# Interaction between *Mycobacterium tuberculosis* and pulmonary epithelium

Olubisi T. Ashiru

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy in the Department of Medical Microbiology, Infection Prevention and Control

University of KwaZulu-Natal, South Africa.

**2013**

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

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## PRESENTATIONS EMANATING FROM THIS THESIS

Ashiru OT, Pillay M and Sturm AW. 2012. Expression of genes coding for putative virulence genes in oxygen deprived Beijing and F15/LAM4/KZN isolates of *Mycobacterium tuberculosis*

- College of Health Sciences Research Symposium on the 12th of September, 2012
- Poster presentation

Ashiru OT, Pillay M and Sturm AW. 2012. Expression of genes coding for iron-regulated heparin binding hemagglutinin (*hbhA*), mycobacterial DNA-binding protein 1 (*mdp1*), ferredoxin (*fxdA*) and heat shock protein (*hspX*) in oxygen deprived Beijing and F15/LAM4/KZN isolates of *Mycobacterium tuberculosis* is up-regulated after exposure to A549 cells

- 3rd South Africa TB/HIV Integration Conference, held in Durban from 12th – 15th June, 2012
- Oral presentation

Ashiru OT, Pillay M, Wesley-Smith J and Sturm AW. 2011. Clinical isolates of *Mycobacterium tuberculosis* induce morphological changes in A549 cells

- 49th Annual Meeting of the Microscopy Society of Southern Africa (MSSA), held in Pretoria from 6th – 9th of December, 2011
- Oral presentation, FIONA GRAHAM PRIZE FOR FIRST TIME ACCEPTED ABSTRACT

Ashiru OT, Pillay M and Sturm AW. 2011. *Mycobacterium tuberculosis* isolates cultured in the presence and absence of oxygen induces cytotoxicity in A549 alveolar epithelial cells

- College of Health Sciences Research Symposium from 14th – 15th September, 2011
- Poster presentation

Ashiru OT, Pillay M and Sturm AW. 2011. *Mycobacterium tuberculosis* isolates cultured in the presence and absence of oxygen induces cytotoxicity in A549 alveolar epithelial cells

- 4th Joint Congress of the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) Congress, held in Durban from 8th – 11th of September, 2011
- Poster presentation

Ashiru OT, Pillay M and Sturm AW. 2008. Adhesion and invasion of clustering and non-clustering strains of *Mycobacterium tuberculosis* in pulmonary epithelial cells

- Astrazeneca College of Health Sciences Research Symposium
- Oral presentation

Ashiru OT, Pillay M and Sturm AW. 2008. Adhesion and invasion of clustering and non-clustering strains of *Mycobacterium tuberculosis* in pulmonary epithelial cells

- 1st South Africa National Tuberculosis (TB) conference held in Durban from 1st – 4th of July, 2008

- Oral presentation, BEST PRESENTATION AWARD

## PUBLICATIONS EMANATING FROM THIS THESIS

Ashiru OT, Pillay M and Sturm AW. 2012. *Mycobacterium tuberculosis* isolates grown under oxygen deprivation invade pulmonary epithelial cells. *Anaerobe Journal* **18(4):**471-4

Ashiru OT, Pillay M and Sturm AW. 2010. Adhesion to and invasion of pulmonary epithelial cells by the F15/LAM4/KZN and the Beijing strains of *Mycobacterium tuberculosis*. *Journal of Medical Microbiology* **59:**528-33

**ACKNOWLEDGEMENTS**

I would like to express my sincere gratitude to the following individuals:

Professor AW Sturm my supervisor for his many contributions such as expertise, constructive criticism, keen observation and guidance in my professional development.

Dr M Pillay for all her contributions to my research

Dr J Wesley-Smith, Mrs. P Maartens, Miss Sharon and Mr. Philip Christopher for the use of the Electron Microscopy Unit, assistance and guidance with the scanning electron microscopy work.

Professor T Naicker and Ms. Denise of the Optics and Imaging Centre for the use of their unit, assistance and guidance with electron microscopy.

Dr Ravesh Singh of the HPP for guidance on the initial molecular techniques

Prof. P. Moodley and all other members of the department of Infection Prevention and Control: for assistance and guidance in various capacities.

My husband, children and family for all their support

Last but definitely not the least, to God the Father, God the Son and God the Holy Ghost - my creator, the lover of my soul, the source of my life and my reason for living

**DEDICATION**

This work is dedicated first to God Almighty – my Father, Creator, Lover of my soul, my all in all - Jehovah Elohim for without whom I could not have accomplished anything of worth and second to the most important people in my life: Tobi, Ade, Sefunmi & my loving husband – you all make it worth it. You will never know how much I love you all.

**TABLE OF CONTENTS** **PAGE**

**LIST OF FIGURES** **PAGE**

**LIST OF TABLES** **PAGE**

## LIST OF ABBREVATIONS

| | |
|---|---|
| A549 | human type II alveolar epithelial cell line |
| AIDS | Acquired immunodeficiency syndrome |
| ANOVA | analysis of variance |
| AT-I | alveolar epithelial type-I |
| ATCC | American Type Culture Collection |
| BBM | human bronchial epithelial cell line |
| BME | β-mercaptoethanol |
| CDC | Centers for Disease Control and Prevention |
| c.f.u. | colony forming unit |
| $CO_2$ | carbon dioxide |
| CR | complement receptor |
| CV | coefficient of variation |
| DMSO | dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DR | Drug resistant |
| ECM | extracellular matrix |
| EMEM | Eagle's minimum essential medium |
| FBS | fetal bovine serum |
| GAGs | glycosaminoglycans |
| G+C | guanine plus cytosine |

| | |
|---|---|
| GTC | guanidinum thiocyanate |
| HBH-A | heparin-binding hemagglutinin |
| HEp-2 | human respiratory epithelial cell |
| HIV | human immunodeficiency virus |
| IGRAs | Interferon-γ release assays |
| IL | Interleukin |
| IS | insertion sequence |
| KZN | KwaZulu-Natal |
| LAM | Lipoarabinomannan |
| LDH | Lactate dehydrogenase |
| LJ | Lowenstein Jensen |
| MABA | Microplate alamar blue assay |
| MCP | monocyte chemotactic protein |
| MDR | multi-drug resistant |
| MDP-1 | Mycobacterial DNA-binding protein 1 |
| Min | minutes |
| MIRU | mycobacterial interspersed repetitive units |
| M.O.I | multiplicity of infection |
| mRNA | messenger ribonucleic acid |
| *M. africanum* | *Mycobacterium africanum* |
| *M. bovis* | *Mycobacterium bovis* |
| *M. canetti* | *Mycobacterium canetti* |
| *M. caprae* | *Mycobacterium caprae* |

| | |
|---|---|
| *M. microti* | *Mycobacterium microti* |
| *M. mungi* | *Mycobacterium mungi* |
| *M. pinnipedii* | *Mycobacterium pinnipedii* |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| NAAT | Nucleic acid amplification tests |
| OADC | oleic acid, albumin, dextrose, catalase |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed Field Gel Electrophoresis |
| PPD | purified protein derivative |
| PVP | polyvinylpyrrolidone |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| SD | standard deviation |
| SEM | Scanning electron microscopy |
| Spoligo | spacer oligonucleotide |
| TB | tuberculosis |
| UV | ultraviolet |
| VNTR | variable numbers of tandem DNA repeats |
| WHO | World Health Organization |
| XDR | extensively drug resistant |
| ZN | Ziehl-Neelsen |

**ABSTRACT**

**Background**

*Mycobacterium tuberculosis* isolates such as the Beijing and F15/LAM4/KZN families dominate in patients. The emergence of extensively drug resistant (XDR) *M. tuberculosis* isolates raises concern. The need to better understand the pathogenesis of *M. tuberculosis* isolates resulted in this work.

**Methods**

*M. tuberculosis* clinical isolates that belonged to the Beijing and F15/LAM4/KZN families, isolates with unique DNA fingerprints and laboratory strains were used. Isolates were grown in the presence of oxygen and then exposed to A549 alveolar and BBM bronchial epithelial cells. The number of bacilli that adhered to the epithelial cells were viewed and counted using light microscopy. Isolates grown in the presence of oxygen and under oxygen deprivation were used for subsequent assays. Invasion of A549 and BBM cells by isolates grown under these different circumstances was investigated. Based on the results, the remaining assays were performed with A549 cells only. Cytotoxicity was quantified using the Cyto Tox96 Non-Radioactive Cytotoxicity Assay kit.
Morphological changes in A549 cells after exposure to the isolates were observed using the scanning electron microscopy (SEM). Real-time quantitative PCR was performed to assess the relative expression levels of four genes potentially associated with virulence

(*hbhA*; *mdp1*; *fdxA*; *hspX*). Results were normalized against *16S* rRNA and *ftsZ* gene transcription and reported as fold difference as compared to H37Rv.

