translocate the alveolar epithelial cell barrier and the subsequent invasion of microglial cells. These findings may contribute to the growing body of knowledge on CNS-TB pathogenesis and associated strain resistance profile.

1.4. Aim of this study

To determine and compare the transmigration of F15/LAM4/KZN, Beijing, F28 and F11 strain families of *M. tuberculosis* across an *in vitro* alveolar epithelial cell barrier and their affinity for microglial cells.

1.5. Objectives of this study

i. To co-culture human alveolar epithelial cells and human microglial cells in a transwell system.
ii. To infect the alveolar epithelial cells with F15/LAM4/KZN, Beijing, F28 and F11 strains.
iii. To analyse the transmigration of these strains across the alveolar epithelial cell monolayer into the microglial cell monolayer.
iv. To determine the intracellular multiplication of these strains in microglial cells.

1.6. Study design

A human microglial cell line was cultured in a 24-well tissue culture plate, then infected with different *M. tuberculosis* isolates for 24 hours. Following incubation, the cells were lysed and plated on Middlebrook 7H11 agar plates to count the number of colony-forming units (CFU) per mL (CFU/mL). The CFUs were counted after three weeks and analysed.

A co-culture of human alveolar epithelial cells and human microglial cells was developed by growing A549 epithelial cells on transwell insert membranes and M059K glial cells in the wells of the transwell plate. The A549 cells were inoculated with various strains of *M. tuberculosis* for 48 hours. The spent culture media was harvested and plated on Middlebrook 7H11 agar plates to perform a CFU count, after three weeks, in order to analyse the ability of the various *M. tuberculosis* strains to transmigrate the A549 epithelial cell monolayer. Following which, the M059K glial cell monolayers were lysed and also plated on Middlebrook 7H11 agar plates to perform a CFU count to further evaluate the ability of the strains to invade the glial cell monolayer following transmigration of an alveolar epithelial cell monolayer.

1.7. Dissertation structure

This dissertation is written in the traditional format, as accepted by the University of KwaZulu-Natal, comprising of four chapters. Chapter one contains the introduction and literature review. Chapter two details the materials and methods employed for the experimental procedures used in this study. Chapter three contains the results obtained, and chapter four comprises of the discussion, which examines the outcomes in the context of what has been published about this topic, limitations, conclusions drawn, as well as recommendations for future studies.

CHAPTER 2: MATERIALS AND METHODS

2.1. Ethics approval

The study's ethics approval was acquired from the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal (Reference number: BE301/17).

2.2. Growth of Mycobacterium tuberculosis strains

All experiments with *M. tuberculosis* were conducted in a Biosafety Level II Laboratory at the Department of Medical Microbiology, University of KwaZulu-Natal. An N95 mask (3M Health Care, St. Paul, Minnesota, USA) and powder-free latex gloves were worn at all times. The *M. tuberculosis* strains were recovered from the culture collection of the Department. These included three strains belonging to the F15/LAM4/KZN (KZN) family, two strains to the Beijing family and one strain each to the F11 family and the F28 family. The laboratory strain H37Rv (ATCC 27294) was used as a reference strain. Table 2.1 shows the drug susceptibility and genotype profiles of the strains used in this study. Multidrug-resistance (MDR) was described as resistance to both isoniazid and rifampicin, which are the major first-line drugs used against TB, and extensive drug resistance (XDR) as resistance to rifampicin and isoniazid, plus resistance to any fluoroquinolone and any one of the three injectable drugs (capreomycin, kanamycin, and amikacin) (World Health Organisation, 2018).

The isolates were retrieved from the – 70 °C freezer and thawed at room temperature. Following this, 100 µL of the suspension of each strain was inoculated in 10 mL of Middlebrook 7H9 broth (Difco Laboratories, Becton, Dickinson and Company, Sparks, USA), supplemented with 10 % of a mixture of oleic acid, albumin, dextrose, catalase (OADC) (Becton, Dickinson and Company, Sparks, USA) and 0.2 % glycerol (Rochelle Chemicals, Johannesburg, South Africa). The broth cultures were incubated at 37 °C in a shaking incubator until an optical density of 1.0 measured at a wavelength of 600 nm (OD_{600nm}) was reached. The H37Rv laboratory strain, MODS 688 and MODS 388 strains reached an OD_{600nm} of 1.0 at day 7 post inoculation while the TF 832, TF 44949 and V4207 strains reached OD_{600nm} of 1.0 at day 8. The TF 1516 and R4933 reached OD_{600nm}

1.0 at day 10 after inoculation into the Middlebrook 7H9 broth. The cultures were subsequently subcultured on Middlebrook non-selective 7H11 (Difco Laboratories, Becton, Dickinson and Company, Sparks, USA) agar plates supplemented with 10 % OADC and 0.5 % glycerol (enriched Middlebrook agar). The plates were sealed with CO_2-permeable plastic bags and incubated at 37 °C for three weeks.

Table 2.1: Strains of *Mycobacterium tuberculosis* used in the study and their susceptibility profiles

Strain number	***Mycobacterium tuberculosis* strain family**	**Resistant to:**	**Susceptibility classification**
TF 1516	Beijing	-	Susceptible
R4933	Beijing	I, R, E, P, S, Eth, A, C, K, O	XDR
V4207	F15/LAM4/KZN	-	Susceptible
MODS 688	F15/LAM4/KZN	I, R, E, P	MDR
MODS 388	F15/LAM4/KZN	I, R, E, P, S, Eth, A, C, K, O	XDR
TF 44949	F28	I, R, E, P	MDR
TF 832	F11/LAM3	-	Susceptible

I isoniazid, R rifampicin, E ethambutol, P pyrazinamide, S streptomycin, Eth ethionamide, A amikacin, C capreomycin, K kanamycin, O ofloxacin, XDR extensively drug-resistant, MDR multidrug-resistant

2.2.1. *Inoculum preparation*

A loop full of *M. tuberculosis* culture grown on enriched Middlebrook 7H11 agar plate was harvested using a disposable plastic loop. To prepare bacterial single-cell suspensions, colonies were harvested into tubes containing six sterile glass beads (Associated Chemical Enterprises, Johannesburg, South Africa) and 6 mL of phosphate-

buffered saline (PBS), pH 7.3 (Oxoid, Basingstoke Hampshire, England). To ensure that bacterial clumps are disrupted, the harvested bacteria were smeared carefully against the side of the tube before introduction into the fluid. The suspension was centrifuged at 3000 x *g* for 20 minutes and resuspended in complete growth DMEM: F12 media. The suspension was subsequently vortexed for 5 minutes and after that allowed to stand for a further 15 minutes to allow the larger bacterial clumps to settle. The top 4 mL of the bacterial suspension was aspirated and transferred into a sterile tube. This aspirated suspension was subsequently passed four times through a sterile 25-gauge needle (Avacare Health, South Africa) followed by filtration through a 5.0 µm Millipore (Millex) filter (Merck Millipore, Tullagreen, Ireland). The measured OD_{600nm} was used to standardise the suspensions. The suspension of each bacterial strain was diluted to obtain the target concentration of 1 X 10^7 bacteria/mL. From the suspensions, 10-fold serial dilutions were made in PBS, pH 7.3, and aliquots of 100 µL of each dilution were cultured on enriched Middlebrook 7H11 agar plates, in triplicate, to verify the number of colony-forming units (CFU) per mL (CFU/mL). These plates were sealed with CO_2-permeable plastic bags and incubated in a 5 % CO_2 incubator at 37 °C for three weeks.

2.3. Culture of cell lines

Two types of cell lines were used in this study; human type II alveolar epithelial cell line A549 (ATCC CCL-185) and human glial cell line M059K (ATCC CRL-2365). Both cell lines were maintained under the same conditions, in 5 % CO_2 in a humidified atmosphere at 37 °C. The medium was changed every 2 or 3 days unless stated otherwise.

The A549 cells were cultured in Eagle's Minimum Essential Medium (EMEM) (Lonza, Walkersville, USA) with 2 mM L-glutamine, 25 mM HEPES and 10 % fetal bovine serum (FBS) (Biowest, Nuaillé, France) added to the media. The M059K cells were cultured in a mix of equal amounts (ratio 1:1 v/v) of Dulbecco's Modified Eagle's Medium and Ham's F12 medium (DMEM: F12) (Lonza, Walkersville, USA) containing 2.5 mM L-glutamine, 15 mM HEPES and 10 % FBS per 500 mL.

Both cell lines were subcultured when 80 – 90 % confluency was reached. The cells were washed three times with 10 mL of warm PBS, pH 7.3. This was followed by addition of

2 mL of a trypsin-versene mixture (Lonza, Walkersville, USA). The trypsin-versene mixture contains 0.5 g/L trypsin and 0.2 g/L versene (EDTA). Cells were incubated with the trypsin-versene at 37 °C for 3 – 10 minutes, depending on the cell type. After incubation cells were observed under an inverted microscope to check if they were detached from the flask. Two mL of FBS was then added to the culture flask to inactivate trypsin. The trypsinised cells were transferred to a 15 mL centrifuge tube and counted to determine the number of viable cells. The desired concentration of viable cells per cm^2 was resuspended in new flasks containing an appropriate aliquot of the complete growth medium appropriate for each cell line.

The Trypan Blue Exclusion assay was used to determine the number of viable cells. In a Trypan Blue Exclusion assay, viable cells are distinguished by the ability to exclude the trypan blue dye due to their intact cell membrane, whereas the dye passes the cell membrane of non-viable cells. Viable cells appear clear and non-viable cells appear blue under a light microscope. A cell count was performed before seeding cells for infection experiments. To perform a cell count, 20 µL of trypsinised cell suspension and 20 µL of a 4 % Trypan Blue (Lonza, Walkersville, USA) dye were mixed in an Eppendorf tube, then 10 µL of the mixture was filled into a Neubauer haemocytometer chamber (Figure 2.1). A Neubauer haemocytometer chamber has nine large squares. The four large squares at the corners, made up of a set of 16 small squares, were used to count viable cells. Viable cells located within each of the four large squares were counted under a light microscope. Cells located on the left and top lines of each square were included in the count, whereas those found on the right and bottom lines were excluded. Cell number was calculated using the following formula:

$$\text{Total no of cells (cells per mL)} = \frac{\text{no. of cells}}{\text{no. of squares}} \times \text{dilution factor} \times 10^4$$

Where no. of cells is the number of viable cells counted within the four large squares, no. of squares represent the four large squares, and the dilution factor is the volume of cell suspension diluted with trypan blue dye that was added to the haemocytometer.

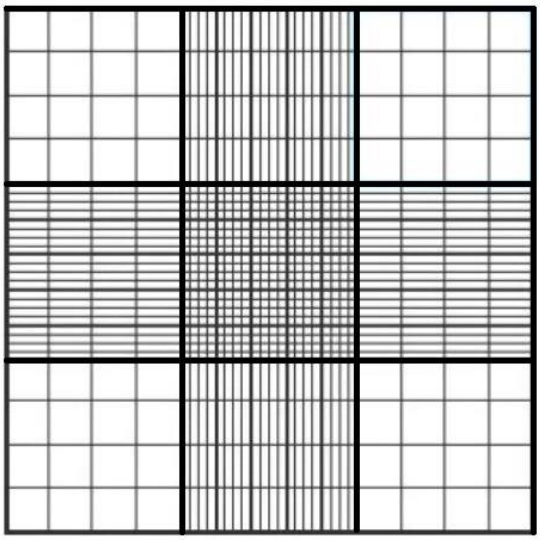

Figure 2.1: Neubauer haemocytometer chamber

2.4. Co-culture of A549 and M059K cell lines

Co-culture of cell monolayers was set up in 24-well plates (Costar, Corning-Incorporated, Kennebunk, USA) with transwell permeable supports (Costar, Corning-Incorporated, Kennebunk, USA) with a membrane pore size of 3.0 µm. The cells were harvested (2.3.) and counted (2.3.). An amount of 2 X 10^4 cells/mL of the A549 cell suspension was seeded onto the insert's membrane and complete growth DMEM: F12 medium was added to the insert to a final volume of 200 µL. Starting from 24 hours after seeding the A549 cells, the transmembrane electrical resistance (TER) of the monolayers in three different wells was monitored daily using a MilliCell ERS Volt/Ohm meter (EMD Millipore Corporation, Billerica, USA). Cells were incubated till stabilisation of the TER was reached. Inserts without cells were included as controls.

A separate 24-well plate was used to seed 1 mL of 1 X 10^5 M059K viable cells. The cells reached confluency three days after seeding the A549 cells. On the day of infection, the inserts seeded with the A549 cells were placed in the 24-well plates seeded with M059K cells to establish the A549/M059K cell co-culture model. The model is shown in Figure 2.2.

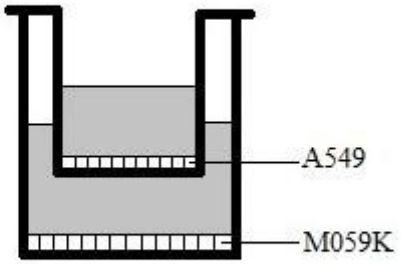

Figure 2.2: The A549/M059K cell co-culture model

2.5. Infection of M059K cells with *Mycobacterium tuberculosis*

Multiplication of *M. tuberculosis* in M059K glial cells was conducted as described before for osteoblasts (Sarkar *et al*., 2016). Confluent monolayers of M059K cells were washed three times with warm PBS, pH 7.3. Following the wash step, 1 mL of DMEM: F12 supplemented with 10 % FBS was added to each well. The cells were then infected with the single-cell suspensions of each *M. tuberculosis* strain, in triplicate wells, at a multiplicity of infection (MOI) of 10 bacteria per glial cell. The cells were incubated for 2 hours at 37 °C in a 5 % CO_2 atmosphere.