**Results**

All isolates adhered to and invaded A549 cells in significantly higher numbers than BBM cells ($P \leq 0.0029$). Isolates grown under oxygen deprivation displayed higher levels of virulence than their aerobic phenotype. Grouped together, the isolates belonging to the Beijing and F15/LAM4/KZN families of strains showed greater adhesion capacity (28%) than isolates with unique DNA fingerprints (5%) ($P<0.05\%$). Three F15/LAM4/KZN isolates (two XDR-variants), were at least twice as invasive ($\geq$33%) as the most invasive Beijing isolate (15%) ($P<0.05$). The highest cytotoxicity level (35.7%) was produced by an XDR-F15/LAM4/KZN strain. SEM revealed bleb-like structures on bacterial cells grown under oxygen deprivation. Beijing and XDR-F15/LAM4/KZN isolates had the highest number of projections (16$\pm$5 per bacillus. The expression levels of all four genes were highest in Beijing and F15/LAM4/KZN isolates grown under oxygen deprivation and exposed to A549 cells.

**Conclusions**

Beijing and F15/LAM4/KZN strains are more virulent and their successful spread might be related to their interaction with alveolar epithelium. *M. tuberculosis* pathogenesis studies should include isolates grown under oxygen deprivation.

# CHAPTER 1 - INTRODUCTION

For centuries, tuberculosis (TB) has taken the lead among infectious diseases that kill humans. A third of the world's population, which is more than two billion people is believed to be infected with its aetiologic agent – *Mycobacterium tuberculosis* (*M. tuberculosis*) (Kochi, 1991).

Robert Koch (1843 – 1910) discovered *M. tuberculosis* in 1882 for which he received the Nobel Prize in 1905. The bacillus is a slender, straight or slightly curved rod when viewed under the scanning electron microscope (Fig. 1.1). It is a slow growing organism, with a doubling time of about 18 hours and colonies are usually visible in 3 – 6 weeks when cultured on complex solid media, such as Lowenstein Jensen (LJ) and Middlebrook media. These colonies are raised, irregularly shaped with a dry wrinkled surface. They have a tough and tenacious consistency (Fig. 1.2).

The presence of mycolates and mycolic acids in the cell wall of *M. tuberculosis* makes its cell wall thick and waxy. This layer of fatty acids also provides considerable resistance to adverse environmental conditions like pH fluctuations, drying and exposure to chemicals, including some disinfectants.

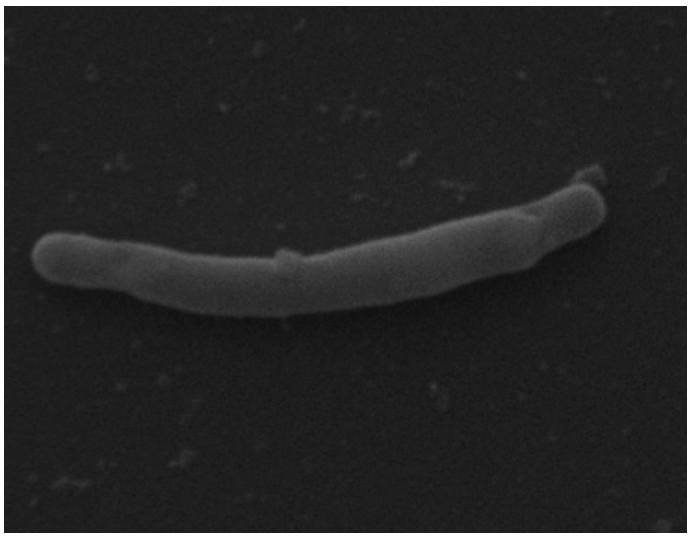

**Figure 1.1 Micrograph of *M. tuberculosis* bacillus Mag X 30000K**

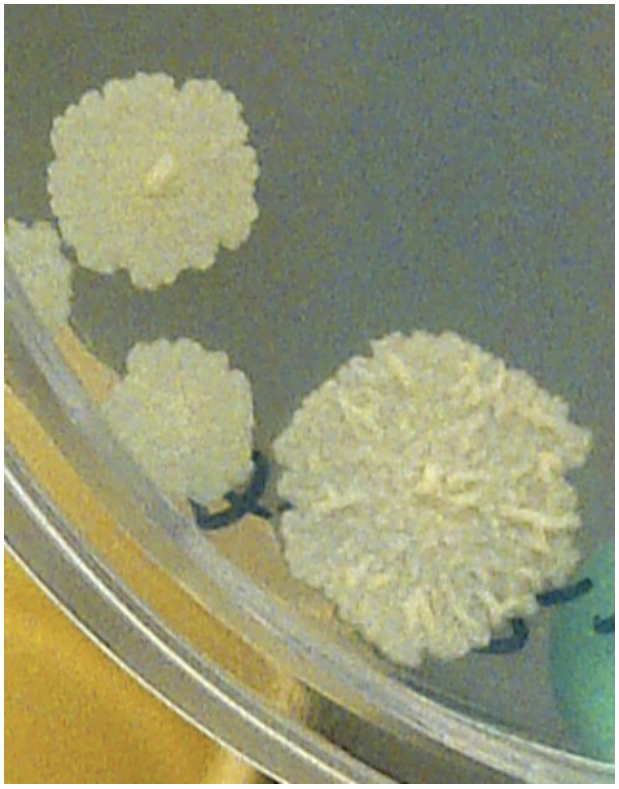

**Figure 1.2 Colonies of *M. tuberculosis***

The nature of its cell wall makes permeability by most staining solutions used for other bacteria impossible. However Robert Koch (1843 – 1910) developed a staining technique which made the bacteria visible. This method is such that the cell remains stained even after exposure to acid and hence mycobacteria are referred to as acid fast bacilli.

Its genome has a high guanine plus cytosine (G+C) content. *M. tuberculosis* hydrophobic nature allows it to float on the surface of aqueous media unless dispersed by detergents such as polyoxythylene sorbitan monooleate, better known as "Tween". *M. tuberculosis* is sensitive to ultraviolet (UV) radiation and heat.

TB is not only caused by *M. tuberculosis* but also by a number of closely related species. These species together are known as the *M. tuberculosis* complex, whose members are *Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium canetti, Mycobacterium microti, Mycobacterium mungi, Mycobacterium pinnipedii* and of cause *M. tuberculosis* (Alexander *et al.* 2010; Tsukamura *et al.* 1985). The following members of the complex can cause human TB: *M. tuberculosis, M. africanum, M. bovis* and *M. canetti* (Cole 2002).

Humans are the natural reservoir of *M. tuberculosis* and *M. africanum* (Wayne and Kubica, 1986). *M. bovis* has a broad host range in mainly but not exclusively hoofed animals (Thoen *et al.* 1984). Voles are the main host of *M. microti* (Wells, 1945), *M. canetti* is found in humans (Koeck *et al*. 2011), *M. caprae* predominant hosts are cattle and humans (Kubica *et al*. 2003; Prodinger *et al*. 2002) but have also been isolated from

wildlife species – namely red deer (Prodinger *et al*. 2002) as well as wild boar (Erlder *et al*. 2004; Machackova *et al*. 2003), *M. pinnipedii* was found in seals (Cousins *et al*. 2003) while the host of *M. mung*i are mongooses (Alexander *et al.* 2010).

## 1.2 Historical aspects of TB

The first clear description of TB was by the ancient Egyptians. While their doctors described clinical features of scrofula and its treatment (medical and surgical), their artists showed spinal TB and its deformity via their paintings. The Egyptians also established the first known sanatorium.

During the time of Hippocrates (460 – 371 B.C.), TB was referred to as 'phthisis', a Greek term for 'wasting away'. Hippocrates considered the disease to have been inherited and non-contagious (Chadwick and Mann, 1978). In the early 20$^{th}$ century, TB was referred to as 'consumption' and Oliver Wendell Holmes, Sr. (1809 - 1894) coined the terms 'white plague' to describe TB. Franciscus (Francois) de la Boë, also known as Sylvius Leyden (1614 – 1672) was the first to use the term 'tubercle' (Johnston, 1993).

The staining method used to identify the bacillus microscopically that was first developed by Robert Koch was improved upon by Koch's student Paul Ehrlich (1854 – 1915) and then Edvard Georg Rindfleisch (1836 -1908) both in 1882. In the same year, Franz Ziehl (1859 – 1926) further improved the method. Then in 1883, Friedrich Karl Adolp Neelsen (1854 – 1898) combined Franz Ziehl and Paul Ehrlich methods. The term "Ziehl-

Neelsen staining" evolved between 1890 and 1893 (Bishop and Neuman, 1970) and is used ever since. In 1895, Wilhelm Konrad Roentgen (1845 – 1923) discovered an advanced investigative method of the thorax using X-rays and this improved the diagnosis of TB (Murray, 2004).

## 1.3 Spread of TB

TB is spread from person to person through the air. When a person with an infectious TB disease coughs or sneezes, tiny droplet nuclei containing *M. tuberculosis* may be expelled into the air. *M. tuberculosis* in these tiny droplet nuclei may then be inhaled into the alveolar space of the lungs. The bacilli may then be phagocytosed by alveolar macrophages (McDonough *et al*. 1993) or they may invade non-professional phagocytic cells such as the epithelial pneumocytes (Mehta *et al*. 1996).

Subsequent passage of these bacilli through the alveolar epithelium has been suggested to be required for the establishment of infection and disease progression (Balasubrananian *et al*. 1994). Non-professional phagocytic cells have been presumed to serve as hiding places for *M. tuberculosis* in persistent infection. The ability of *M. tuberculosis* to gain passage into the lung tissues and back into the airways, through the alveolar epithelium has been suggested to be an important aspect of the spread of TB (Dobos *et al*. 2000).