After 2 hours of incubation, infected cells were washed three times with warm PBS, pH 7.3, and an aminoglycoside protection assay was performed to kill remaining non-adherent extracellular bacteria. To carry out the aminoglycoside protection assay, 1 mL of complete DMEM: F12 media containing 200 µg/mL of amikacin (Sigma-Aldrich, St. Louis, USA) was added to the infected monolayers and incubated for 1 hour at 37 °C in a 5 % CO_2 atmosphere. Afterwards, the media was removed, cultured on enriched Middlebrook 7H11 agar plates in triplicate and incubated for three weeks at 37 °C in a 5 % CO_2 atmosphere to check the removal of extracellular bacteria. To remove the

amikacin, cells were washed three times with warm PBS, pH 7.3. One mL of complete DMEM: F12 media was added to the cells and then further incubated for 24 and 48 hours.

After the indicated time points, cells were washed three times with warm PBS, pH 7.3, and then lysed with 1 mL of 0.1 % Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 20 minutes at 37 °C. Lysates were 10-fold serially diluted, and 100 µL aliquots were cultured on enriched Middlebrook 7H11 agar plates for CFU count as described in 2.2.1 above. The inoculated Middlebrook 7H11 plates were sealed with CO_2-permeable plastic bags and incubated in a 5 % CO_2 incubator at 37 °C for three weeks. The obtained colony counts were used to determine the number of bacteria located within the M059K glial cells. The number of intracellular bacteria was expressed as a percentage of the inoculum using the following formula:

$$\text{Percent invasion} = \frac{\text{CFU of intracellular bacteria}}{\text{CFU of inoculum}} \times 100$$

2.6. Migration experiments

A549 epithelial cells were infected at an MOI of 10 bacteria per epithelial cell. Before infection, the monolayers of both A549 and M059K cells were washed three times with warm PBS, pH 7.3. One mL of DMEM: F12 media supplemented with 10 % FBS was added to wells seeded with M059K cells and 200 µL of the bacterial suspension (~ 10^7 bacteria) of each *M. tuberculosis* strain was added into the inserts above the A549 monolayer. This was done in triplicate. After adding the bacterial suspensions, cells were incubated at 37 °C in a 5 % CO_2 atmosphere.

At 24 and 48 hours after incubation, inserts were removed from the wells and media from the space between the insert and M059K cells was harvested. Six tenfold serial dilutions were made of the harvested media in Middlebrook 7H9 broth, and 100 µL aliquots were cultured on enriched Middlebrook 7H11 agar plates. These plates were sealed with CO_2-permeable plastic bags and incubated in a 5 % CO_2 incubator at 37 °C for three weeks.

Plates with an estimated colony number between 20 and 200 were used for counting to establish the CFU count per mL in undiluted media by multiplying the number of counted colonies with the dilution factor. The M059K monolayers were then washed with warm PBS, pH 7.3, three times to remove remaining media and non-adherent extracellular bacteria. Washed monolayers were lysed with 1 mL of 0.1 % Triton X-100 for 20 minutes at 37 °C. Lysates were serially diluted, and 100 µL aliquots were cultured on enriched Middlebrook 7H11 agar plates for CFU count as described in 2.2.1 above. The obtained colony counts were used to determine the percentage of transepithelial migration of bacteria as well as the combined percent adhesion and invasion of the M059K glial cells. The following formulas were used:

$$\text{Percent transmigration} = \frac{(\text{CFU of bacteria in media} + \text{CFU of adhered bacteria})}{\text{CFU added to insert}} \times 100$$

$$\text{Percent adhesion} = \frac{\text{no. of adhered bacteria}}{\text{no. of transmigrated bacteria}} \times 100$$

CHAPTER 3: RESULTS

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software (version 25) and Microsoft Excel were used to analyse data. Statistical analysis was done using Kruskal-Wallis (Non-parametic ANOVA) Test followed by Dunn's Multiple Comparisons Test. A p-value < 0.05 was considered statistically significant.

3.1. Co-culture of A549/M059K cell model

To examine whether *M. tuberculosis* transmigrates through a polarised intact A549 alveolar epithelial cell monolayer and subsequently adheres to and invade M059K glial cells, A549 and M059K cells were grown in transwell plates with A549 on the transwell membrane and M059K in the bottom of the well as described in 2.3. The structural integrity of the A549 monolayer was determined by measurement of the TER between the upper and lower chamber of the A549 cell-seeded transwell plates. The A549 epithelial cell layer was observed as a single cell monolayer by microscopy. As shown in Figure 3.1 below, the TER of the A549 cells increased from day 1 to day 4, followed by stabilisation until day 6. The TER decreased on day 7, suggesting that there was damage or loss of structural integrity of the A549 cell monolayer. The stabilised A549 cell monolayer grown for six days was used for the transmigration assay. The confluent M059K glial cell monolayers, confirmed by microscopic observation, reached confluency three days after seeding.

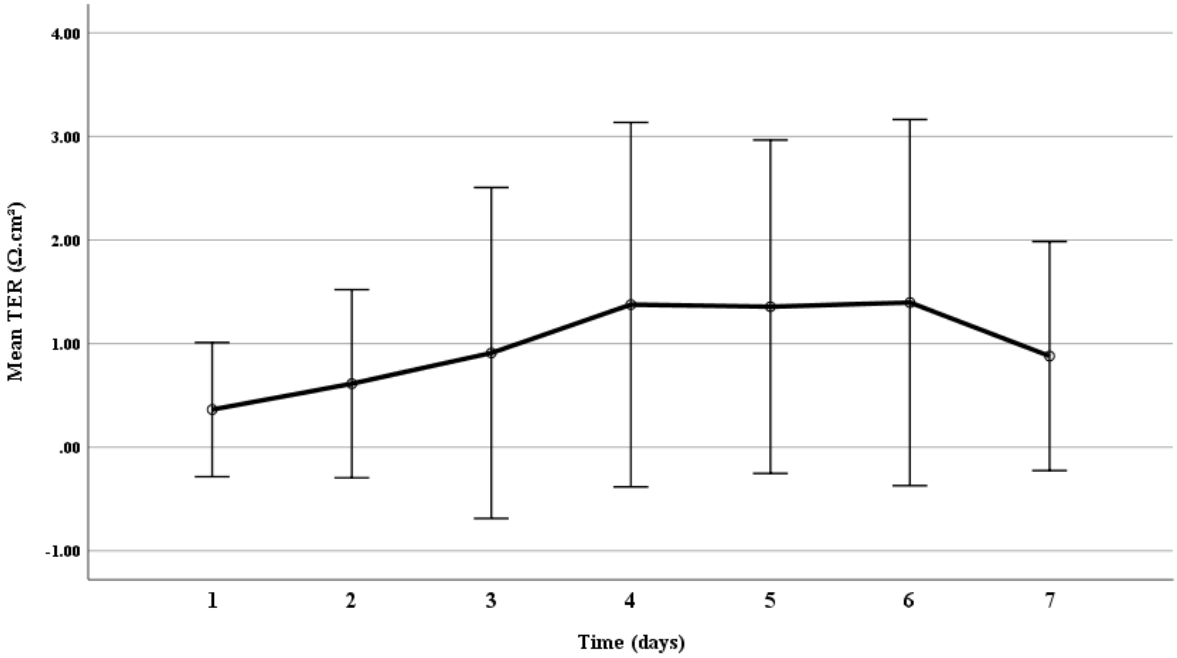

Figure 3.1: Daily measurements of the TER (Ω.cm²) of A549 epithelial cell monolayer monitored in triplicate wells of a transwell plate. Results represent the mean TER (Ω.cm²) of three independent experiments performed in triplicate.