*M. tuberculosis* can affect any organ system , but it primarily affects the lung with 75 percent of active TB cases being pulmonary TB. *M. tuberculosis* was previously

considered to be an obligate aerobe (Todar, 2010), hence, studies have mainly been performed with aerobically grown organisms.

However, studies on this organism need to include bacilli cultured in oxygen deprived environment, as bacilli under this condition *in vitro* remained viable as well as virulent for several years (Corper and Cohn, 1933), and oxygen deprived environments with low redox potentials are found in all tissues throughout the human body. In addition, in granuloma as well as the lining of lung cavities, which are both characteristic pathological features of TB infection, the redox potential is also low, and this result to a hypoxic (oxygen deprived) environment (McClane *et al*. 1999).

*M. tuberculosis* was presumed to be a highly homogenous species with variation in disease presentation and complications as a result of different host responses (Bellamy and Hill, 1998). Development of molecular techniques, have shown otherwise. The use of strain-specific genetic markers made the identification and tracking of distribution and spread of individual strains of *M. tuberculosis* possible.

Restriction fragment length polymorphism (RFLP) typing is the most extensively used differentiation method and this uses the *M. tuberculosis* complex specific insertion sequence IS6110 to differentiate clinical isolates (van Soolingen *et al.* 1999). Polymerase chain reaction (PCR) based typing methods such as mycobacterial interspersed repetitive units (MIRU) and spacer oligonucleotide (spoligo) typing are also been used increasingly

for the typing of isolates. The above typing methods allow *M. tuberculosis* strains to be grouped into strains and strain families with similar DNA fingerprint patterns.

It has been reported that in proportions of TB cases, *M. tuberculosis* isolates from different patients, share highly similar or identical DNA fingerprint patterns, referred to as isolate clustering (Hawkey *et al.* 2003; van Soolingen *et al.* 1999). Clustering has been used to estimate the extent of disease linked to recent transmission (Easterbrook *et al.* 2004; van Deutekom *et al.* 2004) while TB in patients with non-clustering isolates displaying unique DNA fingerprint patterns is considered to be reactivation of latent infection (Easterbrook *et al.* 2004; Wilkinson *et al.* 1997).

The availability of the whole genome sequence of *M. tuberculosis* H37Rv laboratory strain (Cole *et al*. 1998) has made it possible to study the expression profile of the genes in this organism. Information on the expression profile of these genes, would further our understanding of the bacilli and its interaction with its host. Some genes have been suspected to be implicated during the process of infection, while others have been implicated in biological functions unrelated to pathogenesis. Although, the annotated functions of most genes in this organism are still unknown, investigation of their expression profile *in vitro* and during the infection process needs to be done. This will generate useful information on probable targets for existing and new drug therapy.

Nineteen years after the declaration by World Health Organization (WHO) of TB as a global emergency, the first disease to be declared as such in the history of the

organization, the global burden of TB remains overwhelming (Glickman and Jacobs, 2001).

The burden of TB disease was approximately 9.2 million new TB cases and 1.7 million deaths in 2006 (WHO, 2009). TB is responsible for 25 percent of deaths among adults in the developing world. That is more than those caused by diarrhea and malaria combined. The high prevalence of TB in sub-Saharan Africa and its association with the human immunodeficiency virus (HIV) infected individuals makes it an even more important public health concern.

WHO declared that South Africa has one of the worst TB epidemics in the world and the country is rated 3rd among the 22 high TB burden countries (WHO, 2011). This probably is due to the high rates of TB, increasing rates of HIV as well as the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) TB.

The slow growth of *M. tuberculosis*, resulting in difficulty in diagnosis of the disease, and the need for long-term therapy, further complicates this situation. TB is the leading infectious killer of youths and adults in South Africa and it is estimated that TB kills almost one thousand people every month (SAhealthinfo).

In South Africa, KwaZulu-Natal province contributes the highest case burden to the national TB burden. In 2006, the province reported approximately 30 percent of all national TB cases, 25 percent of all new smear positive cases; 42 percent of all MDR-TB

cases and 65 percent of all XDR-TB cases (TB strategic plan SA, 2007-2011). In the same year (2006) the reported incidence of TB in KwaZulu-Natal province was 780/100 000 (WHO, 2009) and Moodley *et al*. (2011) reported that the incidence of culture-confirmed TB was 145/100 000 in the province. They suggested that this showed that over 80% of reported TB cases were not culture-confirmed, lacked drug susceptibility test results and hence, an underestimation of the TB burden in the province (Moodley *et al*. 2011). Unfortunately reports on the TB burden in the other provinces in South Africa after 2006 have not been published to allow for further comparison with data reported by Moodley *et al.* 2011.

Although, there have been and still are on-going efforts to overcome the TB burden, the situation is of major concern. An increased understanding of characteristics of the causative organism at the basic science level would be very informative, as basic science serves as the cornerstone for applied medical science. The study of the interaction between the organism and host cells *in vitro* can potentially lead to a better understanding of the characteristic of *M. tuberculosis* during the infection process. This could further assist in the identification of targets for the development of new treatments and preventive therapies.

Pulmonary TB involves primarily the cells that line the bronchial tree ( bronchi, bronchioles and alveoli). An alveolus (Fig. 1.3) consists of the following cell types surrounding the alveolar space: fibroblasts, capillaries, alveolar macrophages and alveolar epithelia.

Cell to cell interactions are essential in the establishment of infectious diseases. Infections involve a continuous interplay between the microbial pathogen and host cells (Menozzi *et al*. 2002). One of the initial and crucial events in bacterial pathogenesis is the adherence of the microorganism to its target tissue (Delogu and Brennan, 1999).

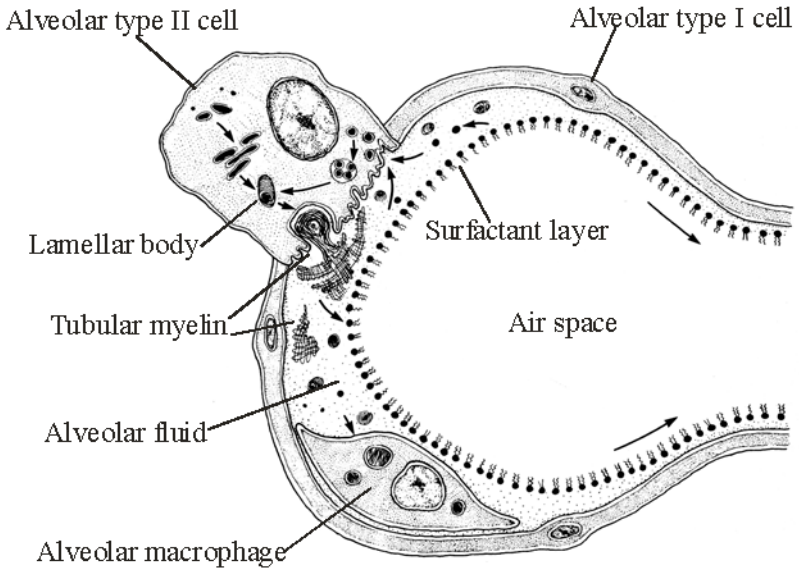


**Figure 1.3 Structure of an alveolus (modified from Hawgood and Clements 1990)**

This adherence step is mediated by specialised microbial surfaced-exposed molecules called adhesins, that recognise complementary receptors on the host cells (Menozzi *et al*. 2002).

Many pathogens, including respiratory pathogens, have been shown to produce heparin-binding adhesins that interact with sulphated glycoconjugates that are present on the surface of most epithelial cells and in the extracellular matrix (ECM) (Yanagishita and Haschall, 1992). *M. tuberculosis* has been reported to express on its surface heparin-

binding hemagglutinin (HBH-A), a 28-kDa protein that binds to glycosaminoglycans (GAGs) and has been identified as a mycobacterial adhesin in the mycobacterium-epithelial cell interaction (Menozzi *et al*. 1998).

It is possible that mycobacteria use multiple adhesins to promote adhesion to non-phagocytes. A number of non-specific DNA-binding proteins are found in association with bacterial chromosomes and are called histone-like proteins (Aoki *et al*. 2004). Mycobacterial DNA-binding protein 1 (MDP-1) is a mycobacteria-specific histone-like protein, which is found in the bacterial cell wall and inside the cell (Shimoji *et al*. 1999; Aoki *et al*. 2004). Aoki *et al* (2004) reported that HBH-A and MDP-1 both bind to GAGs.

Virulence of bacterial pathogens often requires the production and action of both toxins and adhesins. Whereas toxins are generally released by the pathogens into the extracellular milieu and can thus act at distant sites, adhesins typically remain associated with the bacterial surface, allowing the microorganisms to adhere to host determinants or other receptor molecules on the surface and matrix (Rostand and Esko, 1997; Menozzi *et al*. 2002). Toxins have so far not been identified in *M. tuberculosis*.

The expression of several genes have been reported on, some of which encode factors responsible for virulence. The cell division protein of *M. tuberculosis*, coded for by the *ftsZ* gene (Rv2150c) is a cytokinetic protein and a major cell division regulatory molecule (Roy *et al*. 2004; Roy and Ajitkumar 2005). The *hspX* gene (Rv2031c) also known as

*acr* (which encodes α-crystallin) and the *fdxA* gene (Rv2007c) coding for ferredoxin were both reported to belong to the dormancy regulon. They both have also been reported to be induced in oxygen-deprived environments and during latency (Ramage *et al*. 2009; Ohno *et al*. 2003; Sherman *et al*. 2001).

Menozzi *et al*. (2006) reported that the iron-regulated heparin binding hemagglutinin, coded for by the *hbhA* gene (Rv0475) was implicated in extrapulmonary dissemination of *M. tuberculosis*. However, Delogu *et al*. (2006) reported that this same gene played a role during infection of the lung. Both these observations imply a role of this protein in TB pathogenesis. Mycobacterium-specific histone-like protein MDP1, coded for by *mdp1* gene (Rv2986c) also known as *hupB,* has been reported to play a role during stress response and cell wall assembly (Katsube *et al*. 2007). The gene product has also been reported to accumulate during the stationary phase of growth.

## 1.4 Pathogenesis of TB

Pathogenesis of TB is a multi-factorial process. One important factor is the virulence of the infecting strain. Other factors that influence pathogenesis are the infecting dose, which is for *M. tuberculosis* the number of the bacilli inhaled, as well as the immune response of the host to the infection. For infection to occur there must be an interaction between the bacilli and the host cell, whereby the bacilli become established in the host.

When there is a noticeable impairment in the function of the host as a result of the infection a disease is said to occur. This does not always happen and thus not all infection leads to disease. In a host with adequate defences, the presence of the bacilli can be asymptomatic. However, in an immune compromised host (such as HIV-infected individuals), and immune competent hosts, the presence of the bacilli can result in a state of overwhelming disease, which could lead to death. As reported in 2006 by Gandhi *et al*, on the high mortality that occurred in Tugela Ferry region of KwaZulu-Natal province among patients co-infected with TB and HIV.

It has been demonstrated that *M. tuberculosis* invades and survives within human type II alveolar epithelial cells *in vitro* (Bermudez and Goodman, 1996; Lin *et al*. 1998). In these studies and other similar ones (Bermudez *et al*. 2002; Reddy and Hayworth, 2002; Garcia-Perez *et al*. 2003; Garcia-Perez *et al*. 2008) the virulent laboratory strain H37Rv was used together with one or two other strains and clinical isolates. Improvement of the knowledge about the mechanisms employed by *M. tuberculosis* to infect its host will certainly offer new opportunities for the development of both effective therapy and vaccines (Bloom *et al*. 1992; Bermudez *et al*. 2002). The inclusion of clinical isolates that belong to different strain families in similar experiments might yield useful information on this organism.

Several strains of *M. tuberculosis* have been shown to dominate in patients with active disease, suggesting that these are more successful pathogens as compared to other strains. These strains are recognized through genotyping. So far, attempts to find differences

between them have focused on adaptation in macrophages. The other option is that they are more successful, because they are better colonisers of the bronchial tree.

In KwaZulu-Natal, there are 2 dominant families of strains among the drug resistant isolates: the ubiquitous Beijing family and the F15/LAM4/KZN family which is only found locally. The latter has evolved since 1995 from a single genotype to a family of closely associated types (Pillay and Sturm, 2007). A recent development is its progression into XDR isolates that spreads rapidly among HIV-infected and non-infected patients in the province. Gandhi *et al* (2006) reported that TB patients co-infected with HIV had high mortality. These suggest increased virulence.

Non-professional phagocytic cells such as epithelial cells do not normally ingest microbes or large particles, and therefore, have proved to be excellent models for studying events entirely driven by the pathogen (Alonso and Portillo, 2004). The work leading to this thesis aimed at studying differences in interaction of mycobacterial isolates with different RFLP signature with alveolar epithelium. The obvious approach should have been to expose both types of epithelial cells to *M. tuberculosis*. However, alveolar type 1 (AT-1) cells cannot be grown *in vitro*. Hence, the human alveolar type II epithelial cell line (A549 – ATCC CCL 185) and human bronchial epithelial cell line (BBM – CRL 9482: a derivative of BEAS-2B cells) were used.

Infection assays were done using representative clinical isolates of the Beijing and F15/LAM4/KZN families and compared these with isolates with unique fingerprints as

well as with the laboratory strains H37Rv and H37Ra. Experiments were performed with organisms cultured in the presence of oxygen, as well as under oxygen deprived conditions. Light microscopy and scanning electron microscopy were used to investigate the differences in adhesion and invasion characteristics of the organisms. Furthermore, the expression profile of selected genes potentially associated with virulence, were investigated as well as the possible production of cytotoxins.

The aims and objectives of the study are as follows:

- To establish differences in adhesion capacity to A549 alveolar and BBM bronchial epithelial cells between clustering and non-clustering strains of *M. tuberculosis*
- To establish differences in adhesion capacity to A549 alveolar and BBM bronchial epithelial cells between *M. tuberculosis* isolates belonging to the Beijing family, the F15/LAM4/KZN family and the XDR-variant of the F15/LAM4/KZN.
- To establish differences in the internalization in A549 alveolar and BBM bronchial epithelial cells between these groups of isolates of *M. tuberculosis* in the presence of oxygen and under oxygen limited conditions
- To compare the expression profile of *hspX, fdxA, hbhA* and *mdp1* genes after normalization with 16S and *ftsZ* genes before and during the exposure to epithelial cells among *M. tuberculosis* isolates grown with and without oxygen.

- To investigate the cytotoxic effect on A549 alveolar epithelial cells by *M. tuberculosis* isolates grown with and without oxygen using the lactate dehydrogenase (LDH) assay
- To investigate the ultrastructural changes that *M. tuberculosis* isolates induce in A549 alveolar epithelial cells

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# CHAPTER 2 – LITERATURE REVIEW

## 2.1 Origin of *Mycobacterium tuberculosis*

Knowing the true origin of an organism can be of benefit in understanding the way this organism interacts with its environment. Until recently it was thought that *M. tuberculosis* originated as a result of evolution in *Mycobacterium bovis*, as human tuberculosis (TB) was presumed to have originated from the domestication of cattle (Stead *et al*. 1995). However, investigations of the distribution of deletions and insertions patterns in the *M. tuberculosis* complex genome led to the conclusion that *M. bovis* is not the ancestor of *M. tuberculosis* (Mostowy and Behr, 2005), but that both have independently evolved. Another study suggested that *M. tuberculosis* could have evolved from *Mycobacterium canetti* and not *M. bovis* (Brosch *et al*. 2002). Mostowy and Behr (2005) further stated that genomic records suggest that humans either directly or indirectly brought tuberculosis to the farm.

TB is among the oldest known human diseases and *M. tuberculosis* is as old as the disease it causes. Evidence for this is found in bones (Daniel *et al.* 1994) and soft tissues of ancient human remains (Zink *et al.* 2003). TB is believed to have existed in Egypt during the pre-Pharaonic dynasties. More than 3000 years old Egyptian mummies found in Thebes were reported with lesions in the chest and bones suggestive of TB (Nerlish *et al.* 1997).

## 2.2 Classification of *M. tuberculosis*

The classification of an organism begins with its identification. This enables different individuals at different geographic locations identify an organism as the same. As with all organisms, identification of mycobacteria has traditionally been performed through phenotypic characteristics. These include morphological and biochemical characteristics as well as antigenic make-up. More recently genotypic characterization has been developed. This allows a species to be subdivided into genetically related families. As a result, isolates from patients can be classified as belonging to a well characterized family within the species *M. tuberculosis*. These developments resulted in the recognition that some families are more effectively spreading within the human population than others (Hanekom *et al*. 2007). This motivated researchers to study organisms that belong to a particular family and compare characteristics among families to better understand these differences, as well as to overcome the challenges these organisms pose to our health and environment.

### 2.2.1 Phenotypic characteristics

#### 2.2.1.1 Staining methods

The staining of bacteria resulted in a first classification. Using the Gram staining technique, bacteria can be classified as Gram positive or Gram negative. This technique

was discovered by Hans Christian Gram (1850 – 1938) - a Danish scientist - in 1884, and has stood the test of time. This technique enables the bacteria to be classified, based on the structure of its cell wall. Though a popular method used to classify bacteria, it is not a widely accepted classification criteria for *M. tuberculosis* because the bacillus weakly stains Gram positive. Therefore, acid fast staining methods such as Kinyoun staining that was first described in 1915 by J. J. Kinyoun as well as Ziehl-Neelsen (ZN) staining are used to establish the cell wall characteristics of mycobacteria. These methods are based on the ability of mycobacteria to resist de-staining because of its lipid rich cell wall.

2.2.1.2 Growth requirement

Bacteria that require oxygen to grow are classified as aerobic, while those that grow in the absence of oxygen are classified as anaerobic. *M. tuberculosis* has been previously classified as an obligate aerobe however as far back as 1933 Corper and Cohn questioned this. They reported that out of a total of 56 *in vitro* cultures of human and bovine isolates, 24 contained viable bacilli after 12 years of incubation in sealed containers (Corper and Cohn, 1933) in which the oxygen initially present would have been depleted over time. Later studies confirmed this finding (Wayne and Lin, 1982; Sherman *et al*. 2001; Rustad *et al*. 2009; Ramchandra and Sturm, 2010). In addition, sequencing of the full genome of reference strain H37Rv revealed the presence of genes coding for enzymes involved in anaerobic metabolism (Cole *et al*. 1998). Subsequently it was shown that in the absence of oxygen, these genes are expressed (Ramchandra and Sturm, 2010). Hence the

classification of *M. tuberculosis* as a facultative anaerobe rather than an obligate aerobe should now be fully embraced.