3.2. Invasion of M059K glial cells by *Mycobacterium tuberculosis* strains

To determine whether the extensively drug-resistant F15/LAM4/KZN (KZN-XDR), multidrug-resistant F15/LAM4/KZN (KZN-MDR), drug-susceptible F15/LAM4/KZN (KZN-S), extensively drug-resistant Beijing (Beijing-XDR), drug-susceptible Beijing (Beijing-S), drug-susceptible F11 (F11-S) and multidrug-resistant F28 (F28-MDR) strains of *M. tuberculosis* invade glial cells, glial cells were infected with each strain and incubated for 24 hours after which the extracellular bacteria were removed (2.5). The 7H11 agar plates that were inoculated with the discarded media following the amikacin protection assay had no growth (2.5), confirming that only internalised bacilli were reported. Figure 3.2 shows the invasion rates of the different strains. The laboratory H37Rv strain invaded the M059K glial cells at a rate of 8 %. There was no significant difference in invasion rate between the KZN-XDR and F28-MDR strains and the H37Rv strain ($p = 0.28$ and $p = 0.10$, respectively). However, there was a significant difference in invasion rate between the KZN-S ($p < 0.001$), F11-S ($p = 0.0001$), Beijing-XDR ($p =$

0.0041), KZN-MDR (p = 0.0202) and Beijing-S (p = 0.0489) strains and the H37Rv strain.

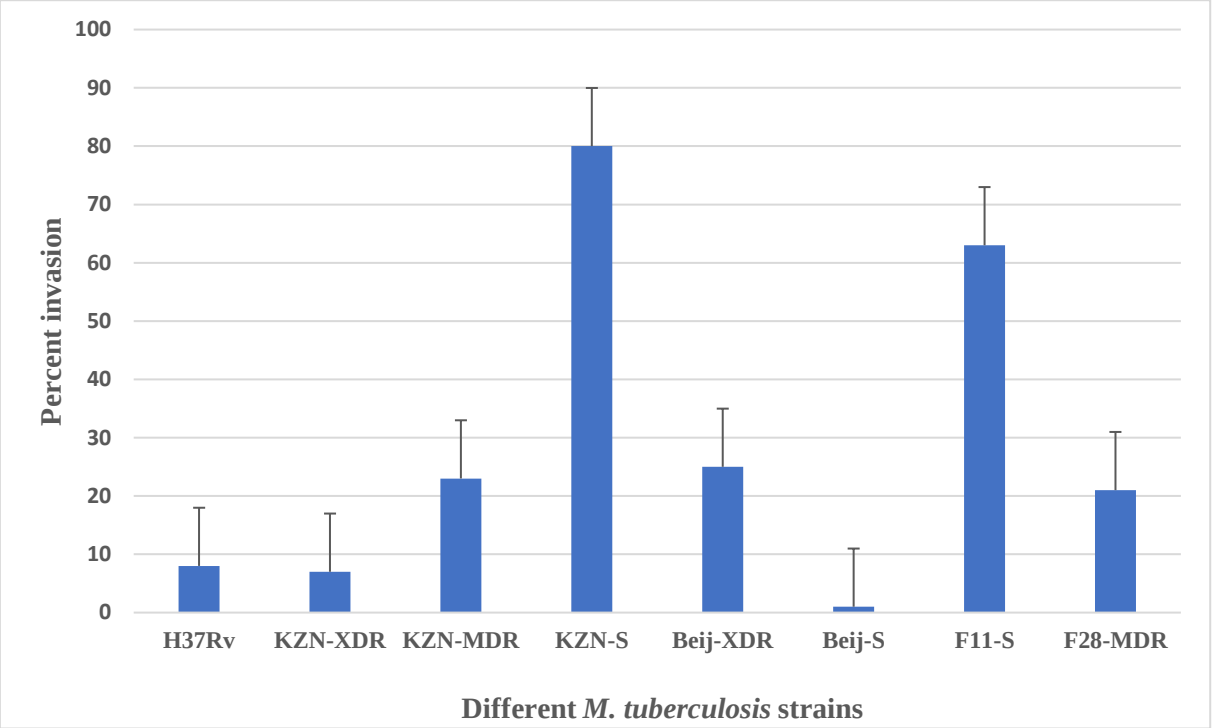

Figure 3.2: Percent invasion of M059K glial cells by different *M. tuberculosis* strains after 24 hours of incubation.

3.3. Transepithelial migration and subsequent adhesion and invasion of M059K glial cells by different strains of *M. tuberculosis*

All the *M. tuberculosis* strains used in the study were able to migrate through the A549 epithelial cell monolayer and subsequently adhere to and invade the M059K glial cells. The transmigrations of the KZN-XDR, KZN-MDR, KZN-S, Beijing-XDR, Beijing-S, F11-S and F28-MDR strains were evaluated in comparison with the H37Rv laboratory strain. Figure 3.3.1 shows the percent transmigration of the different strains over 24 and 48 hours. At the 24-hour time point, the Beijing-XDR strain displayed the highest percent transmigration when compared to all other strains, followed by the KZN-S and the KZN-XDR strains. However, when compared to H37Rv, statistically significant differences

were observed for the Beijing-XDR ($p = 0.004$) and KZN-S ($p = 0.03$) strains only. Although the F11-S, KZN-MDR and F28-MDR strains demonstrated neglectable transmigration rates, those of the F11-S and KZN-MDR strains were significantly different from that of the H37Rv laboratory strain ($p = 0.0374$ and $p = 0.004$, respectively). There was no significant difference in transmigration rate between the F28-MDR ($p = 0.15$) and KZN-XDR ($p = 0.16$) strains and the H37Rv strain. No cells of the Beijing-S strain were found in the basolateral fluid at the 24-hour time point.

After 48 hours of incubation, the transmigration rate of the H37Rv strain remained stable. In contrast, there was a decrease in the number of bacteria of the KZN-S and Beijing-XDR strains. The KZN-XDR strain was the only strain which displayed a slight increase in the number of bacteria. Although the Beijing-XDR strain showed a decrease in the percentage of the transmigrated organisms at this time point, its transmigration rate remained the highest amongst all other strains. The transmigration of KZN-MDR, F11-S and F28-MDR did not increase while the Beijing-S strain still did not show any sign of transmigration. The KZN-S and F11-S strains did not show a statistically significant difference when compared to the H37Rv ($p = 0.18$).