### 2.2.2 Conventional identification

#### 2.2.2.1 Runyon classification

Ernest Runyon (1903-1994) introduced the Runyon classification of non-tuberculous mycobacteria in 1959. This classification is based on the production of pigment and rate of growth of the organisms. The slow growing mycobacteria make up the first three groups and the fourth group comprise of the rapid growing non-tuberculous mycobacteria.

##### 2.2.2.1.1 Runyon group I

These mycobacteria are referred to as photochromogens. They produce non pigmented colonies when grown in the dark and these colonies become pigmented with a yellow colour only after exposure to light. *Mycobacterium kansasii*, *Mycobacterium marium*, *Mycobacterium simiae* and *Mycobacterium asiaticum* are all photochromogens.

##### 2.2.2.1.2 Runyon group II

The mycobacteria in this group are referred to as scotochromogens. They produce yellow pigmented colonies when grown in the dark and orange pigmented colonies when grown in the light. Some strains have been shown to increase pigment production on continuous exposure to light. *Mycobacterium scrofulaceum*, *Mycobacterium gordonae*, *Mycobacterium szulgai*, *Mycobacterium xenopi*, *Mycobacterium celatum* and *Mycobacterium flavescens* are all scrotochromogens.

2.2.2.1.3 Runyon group III

These mycobacteria are also referred to as nonchromogens because they produce colourless or pale yellow pigmented colonies. These pale yellow or tan pigments do not intensify upon exposure to light. *Mycobacterium avium-intracellulare*, *Mycobacterium paratuberculosis*, *Mycobacterium terrae*, *Mycobacterium shimoidae* and *Mycobacterium genavense* are all nonchromogens.

2.2.2.1.4 Runyon group IV

These mycobacteria form colonies within five to seven days and are referred to as rapid growers. They do not produce pigment. *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium peregrinum*, *Mycobacterium abscessus* and *Mycobacterium thermoresistible* are all rapid growers.

2.2.2.2 Conventional classification of *M. tuberculosis*

In the late 1800s the cause of tuberculosis was a bacterium later named *M. tuberculosis*. Its placement in the genus Mycobacterium by Lehamann (1858 - 1940) and Neumann (1868 - 1952) in 1896 initiated its classification (Shinnick and Good, 1994).

Table 2.1 Classification of *M. tuberculosis*

| | |
|---|---|
| Kingdom | Prokaryota |
| Phylum | Actinobacteria |
| Class | Actinobacteria |
| Subclass | Actinobacteridae |
| Order | Actinomycetales |
| Suborder | Corynebacterineae |
| Family | Mycobacteriaceae |
| Genus | Mycobacterium |
| Species group | *Mycobacterium tuberculosis* complex |
| Species | *Mycobacterium tuberculosis* |

### 2.2.3 Genotypic characteristics

Bacteria are also identified based on their genetic makeup. This method is presently considered to be the most precise method when compared to the conventional identification methods currently in use. There are various approaches to genotypic identification. This method is based on nucleic acid either DNA or RNA obtained from the organism. Hence, the ability to extract or isolate good quality and pure DNA/RNA has major influence on the end result. Advancements in mycobacterial genetics have positively influenced the correct identification and classification of particular strains as well as species.

#### 2.2.3.1 Nucleic acid sequence analysis

Mycobacterial identification through nucleic acid sequence analysis is an approach that involves the amplification and sequence analysis of parts of the mycobacterial genome. The ribosomal RNA (rRNA) gene sequence of prokaryotic organisms is highly conserved. Therefore, the sequence of the 16S rRNA component is used in the identification of an organism as well as in defining its evolutionary relationship with other organisms by the establishment of a phylogenetic tree (Rogall *et al*. 1990; Springer *et al*. 1996; Kirschner and Bottger, 1998; Cloud *et al*. 2002).

The whole genome of an organism can also be sequenced and a wealth of information on the DNA can be obtained from this, about the organism. The first full genome sequencing

and annotation of *M. tuberculosis* H37Rv was performed by Cole *et al* (1998) at The Institute for Genomic Research (Fleischmann *et al.* 2002) and this sequence is used to compare the sequence of other laboratory strains and clinical isolates. Other milestones allowing better understanding of the biology of mycobacteria was the development of methods to manipulate mycobacterial genomes by the Gicquel and Jacobs laboratories (Glickman and Jacobs, 2001; Pelicic *et al*. 1998).

2.2.3.2 Molecular typing

Molecular typing helps to differentiate strains within the same species. Pulsed Field Gel Electrophoresis (PFGE) is widely used in epidemiological studies of pathogenic organisms but this is not applicable for genotyping of *M. tuberculosis.* In 1993 Van Embden *et al* published a reproducible method to establish restriction fragment length polymorphism (RFLP) and this has become the gold standard of genotyping for the *M. tuberculosis* complex. This involves determination of the number and the position of the insertion sequence (IS) 6110 within the chromosomal DNA of *M. tuberculosis* isolates (Van Soolingen *et al*. 1999).

Polymerase chain reaction (PCR) based typing methods such as spacer oligonucleotide typing (spoligotyping) (Hermans *et al.* 1992; Sola *et al*. 1999) variable numbers of tandem DNA repeats (VNTR) (Haas *et al.* 1993; Frothingham and Meeker-O'Connell, 1998) and mycobacterial interspersed repetitive units (MIRU) (Mazars *et al.* 2001) are also being used frequently for the rapid typing of isolates. The use of molecular

subtyping techniques such as RFLP and spoligotyping has enabled the further identification of different strains of *M. tuberculosis.* Table 2.2 contains a list of global strain families of *M. tuberculosis* (Ashiru *et al*. 2010).

Table 2.2 Global strain families of *Mycobacterium tuberculosis*

| Strain/family | Reference |
|---|---|
| CAS | (Sreevatsan *et al.* 1997 ; Al-Hajoj *et al.* 2007) |
| EAI | (Sreevatsan *et al.* 1997 ; Al-Hajoj *et al.* 2007) |
| MANU family | (Sreevatsan *et al.* 1997 ; Al-Hajoj *et al.* 2007) |
| CDC 1551 | (Valway *et al.* 1998) |
| T family | (Sola *et al.* 2001 ; Nouvel *et al.* 2006) |
| Beijing family | (Glynn *et al.* 2002) |
| F28 | (Streicher *et al.* 2004) |
| Haarlem family | (Mardassi *et al.* 2005) |
| LAM3/F11 | (Nicol *et al.* 2005) |
| F15/LAM4/KZN | (Pillay and Sturm, 2007) |
| U family | (Dou *et al.* 2008) |

## 2.3 Historical background of the name *M. tuberculosis*

The name of an organism is one of the basic means used to identify and refer to it. As with some of the aspects of this bacterium, the naming had its share of drama. Although, discovered by Robert Koch (1843 – 1910), he in his first (1882) and second (1884) papers on the etiology of tuberculosis, did not give a generic name or a species name according to the Linnaean binary combination rule, but rather used various vernacular names, such as Tuberkel-Virus, Tuberkelbacterien and Tuberkelbacillen (Lessel, 1960) to refer to this organism.

This gave other scientists in the field an opportunity to name this organism. The first of the names given was *Bacterium tuberculosis*, by Wilhelm Zopf (1846 - 1909) in 1883. Thereafter, Edward Emanuel Klein (1844 – 1925) assigned it to the genus Bacillus and named it *Bacillus tuberculosis*. This was followed in 1886 when Lutz Adolph (1855 - 1940) transferred it to a new genus he called Coccothrix, while Elias Metschnikoff (1845 - 1916) in 1888 proposed and placed it in the genus Sclerothrix. However, it was Karl Lehmann (1858 - 1940) together with Rudolf Neumann (1868 - 1952) who in 1896 initiated the inclusion of *M. tuberculosis* as well as *Mycobacterium leprae* in the genus Mycobacterium. Following this, S. Orla-Jensen (1870 - 1949) in 1909 tried to substitute Mycobacterium with Mycomonas; while in 1938 an attempt was made to place it among the Eumycetes (Lesser, 1960). None of these proposals were accepted.

## 2.4 Clinical manifestations and epidemiology

TB is primarily a disease of the lungs. However, TB can affect any organ in the body. Therefore, active TB in humans can be either pulmonary or extrapulmonary disease. TB is further classified as primary and secondary or reactivation disease. Reactivation disease follows latent infection.

### 2.4.1 Stages of TB development

Following infection, there are a number of stages in the development of TB. Not all infected individuals develop all stages. This is dependent on the status of the immune system of the patient.

#### 2.4.1.1 Early stage

This stage follows the exposure of the individual to the bacilli. The immune system fights the infection, resulting in minimal or no symptoms and this may not develop further into an active disease. In this stage patients are not infectious

#### 2.4.1.2 Second stage

The second stage is referred to as early progressive (active) disease. This occurs if the initial infection is not controlled by the immune system. Patients often have non-specific symptoms and they can develop a non-productive cough.

#### 2.4.1.3 Third stage

This stage is called late primary progressive (active) disease. During this phase symptoms and signs are suggestive of TB and the cough is productive. Patients in this stage of disease are infectious and should be managed accordingly.

#### 2.4.1.4 Fourth stage

This stage is the latent disease, in which the bacilli are still present in the patient but, there is no symptom and the patient does not feel sick. Latent disease develops when patients recover from primary progressive disease or directly out of early stage disease.

### 2.4.2 Pulmonary TB

Pulmonary TB accounts for 75 percent of the total TB case burden. The classic symptoms of pulmonary TB include some of the following: productive cough – sometimes with bloody phlegm, shortness of breath, chest pain, fever, night sweats, and weight loss (Sterling *et al.* 2010). However, according to Bassett *et al*. 2009, in cases where the

patient is immunocompromised the disease might be asymptomatic or minimally symptomatic.

When a branch of a pulmonary artery in a tuberculous cavity is affected by external erosion and weakening of the pulmonary artery wall, a tuberculous pulmonary artery mycotic aneurysm, known as Rasmussen aneurysm, occurs. This may result in rupture and haemorrhage. Other complications of pulmonary tuberculosis are bronchiectasis and bronchopleural fistula (Kim *et al.* 2001).

In late pulmonary tuberculosis, complete destruction of the whole lung is not uncommon. TB may promote lung cancer and vice versa, bronchogenic carcinoma can result in reactivation of TB by the erosion of an encapsulated focus and decrease of patient's resistance (Snider and Placik, 1969; Kim *et al*. 2001).

### 2.4.3 Extrapulmonary TB

Extrapulmonary TB disease can present in various forms, depending on the part of the body it affects. This includes pleural TB, TB meningitis, spinal TB (also known as Pott's disease), TB of the skin (also known as lupus vulgaris), genito-urinary TB and TB peritonitis

### 2.4.4 Miliary TB

Miliary TB occurs when there is haematogenic spread of the bacteria throughout the body. This condition manifests with fever, severe weight loss and general progressive deterioration. If treatment is not initiated without delay, the patient will die. The patient's sputum microscopy is usually negative, with 60 – 90 percent of the patients having a normal white blood cell count (Van den Enden, 2004).

## 2.5 Laboratory diagnosis

Laboratory diagnosis of TB is performed on representative specimen from individual TB patients. This specimen is collected from the site of infection; either the lung or body fluids or appropriate body tissue. It is possible to perform immunological tests such as the Mantoux test on the skin and Interferon-γ release assays (IGRAs) on the isolates from the collected specimens. An x-ray of the chest can be used as diagnostic tool for miliary TB.

Drug susceptibility testings are also carried out using either the 1 % proportional methods or Micro-plate assays (MABA, Nitrate reductase, MTT). Serological tests are also being promoted commercially, but they are unreliable (Dowdy *et al*. 2011)

### 2.5.1 Microscopy

The detection limit with standard microscopy is approximately 10, 000 bacteria per 1 mL of sputum. Differential staining of mycobacteria is based on their acid fastness due to the mycolic acids that are present in the cell wall. The Ziehl-Neelsen (ZN) staining method is often used. The specimen is stained with phenol (formerly called carbol) fuchsin solution, which gives the bacilli a red colour. Acid alcohol (95 percent ethanol with 3 percent hydrogen chloride) is then applied to the specimen, to decolourise the smear, with only the mycobacteria retaining the red colour. Methylene blue or malachite green is then used to counterstain, as a microscopic preparation is easier to read if the background is stained with a different colour. ZN staining of pleural fluid, cerebrospinal fluid, ascites fluid and other specimens from extrapulmonary sites has very low sensitivity.

In place of the ZN staining method, a fluorochrome based technique such as auramine-rhodamine can also be used. Here the blanching, with acid alcohol is followed by counterstaining with potassium permanganate. The bacilli then fluoresce under ultra violet light against a black background.

### 2.5.2 Polymerase Chain Reaction (PCR)

#### 2.5.2.1 Nucleic acid amplification tests (NAAT)

NAAT are used for diagnosis of TB. There are different tests using different mycobacterial nucleic acid sequences available commercially. An example is the amplified mycobacterium tuberculosis direct test (MTD, Gen-Probe) with a sensitivity of

92 percent and specificity of 99 percent for smear-positive patients (Guerra *et al.* 2007). The Cepheid GeneXpert system is another example of NAAT which is rapid and simple to use and using the Xpert MTB/RIF assay it is able to detect rifampicin resistance in *M. tuberculosis* from sputum samples with a specificity of 100 percent for smear-positive patients. However, the Xpert MTB/RIF assay have variable levels of specificity and sensitivity (Helb *et al.* 2010).

## 2.6 The biology of *M. tuberculosis*

### 2.6.1 Morphology and physiology of the cell wall

As earlier mentioned, *M. tuberculosis* bacillus is rod shaped. The cell wall is made up of peptidoglycan-arabinogalactan polymer bounded covalently with mycolic acids, various extractable lipids (Barry *et al.* 1998; Daffe and Draper, 1998) as well as pore-proteins (Niederweis, 2003) (Fig. 2.1). The cell envelope houses most of the lipid present in the organism, thereby creating a waxy layer that is an impermeable barrier to noxious compounds and responsible for its staining properties. Lipoarabinomannan (LAM), phthiocerol dimycocerate and trehalose dimycolate are some of the lipids, which are linked non-covalently with the cell membrane and might be involved in virulence of *M. tuberculosis* (Glickman and Jacobs, 2001).

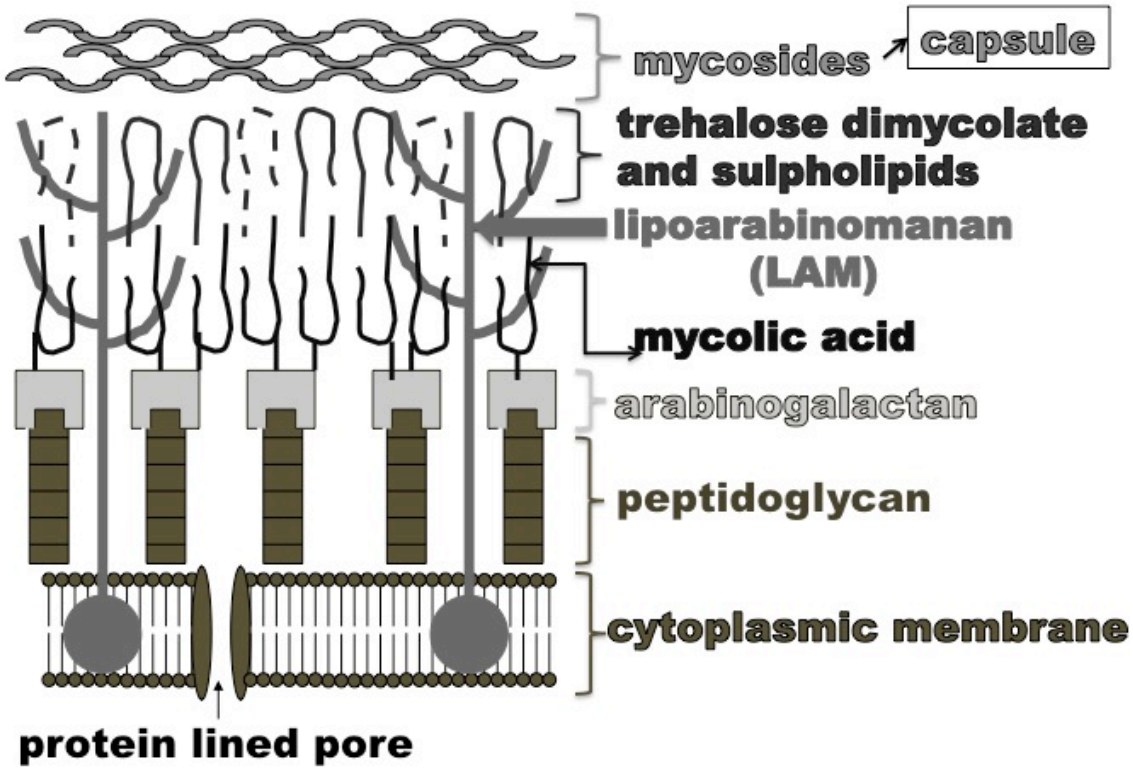


**Figure 2.1** ***Mycobacterium tuberculosis*** **cell wall showing the different layers**

The cord factor has also been reported to be involved in cytokine-mediated events (Indrigo *et al.* 2003; Devergne *et al.*1992). Chatterjee (1992) reported that LAM induces the release of tumor necrosis factor from macrophages. The cell envelope of mycobacteria have been purposed to be an important contributing factor to their resistance to antimicrobial drugs (Brennan and Nikaido, 1995).

## 2.7 Pathogenesis

Successful pathogens exploit the host often by interaction with the host cells, in a way that only the pathogen benefits from this relationship. The pathogen's aim is to obtain its

nutrients, physiochemical environment, respiratory as well as other metabolic needs from the host cell (Goering *et al*. 2008). This process of the pathogen obtaining its requirements from the host cell is often at zero energy cost to the pathogen, resulting in the pathogen using its own resources to replicate. *M. tuberculosis* has and continues to successfully accomplish this goal to the detriment of its host.