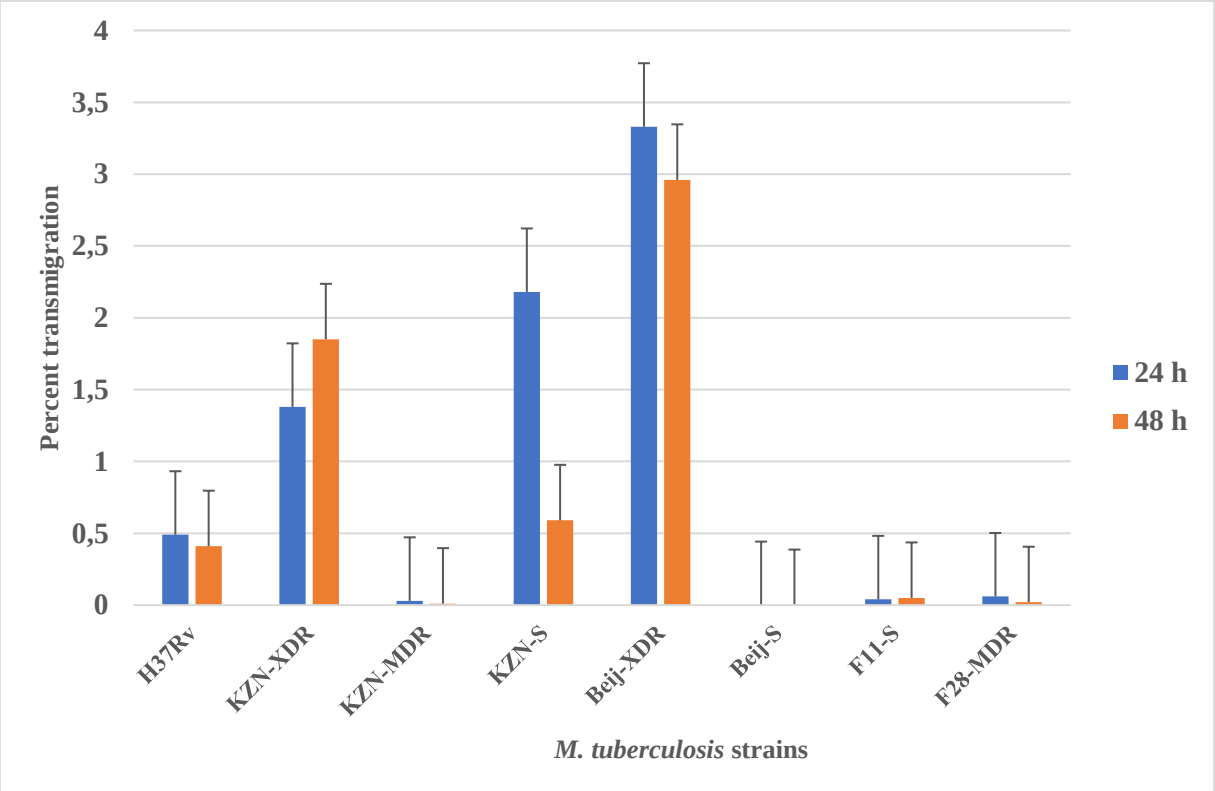

Figure 3.3.1: Percent transepithelial migration of different *M. tuberculosis* strains. Blue bars represent the 24-hour time point, and orange bars represent the 48-hour time point.

Figure 3.3.2 shows the combined adhesion and invasion of the M059K glial cells after migration through the A549 epithelial cell monolayer. All *M. tuberculosis* strains that did transmigrate retained their affinity for glial cells. The results were similar for all strains, with an increase in the percent adhesion and invasion from 24 hours to 48 hours of incubation. At 24 hours, the H37Rv laboratory strain was found to adhere to and invade glial cells at a rate of 7 %. The Beijing-XDR strain displayed a significantly high adhesion and invasion capacity among all the strains ($p = 0.03$). The KZN-S and F11-S strains displayed an equal adhesion and invasion capacity of M059K glial cells at a rate of 8 %, with no significant difference to that of the H37Rv strain ($p = 0.38$ and $p = 0.37$, respectively). The KZN-XDR, KZN-MDR and F28-MDR strains demonstrated significantly lower combined adhesion and invasion rates of 2 % ($p = 0.005$), 0.3 % ($p <$

0.001) and 2 % (p = 0.002), respectively. The Beijing-S strain showed no adhesion and invasion capacity following transmigration.

The adhesion and invasion rate of all strains increased after 48 hours of incubation. The Beijing-XDR strain still displayed a significantly high adhesion and invasion rate at 93 % (p < 0.001). The adhesion and invasion capacity of the KZN-XDR, KZN-S and F11-S strains were also significantly higher (p = 0.0003, p = 0.0002 and p = 0.048, respectively) than that of the H37Rv strain. There was no significant difference between adhesion and invasion capacity of the F28-MDR (p = 0.21) and KZN-MDR (p = 0.15) strains when compared to the H37Rv strain.

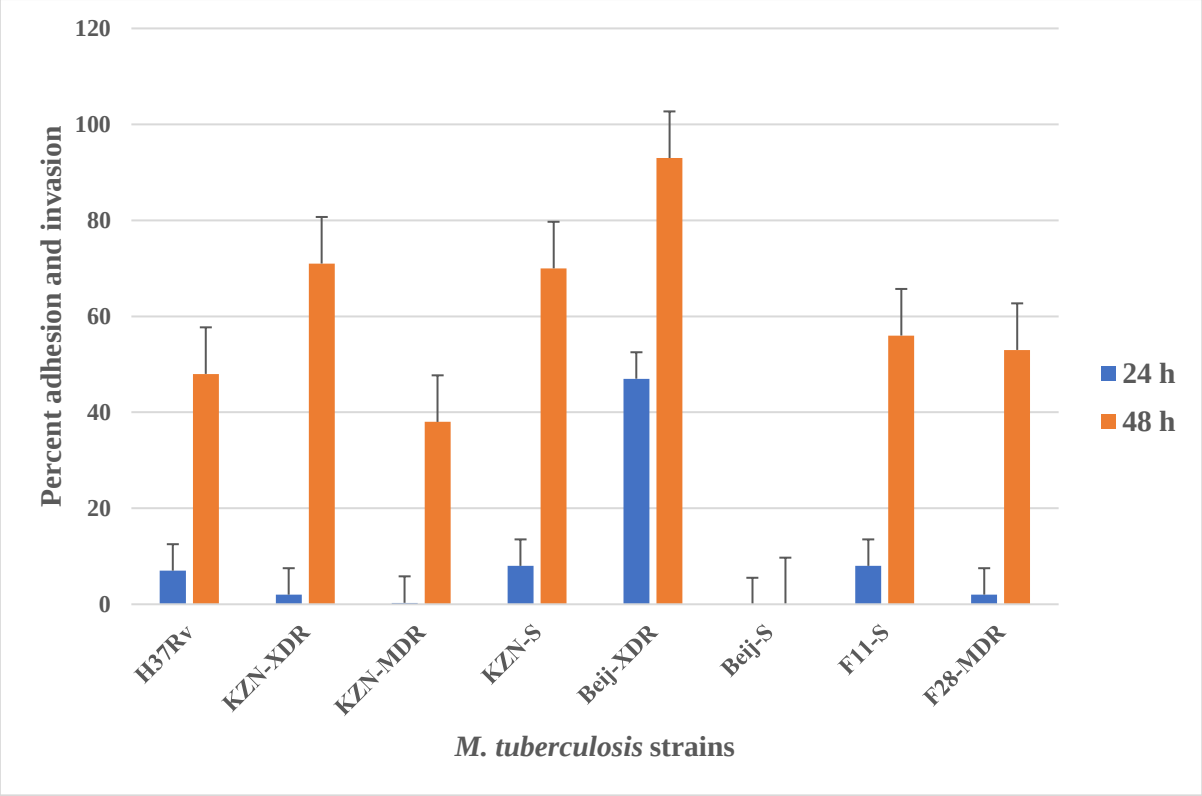

Figure 3.3.2: Combined percent adhesion and invasion of M059K glial cells by different *M. tuberculosis* strains following transepithelial migration. Blue bars represent the 24-hour time point, and orange bars represent the 48-hour time point.