### 2.7.1 Pathogen interaction with host cell

*M. tuberculosis* usually gains access into the body through the respiratory tract (Riley *et al.* 1995) where it attaches to the host cell surface molecules. The ability of the pathogen to recognize these surface molecules allows its attachment to the host cell.

#### 2.7.1.1 *M. tuberculosis* and alveolar macrophages

The alveolar macrophages have been suggested to constitute the main initial defense of the host in pulmonary TB infection. Many investigations have been preformed with macrophages and *M. tuberculosis* bacilli to understand the various steps involved in the infection process. The macrophages are able to inhibit bacillary growth by phagocytosis (Riley, 1996).

*M. tuberculosis* has been reported to gain entry into macrophages by being engulfed in a membrane-bound vacuole referred to as a phagosome (Schlesinger, 1996). The bacilli bind to the cell surface molecules such as the integrin family complement receptors (CR)

such as CR1, CR3 (Ahearn and Fearon, 1989) CR4 as well as mannose receptors (Schlesinger, 1993; Schlesinger, 1996b). Different strains of *M. tuberculosis* may interact with CR3 through a different mode (Ernst, 1998). Virulent strains (H37Rv and Erdman) were reported to bind to mannose receptors, while the avirulent strain (H37Ra) did not (Schlesinger *et al.* 1996).

The terminal mannose residues on LAM are used by the mannose receptors on the macrophage to mediate binding of *M. tuberculosis* to macrophages (Schlesinger *et al.* 1994). Surfactant protein A receptor (Downing *et al.* 1995; Gaynor *et al.* 1995) and scavenger receptor (Zimmerli *et al.* 1996) have also been reported to mediate the bacilli binding. Complement and mannose receptors expression by macrophages, have been reported to be influenced by cytokines (Barnes *et al.* 1994; Wright *et al.* 1986).

2.7.1.2 *M. tuberculosis* and alveolar epithelial cells

Until recently, investigations on *M. tuberculosis* interaction with epithelial cells have received less attention in comparison to the bacilli's interaction with macrophages. Some studies had earlier reported that mycobacteria had the ability to bind to and invade epithelial cells but used epithelial cells that were not of lung origin (Shepard, 1955; Mapother and Sanger 1984; Bermudez and Young, 1994).

McDonough and Kress (1995) used A549 alveolar epithelial cells and reported that *M. tuberculosis* adhered to and invaded the A549 alveolar epithelial cells. They observed

that the virulent strains of *M. tuberculosis* that they used were cytotoxic to the A549 alveolar epithelial cells. They also reported that intracellular passage of the bacilli in macrophages influenced the interaction between microbe and macrophages in subsequent experiments (McDonough and Kress, 1995).

The notion that *M. tuberculosis* bacilli, bind to and invade alveolar epithelial cells was investigated by Bermudez and Goodman (1996) as well. They also used the A549 alveolar epithelial cells but did not passage the *M. tuberculosis* bacilli in macrophages. They reported that the *M*. tuberculosis bacilli invaded the A549 alveolar epithelial cell efficiently and that there was intracellular replication of the bacilli in the A549 alveolar epithelial cells (Bermudez and Goodman, 1996).

Mehta *et al.* (1996) also reported on the interaction of *M. tuberculosis* with respiratory epithelial cell, when they compared the intracellular replication of *M. tuberculosis* in macrophages and epithelial cells. They observed that the bacilli invaded the macrophages more in comparison to the respiratory epithelial cells, but there was a higher replication of the bacilli in the respiratory epithelial cells (Mehta *et al.* 1996).

It has been reported that chemokines, are produced by *M. tuberculosis*-infected epithelial cells and the authors suggested that the alveolar epithelial cells may play a role in the inflammatory response in human TB (Lin *et al.* 1998). *M. tuberculosis*-infected alveolar epithelial cells were reported to produce monocyte chemotactic mRNA for MCP-1 and IL-8 (Lin *et al.* 1998; Wickremasinghe *et al.* 1999).

One major short fall of all the above mentioned studies on the interaction between *M. tuberculosis* and respiratory cells is the lack of the inclusion of fresh clinical isolates.

### 2.7.2 Cytotoxicity induction by *M. tuberculosis*

Cytotoxicity is the ability of pathogen or chemical compound to induce death in a host cell and this might result from apoptosis, necrosis or both. Apoptosis and necrosis are types of cellular death mechanisms. The death of the cell is essential in cellular turnover and homeostasis of multicellular eukaryotes (Miyairi and Byme, 2006) and also occurs during elimination of invading organisms (Fink and Cookson, 2005).

#### 2.7.2.1 Apoptosis

Apoptosis is a programmed cell death. This is a physiological process whereby unwanted cells are eliminated without damage to neighbouring cells (Boatright and Salvesen, 2003). This occurs as part of the developmental and other normal biological processes.

2.7.2.2 Necrosis

Necrosis is an accidental cell death. This is a physiological process that takes place when cells are exposed to serious physical or chemical insult. Necrosis is characterized by swelling of the organelle as well as the cell. This is a non-reversible process that leads to the bursting of the cells with the release of its contents (Fink and Cookson, 2005).

Tissue damage has been noted to play a role in granuloma liquefaction and lung cavitation (Dannenberg, 1982; Dannenberg *et al.* 1994). Disruption and destruction of respiratory alveolar epithelium occurs during disease progression of TB, whereby the bacilli gain passage back into the airways through the epithelium for dissemination into a new host (Vanham *et al.* 1997).

McDonough and Kress (1995) observed that virulent laboratory strain H37Rv was cytotoxic to both human lung epithelial cells and murine macrophages. They also reported that the cytotoxicity was dependent on the bacilli load and duration of exposure. However, they were not able to determine the precise mechanism of the cytotoxicity that they had reported.

Dobos *et al.* (2000) was able to report that the mode of cytotoxicity induced by *M. tuberculosis* infection in A549 epithelial cells was necrosis. They also observed that cytotoxicity is specific to the virulence of the bacilli (McDonough and Kress, 1995) and exposure to streptomycin inhibited necrosis (Dobos *et al.* 2000).

### 2.7.3 Induced ultra-structural morphological changes by *M. tuberculosis*

Reddy and Hayworth (2002) reported that the epithelial cells actively participated in the interaction between *M. tuberculosis* and human respiratory epithelial (HEp-2) cells. They observed that the binding of the bacilli caused membrane ruffling, which resulted in long thin projections emanating from the HEp-2 cell surface.

These thin projections were reported to have entrapped the bacilli and formed loops around them on the surface. Internalization of the bacilli occurred in membrane bound vesicles, which were reported to have formed beneath the bacillary attachment locus. This attachment mechanism is referred to as trigger mechanism and they noted that this also has been reported with other bacteria such as *Salmonella* (Finlay and Falkow, 1990) and *Shigella* (Clerc and Sansonetti, 1987).

Garcia-Perez *et al.* (2003) confirmed the infectivity and intracellular proliferation of the virulent laboratory strain H37Rv in A549 alveolar epithelial cells. They used transmission and scanning electron microscopy to show that the ultrastructural changes in the infected A549 alveolar epithelial cells resembled macropinocytosis as described for *Haemophilus influenza* (Ketterer *et al.* 1999). They also reported that the changes were similar to the observation by Reddy and Hayworth (2002) earlier mentioned.

### 2.7.4 Gene expression

As mentioned above the development by Gicquel and Jacobs laboratories (Glickman and Jacobs, 2001; Pelicic *et al.* 1998) of methods for genetic manipulation of mycobacteria have facilitated the advancement in analysis of mycobacteria at the molecular level. The sequencing of the whole genome of the laboratory virulent strain H37Rv (Cole *et al.* 1998) and some other strains from different parts of the world have made available information that is being used to investigate the physiology and pathogenicity of this organism.

Manganelli *et al.* (1999) analysed mRNA levels of 10 sigma factors using H37Rv exposed to different environmental conditions. They observed that though all the mRNA transcripts of the 10 sigma factors were expressed during the exponential growth phase, the expression level varied. They were able to identify three heat shock responsive sigma factor genes – *sig*B, *sig*H and *sig*E. They also observed a down regulation of mRNA transcript of *sig*C under specific conditions.

Delogu *et al.* (2006) investigated the role of the gene coding iron-regulated heparin-binding hemagglutinin protein (*hbhA*) expression during the immunopathogenetic process of TB, *in vitro* and *in vivo*. They used the Erdman strain of *M. tuberculosis* and reported that a major finding in their study was that the *hbhA* gene was specifically, up regulated by the bacilli in the lung but not in the spleen during the early steps of infection.

## 2.8 Epithelial Cells

Epithelial cells are non-professional phagocytic cells and are presumed to be hiding places for the microorganisms. Both the alveolar and bronchial epithelial cells could potentially be involved in the pathogenesis of TB.

### 2.8.1 Alveolar epithelium

The alveolar epithelium comprises two specialized epithelial cell types; the terminally differentiated squamous alveolar epithelial type-1 (AT1) cell, which constitutes approximately 93 percent of the alveolar epithelial surface area and the surfactant-producing cuboidal alveolar epithelial type-11 (AT11) cells; which account for the remaining 7 percent of the alveolar surface area (Crapo *et al.* 1982).