3.4. Adhesion and invasion of M059K glial cells by different *M. tuberculosis* strains

Using the invasion rates established in paragraph 3.2, the combined adhesion and invasion following transmigration at 48 hours was split into these two parts. The results are shown in Table 3.1. A significant difference in adhesion capacity was observed between the KZN-XDR ($p = 0.10$) and KZN-MDR ($p = 0.000$) strains in comparison to the H37Rv strain. There was no significant difference between the adhesion of KZN-S ($p = 0.999$), Beijing-XDR ($p = 1.0$), F11-S ($p = 0.110$) and F28-MDR ($p = 0.984$) strains and that of the H37Rv strain. No adhesion and invasion were observed for the Beijing-S strain. Comparisons of the invasion capacity of each strain with that of the H37Rv strain showed a significant difference for the KZN-MDR and F11-S strains only ($p = 0.000$).

Table 3.1: Adhesion and invasion of glial cells by *M. tuberculosis* strains after the transmigration of an A549 monolayer represented by CFU counts in APPENDIX 2.

***M tuberculosis* strain**	**Adhesion**	**Invasion**
H37Rv	**4580 (92 %)**	**421 (8 %)**
KZN-XDR	**35638 (93 %) ***	**2473 (7 %)**
KZN-MDR	**69843 (78 %) ***	**20268 (22 %) ***
KZN-S	**290 (20 %)**	**1139 (80 %)**
Beijing-XDR	**8008 (75 %)**	**2725 (25 %)**
Beijing-S	**0**	**0**
F11-S	**26568 (37 %)**	**45654 (63 %) ***
F28-MDR	**10971 (79 %)**	**2896 (21 %)**

*$P < 0.05$

CHAPTER 4: DISCUSSION

For CNS-TB to develop, *M. tuberculosis* needs to migrate from the alveoli of the lung to the CNS. *Mycobacterium tuberculosis* disseminates to other parts of the body by migration through or destruction of lung tissue (Danelishvili *et al*., 2003; Ryndak *et al*., 2016). It has not been previously established that strains of *M. tuberculosis* differ in their ability to migrate from the lung to the brain. In this study, we evaluated the rate at which strains of *M. tuberculosis*, with different genotypes and drug resistance profiles, migrate through an alveolar epithelial cell monolayer and subsequently adhere to and invade a glial cell monolayer. We used a co-culture of alveolar epithelial cells and glial cells in a transwell system. Alveolar epithelial cells seeded on the insert membrane represented the lung side, glial cells seeded in the bottom of the well below the insert represented the brain, and the intermediate space represented the circulation.

Various methods are used to evaluate the structural integrity and permeability of *in vitro* cultures of epithelial and endothelial cell monolayers or bilayers. The transepithelial/ transendothelial electrical resistance (TEER) is the most widely used method to assess the integrity of tight junctions in these cells (Wilhelm *et al*., 2011; Odijk *et al*., 2014; Chen *et al*., 2015; Paradis *et al*., 2016; Srinivasan *et al*., 2016). As described in the literature, the TEER method is reliable as it provides real-time measurements without damaging the cells. Measurements are carried out during the different stages of growth and differentiation of cells. Therefore, TEER values indicate the integrity of monolayers and thus the formation of tight junctions. Varying TEER values for different cell types and even for the same cell types in various studies have been reported (Hermanns *et al*., 2004; Barar *et al*., 2005; Hermanns *et al*., 2009; Klein *et al*., 2013). These differences may be a result of factors such as temperature, the type of medium used and the cell passage number (Srinivasan *et al*., 2016). A general observation made is that TEER is higher in bilayers than in monolayers. Other methods used to assess integrity of *in vitro* barrier models include the use of electron microscopy and immunostaining of proteins characteristic of tight junctions using radioactive and nonradioactive labelled markers (Hermanns *et al*., 2004; Barar *et al*., 2005; Hermanns *et al*., 2009; Klein *et al*., 2013). While the use of labelled radioactive markers gives good sensitivity, their shortfall is that they have a short shelf-life and cannot be stored for long periods (Srinivasan *et al*., 2016). On the other hand, nonradioactive labelled markers such as dextrans have reduced

sensitivity (Srinivasan *et al*., 2016). It is suggested that these compounds can affect barrier integrity by interfering with transport processes and that the tested cells cannot be used for further experiments. In our study, we used the TEER method to assess A549 alveolar epithelial cell monolayer integrity. The TEER of A549 alveolar epithelial cell monolayer was measured daily until a stable value was detected. We observed that the A549 cell monolayer reached stability six days after seeding, then a drop in TEER from the seventh day indicated loss of monolayer integrity.

In this study, results indicate that there are indeed differences in the invasion rate of glial cells between *M. tuberculosis* strains with different genotypes and resistance profiles. The Beijing strain showed an increase in invasion rate with an increasing level of resistance. On the contrary, the F15/LAM4/KZN strain showed a decrease in invasion rate with an increasing level of resistance. These findings are different from those reported for osteoblasts (Sarkar *et al*., 2016). The F28 strain showed a similar invasion rate as the MDR F15/LAM4/KZN strain. This may be because both are MDR strains and thus may have similar changes in their genome affecting invasion of these cells. Despite the XDR characteristic of the Beijing strain, it showed a similar invasion rate as the MDR strains. This suggests that the XDR Beijing strain may possess the same virulence factors as the MDR F15/LAM4/KZN and F28 strains in the pathogenesis of CNS-TB. Overall, the susceptible F15/LAM4/KZN strain demonstrated the highest invasion capacity of glial cells and the susceptible Beijing strain demonstrated the lowest. These findings indicate that the susceptible F15/LAM4/KZN strain, as well as the susceptible F11 strain, might be more likely to cause CNS-TB than the other strains while the susceptible Beijing strain is less virulent in CNS-TB. However, this needs further investigation based on strain-specific resistance profiles and associated virulence factors.

Differences in transepithelial migration were observed among the different strains. These differences were shown by the number of bacteria recovered from media collected below the transwell inserts following infection of A549 cells. We, therefore, consider the possibility of leakages in the insert membranes. We observed that the susceptible and XDR variants of the F15/LAM4/KZN strain transmigrate the alveolar epithelial cell monolayer more efficiently than the MDR variant. This is highly likely for the XDR variant as it has been established that highly resistant isolates are cytotoxic on A549 cells (Ashiru & Sturm, 2015). As for the decrease in the number of bacteria observed from 24

to 48 hours for the susceptible F15/LAM4/KZN isolate, we cannot come up with an explanation. If there was a problem with the integrity of the A549 monolayer or the membrane, one should expect the opposite. More rapid invasion into the glial cells can be ruled out as well since these cells are lysed before counting. We consider this isolate to have a similar transmigration rate as the laboratory H37Rv strain. For the Beijing strain, the XDR variant showed a high transmigration rate, while the susceptible variant showed no transmigration ability. Similar to the MDR F15/LAM4/KZN, the F28 and F11 strains showed a very low dissemination ability. Altogether, the XDR variants demonstrated a greater dissemination ability while the MDR variants demonstrated a lesser dissemination ability. We conclude that *M. tuberculosis* isolates are able to pass through the epithelial lining of the alveoli to other body sites by transepithelial migration. Also, these results show that the XDR isolates pass A549 cells very effectively. This is in keeping with what has been formerly reported by our group (Ashiru & Sturm, 2015).

The BBB maintains homeostasis of the CNS. It is a physiological barrier that regulates the transport of different substances and molecules between the blood and the brain, and also plays a role in protection against pathogens (Wilhelm *et al*., 2011; Paradis *et al*., 2016; Campisi *et al*., 2018). Different components make up the BBB. Brain endothelial cells held together by tight junctions are the major component of the BBB. However, these cells function together with a well-organised structure made up of astrocytes, pericytes, microglia, as well as a basement membrane (Wilhelm *et al*., 2011; Campisi *et al*., 2018). The basement membrane is made up of structural proteins such as the extracellular matrix proteins collagen, laminin, fibronectin and proteoglycans (Wilhelm *et al*., 2011). All these components are involved in the structural integrity of the BBB. Various models have been implemented to improve understanding of the physiology and function of the BBB. Most of these models involve the co-culture of brain endothelial cells and astrocytes or pericytes and in seldom microglial cells. Microglial cells belong to the macrophage lineage of cells and play a role in the immune response. Their presence in the BBB may be the target for macrophage-pathogens like *M. tuberculosis* to enter the meningeal space. Various study models have shown the immunological role of these cells in the CNS (Rock *et al*., 2005; Yang *et al*., 2007; Hernandez Pando *et al*., 2010; Cannas *et al*., 2011; Be *et al*., 2011; Qin *et al*., 2015; Francisco *et al*., 2015; Tucker *et al*., 2016).

Rock *et al*., (2005) and Yang *et al*., (2007) have proven that *M. tuberculosis* selectively infects microglial cells in comparison to astrocytes.

Tuberculosis of the CNS is a fatal form of extrapulmonary TB. It is believed that after the deposition of *M. tuberculosis* in the alveoli of the lung, the bacilli disseminate to other body sites through bacteraemia (Rom & Garay, 2004). Whether disease develops or not depends on both the host's immune response and bacterial virulence factors. From the circulation, *M. tuberculosis* must cross the BBB to cause CNS-TB. It is believed that *M. tuberculosis* crosses the BBB either as free bacilli or through infected macrophages (Jain *et al*., 2006). We hypothesised that different strains of *M. tuberculosis* would disseminate from the lung to the brain at different rates, using glial cells as the macrophages used for transport through the BBB. We, therefore, co-cultured alveolar epithelial cells and glial cells in a transwell system to mimic the natural development of CNS-TB.

A significant increase in percent adhesion and invasion of glial cells was observed from 24 hours to 48 hours following transepithelial migration of all strains. These low adhesion and invasion rates (< 10 %) are consistent with the low transmigration rates (< 5 %) observed. Our results indicate that XDR strains are the most invasive following transepithelial migration, followed by the susceptible strains and then the MDR strains. We further explored adhesion to glial cells and invasion of glial cells separately. We found out that both XDR and MDR isolates have high adhesion rates and low invasion rates during the period of investigation (48 hours), while the susceptible isolates have low adhesion rates and high invasion rates. We suggest that isolates with high adhesion and low invasion rates may need more time to invade or may use different adhesion molecules or ligands that do not trigger an invasion. This needs further investigation.

The invasion capacity of *M. tuberculosis* into glial cells by organisms that have passed through the alveolar epithelial cells could differ from that of bacteria directly inoculated onto the glial cells. This could be due to structural changes on the surface of the bacteria while migrating through the epithelium. Therefore, there is a possibility that using the percent invasion without transmigration to calculate invasion from the combined adhesion and invasion figures may not be correct. However, the low number of bacteria that transmigrated did not allow for reliable differentiation between adhesion and invasion at that point. Our model does not include all the tissue layers involved in the

migration of *M. tuberculosis* from the alveoli to the brain. Further studies on the pathogenesis of CNS-TB should use extended models.

To our knowledge, this is the first study to report on epithelial transmigration and subsequent invasion of glial cells involving different strains of *M. tuberculosis*. Very few studies based on the association between adhesion and invasion of cells and the susceptibility profile of different *M. tuberculosis* strains exist. Such studies have only been reported from our research group for osteoblasts (Sarkar *et al*., 2016) and alveolar and bronchial epithelial cells (Ashiru & Sturm, 2015).

In conclusion, our results provide evidence that there are differences between strains of *M. tuberculosis* and that the changes in the genome that result in resistance also affect transepithelial migration to other body sites.

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APPENDICES

APPENDIX 1: METHODOLOGY

1.1. Growth media

1.1.1. *Middlebrook 7H9 broth*

To prepare 1 L of Middlebrook 7H9 broth, 4.7 g of Middlebrook 7H9 powder was dissolved in 900 mL of distilled water containing 2 mL glycerol and autoclaved for 15 minutes at 121 °C. After autoclaving, the media was cooled down to 55 °C and aseptically supplemented with 100 mL OADC. In order to confirm sterility prior to use, two drops of the broth were inoculated onto a blood agar plate and incubated overnight.

1.1.2. *Middlebrook 7H11 agar (Non-selective media)*

To prepare 1 L of Middlebrook 7H11 agar plates, 19 g of Middlebrook 7H11 powder was dissolved in 900 mL of distilled water containing 5 mL glycerol. The media was autoclaved for 15 minutes at 121 °C. After autoclaving, the media was placed in a water bath and cooled to 55 °C. After cooling, the media was aseptically supplemented with 100 mL OADC and rapidly poured into 90 mm Petri dishes. Each Petri dish received between 20- and 22-mL agar. Once the media had cooled and solidified, sterility was confirmed by placing one plate in a CO_2 incubator overnight. These plates were used to obtain the CFU count.

APPENDIX 2: CFU COUNTS

2.1. Bacterial CFU counts obtained before the infection of A549 cells in the transmigration experiments.

Strain number	Inoculum CFU (CFU/ mL) (Transmigration experiments)
H37Rv	2.6×10^6
MODS 388	2.88×10^6
MODS 688	1.96×10^9
V4207	3.48×10^5
R4933	3.9×10^5
TF 1516	1.78×10^5
TF 832	2.38×10^8
TF 44949	1.68×10^8

2.2. Bacterial CFU counts obtained from the media collected after the infection of A549 cells in the transmigration experiments.

Transmigration CFU						
Strain number	24 hours			48 hours		
H37Rv	12750	11400	11750	5600	5200	5250
	13950	12750	13100	7300	6100	5900
	10850	9800	9700	3900	4300	4600
(KZN-XDR)	32000	31000	34000	19800	19800	17200
	47000	47000	41000	15850	15550	14400
	40000	42000	36000	11900	11300	11600
(KZN-MDR)	640000	690000	710000	140000	145000	143000
	360000	380000	370000	140000	145000	143000
	550000	590000	370000	140000	145000	143000

	8100	8900	7600	580	490	570
(KZN-S)	7700	6750	6450	540	540	770
	7300	4600	5300	820	690	590
	8800	9250	8350	680	760	980
(Beij-XDR)	4700	5200	4800	680	760	980
	6750	7225	6575	680	760	980
	0	0	0	0	0	0
(Beij-S)	0	0	0	0	0	0
	0	0	0	0	0	0
	115000	112000	107000	57000	54500	57500
(F11-S)	117000	103000	101000	57000	54000	63000
	74000	58000	60000	57000	55000	52000
	81000	75000	75000	11400	12500	13700
(F28-MDR)	97000	93000	95000	11400	12500	13700
	144000	161000	104000	11400	12500	13700

2.3. Bacterial CFU counts obtained from the glial cells lysed after the infection of A549 cells in the transmigration experiments.

Combined Adhesion and Invasion CFU						
	24 hours			48 hours		
	690	785	760	9150	4120	4760
H37Rv	1170	1065	1170	7230	6435	1235
	935	930	970	4335	3940	3800
	380	470	360	39000	37000	33000
KZN-XDR	1180	1600	1500	29000	30000	34000
	380	440	510	54000	38000	49000
	1250	1405	1245	124000	116000	110000
KZN-MDR	1020	940	980	60000	53000	56000
	1480	1870	1510	94000	87000	111000

	360	**440**	**510**	**1260**	**1530**	**1360**
KZN-S	**1200**	**1100**	**900**	**1340**	**1350**	**1480**
	310	**330**	**280**	**1520**	**1470**	**1550**
	8000	**7850**	**6400**	**10400**	**11900**	**11000**
Beij-XDR	**5200**	**4100**	**5250**	**12400**	**9900**	**9900**
	6600	**5975**	**5825**	**11100**	**10000**	**10000**
	0	**0**	**0**	**0**	**0**	**0**
Beij-S	**0**	**0**	**0**	**0**	**0**	**0**
	0	**0**	**0**	**0**	**0**	**0**
	3700	**3900**	**2900**	**68000**	**68000**	**88000**
F11-S	**7500**	**7400**	**7750**	**71000**	**68000**	**63000**
	11300	**10900**	**12600**	**72000**	**77000**	**75000**
	1960	**1880**	**1920**	**12800**	**14800**	**12500**
F28-MDR	**1210**	**910**	**1060**	**13650**	**14250**	**13700**
	1300	**1130**	**1440**	**14500**	**13700**	**14900**

2.4. Bacterial CFU counts obtained before the infection of M059k glial cells in the invasion experiment.

Strain number	**Inoculum CFU (CFU/ mL) (Adhesion experiment)**
H37Rv	**5.75×10^4**
MODS 388	**1.0×10^5**
MODS 688	**3.6×10^4**
V4207	**7.75×10^4**
R4933	**1.525×10^5**
TF 1516	**2.025×10^4**
TF 832	**1.4×10^5**
TF 44949	**4.275×10^4**

2.5. Bacterial CFU counts obtained after the infection of M059K glial cells in the invasion experiment.

Adhesion CFU			
Strain number	**24 hours**		
H37Rv	**4250**	**5400**	**5200**
	4950	**3600**	**3300**
	5150	**5950**	**5750**
KZN-XDR	**6000**	**7650**	**4200**
	5400	**6000**	**6250**
	7850	**7400**	**7650**
KZN-MDR	**8200**	**6950**	**8500**
	8150	**8650**	**8150**
	8150	**7800**	**8325**
KZN-S	**60000**	**60000**	**52000**
	62500	**66000**	**61000**
	72500	**66500**	**55500**
Beij-XDR	**38500**	**47000**	**37500**
	35500	**30500**	**39500**
	39000	**46500**	**34500**
Beij-S	**200**	**200**	**50**
	150	**250**	**50**
	50	**100**	**200**
F11-S	**78500**	**80000**	**81000**
	97500	**95000**	**91500**
	93500	**82500**	**97000**
F28-MDR	**8400**	**8400**	**8400**
	9300	**9450**	**9100**
	9100	**9500**	**8700**

APPENDIX 3: ETHICS APPROVAL

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 July 2017

Ms T Nkwagatse (217050912)
Discipline of Microbiology
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
nkwagatsetaetso@gmail.com

Dear Ms Nkwagatse

Protocol: Variation in mycobacterium tuberculosis quinolone-resistant mutants obtained in vitro and number and position of gyrA and gyrB mutations.Degree: MMedSc
BREC Ref No: BE301/17

EXPEDITED APPROVAL

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 17 May 2017.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 30 June 2017 to BREC letter dated 12 June 2017 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 11 July 2017.

This approval is valid for one year from 11 July 2017. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be RATIFIED by a full Committee at its next meeting taking place on 08 August 2017.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

cc supervisor: willem.sturm@gmail.com
cc postgraduate administrator: dushradhp@ukzn.ac.za

UNIVERSITY OF
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Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

23 July 2018

Ms T Nkwagatse (217050912)
Discipline of Microbiology
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
nkwagatsetaetso@gmail.com

Dear Ms Nkwagatse

Protocol: Transepithelial migration of mycobacterium tuberculosis strains through an in vitro tissue culture bilayer..
Degree: MMedSc
BREC Ref No: BE301/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	11 July 2018
Expiration of Ethical Approval:	10 July 2019

I wish to advise you that your application for Recertification received on 10 July 2018 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 14 August 2018.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc supervisor: willem.sturm@gmail.com
cc postgraduate administrator: dudhrajhp@ukzn.ac.za

APPENDIX 4: TURNITIN ORIGINALITY REPORT

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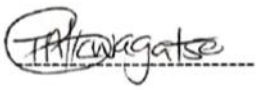

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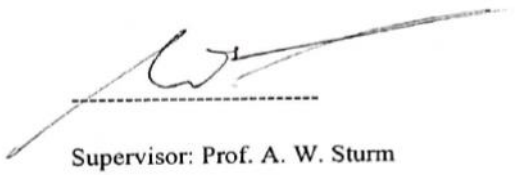

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