It has been estimated that in an average human male there are 28,000 AT1, 1,400 AT11 and 50 to 100 alveolar macrophages per alveolus (Crandall and Kim, 1991). Thus, there is the likelihood for an inhaled *M. tuberculosis* bacillus to come into contact first with the epithelial cells lining the alveolar space (Birkness *et al.* 1999). This strongly supports the need to investigate thoroughly the interaction of the bacilli with the epithelial cells.

The alveolar epithelial cells have been suggested to provide a niche for the pathogen, where it is able to avoid the hostile environment in the macrophage, replicate and

colonize (Bermudez and Goodman, 1996). The epithelial cells also serve as *in vitro* models to study infection processes where the host cell offers little or no resistance to the bacilli activity (Alonso and Portillo, 2004).

Of the 2 alveolar epithelial cell types, only ATII can successfully be grown in vitro.

### 2.8.2 Bronchial epithelium

Bronchial epithelium is a pseudo-stratified columnar epithelium. All cells are attached to the basal membrane but have different heights. This makes it look like two layers, while in reality it is only one. There are three cell types: mucous producing goblet cells, surfactant producing Clara cells and ciliated cells that transport the mucous with trapped particles in an upward direction (Gruenert *et al*, 1995; Ayers and Jeffer, 1988).

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# Chapter 3.

**Adhesion to and invasion of pulmonary epithelial cells by the F15/LAM4/KZN and Beijing strains of *Mycobacterium tuberculosis***

**Olubisi T. Ashiru, Manormoney Pillay, A. Willem Sturm**

*Journal of Medical Microbiology* 59, 528-533 (2010)

**ABSTRACT**

Globally, specific genotypes of *Mycobacterium tuberculosis* have been shown to dominate in patients, suggesting that these are more successful pathogens. One such genotype, the F15/LAM4/KZN (KZN) family of *M. tuberculosis* has predominated in KwaZulu-Natal, South Africa since the early 1990's. This strain recently evolved from multi-drug to extensive drug resistance (XDR). The ability of *M. tuberculosis* strains belonging to the Beijing family, the KZN family, strains with unique DNA fingerprint patterns and laboratory strains (H37Rv and H37Ra) to adhere to, and invade a human alveolar (A549) and a human bronchial (BBM) epithelial cell line was investigated. All strains displayed greater adhesion to and invasion in A549 as compared to BBM. The Beijing and KZN strains combined showed greater adhesion (28%) than the unique strains (5%) ($P<0.05$). The XDR variant of KZN invaded A549 cells more effectively than the other isolates. These results suggest that the successful spread of the Beijing and KZN strains might be related to their interaction with alveolar epithelium.

## 3.1 INTRODUCTION

South Africa is fifth among the 22 high tuberculosis (TB) burden countries, having incidence and mortality rates of 948 and 230, respectively, per 100 000 population in 2007 (WHO, 2009). This may be attributed to the increasing rates of HIV co-infection (CDC, 2008). Strain-specific molecular markers (Van Embden *et al.* 1993) have identified TB patients with isolates of *Mycobacterium tuberculosis* that share highly similar DNA fingerprint patterns (Van Soolingen *et al.* 1999; Hawkey *et al.* 2003) (Table 2.2). These dominate in patients in different geographic regions, suggesting that shared fingerprints may be a marker of increased transmission efficacy and might indicate recent transmission (Weis *et al.* 2002), while infection with unique DNA fingerprint patterns suggests TB reactivation.

In KwaZulu-Natal, two families of strains dominate: Beijing and F15/LAM4/KZN (KZN). The latter has evolved since the early 1990s into a family of closely related strains accompanied by its progression from a multidrug-resistant to an extensively drug-resistant (XDR) strain (Pillay and Sturm, 2007). The rapid spread of this strain with concomitant high mortality among HIV-infected patients in the Tugela Ferry region of KwaZulu-Natal province (Gandhi *et al.* 2006) is suggestive of its increased virulence.

Pulmonary epithelial cells may be the first barrier that the organism faces (Pessolani *et al.* 2003), as there is a predominance of epithelial cells in the lung (Crandall and Kim, 1991). *M. tuberculosis* has been shown to adhere to, invade, as well as replicate within

pulmonary epithelial cells (Bermudez and Goodman, 1996; Bermudez *et al.* 2002; Hall-Stoodley *et al*. 2006). We hypothesized that the effective transmission of the Beijing and KZN strain families results from a greater capacity than strains with unique DNA fingerprints to adhere to and invade the pulmonary epithelial cells.

## 3.2 METHODS

### 3.2.1 Strains and growth condition

Three isolates of the Beijing family, six of the KZN family of strains, including three XDR, and six isolates with unique DNA fingerprints were obtained from the culture collection of the Medical Microbiology Research Laboratories, University of KwaZulu-Natal, Durban. Laboratory strains of *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177) were also included. The isolates were cultured under aerobic conditions on Middlebrook 7H11 complete agar media (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma) at 37$^{o}$C for approximately 3 weeks.

### 3.2.2 Preparation of inocula

Cultures were harvested into 14 mL PBS containing 0.05% Tween 80 and glass beads (3 mm diameter). The suspensions were vortexed for 5 min, after which larger clumps were

allowed to settle for 15 min. To obtain a single cell suspension, the top 6 mL was aspirated 10 times through a 26-gauge needle (Stokes *et al*. 2004) and passed through a 5 mm Millipore filter. Aliquots of a 10-fold serial dilution of the mycobacterial suspension were plated on Middlebrook 7H11 agar in triplicate to determine the number of c.f.u. per 100 epithelial cells used in the adhesion experiments.

### 3.2.3 Epithelial cell lines

The human type II alveolar epithelial cell line A549 (ATCC CCL 185) and a human bronchial epithelial cell line, BBM (CRL 9482 – a derivative of BEAS-2B), were used in the adhesion and invasion assays. A549 cells were maintained in Eagle's Minimum Essential Medium (Biowhittaker-Lonza) containing Earle's Balanced Salt Solution and 25 mM HEPES supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen) and 2 mM L-glutamine. BBM cells were maintained in Bronchial Epithelial Basal Medium (Lonza) supplemented with Bronchial Epithelial Growth Medium (Lonza). Cells were maintained following standard tissue culture practice (Bermudez and Goodman, 1996). After trypsinization, viable cells were seeded onto glass coverslips (12 mm diameter) in a 24-well tissue culture plate at a concentration of $10^5$ cells/mL and incubated for ±40 h at 37$^{o}$C in a 5% $CO_2$ atmosphere.

### 3.2.4 Adhesion assay

Cells were inoculated with the *M. tuberculosis* suspensions at an m.o.i. of 9–12 bacteria per epithelial cell and incubated at 37$^{o}$C in a 5% $CO_2$ atmosphere for 1 h. Thereafter, the cells were washed and fixed with 95% ethanol. The coverslips were mounted on microscope slides using distyrene plasticizer xylene with the infected side facing upward. The slides were left overnight at room temperature and then stained by the Ziehl–Neelsen staining method. The acid-fast bacilli per 100 epithelial cells were counted using light microscopy and the percentage adhesion was calculated using the number of bacteria added per 100 cells as the denominator. A total of 300 epithelial cells were examined in each reported attachment assay. To ascertain that the bacilli do not attach to glass, coverslips without epithelial cells were also inoculated and incubated as described above.

### 3.2.5 Invasion assay

The method of Bermudez and Goodman (1996) was used with minor modifications. After inoculation with *M. tuberculosis* suspension at an m.o.i. of 9–12 bacteria per epithelial cell, the cells were incubated at 37$^{o}$C in a 5% $CO_2$ atmosphere for 2 h. Following this, the cells were incubated for 1 h at 37$^{o}$C in 5% $CO_2$ in the presence of amikacin (100 mg/mL; Sigma). Medium was removed and inoculated on Middlebrook 7H11 plates to confirm killing of extracellular organisms. The amikacin was removed by washing three times with PBS and the cells were lysed with 0.5 mL 0.1% Triton X-100 in distilled water for

20 min. The harvest was suspended in 0.5 mL Middlebrook 7H9 broth and plated on Middlebrook 7H11 agar to determine the number of viable internalized organisms.

### 3.2.6 Statistical analysis

Microsoft Excel as well as Graphpad Instat software were used to determine whether there were significant differences in the adhesion and invasion abilities among the different strains. All comparisons were analysed initially by a one-way analysis of variance (ANOVA) and thereafter by Tukey–Kramer multiple comparisons test. All experiments were performed three times in triplicate for each isolate to assess intra-assay variability. Inter-assay variability was calculated to determine differences among the isolates belonging to the same strain or family.

### 3.2.7 Ethics approval

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference no. H039/06).

## 3.3 RESULTS AND DISCUSSION

Successful strains of *M. tuberculosis* such as the Beijing (Glynn *et al*. 2002) and KZN (Pillay and Sturm, 2007) strains are capable of infecting large numbers of individuals in

the same time period and therefore form genotype clusters. We hypothesized that the ability of the Beijing and KZN strains to adhere to and invade pulmonary epithelial cells exceeds that of strains with unique fingerprints, suggesting that they are more effective pathogens since they are better able to colonize the lung. All isolates adhered to and invaded the A549 cells much more effectively than the BBM cells.

### 3.3.1 Adhesion of *M. tuberculosis* isolates to the A549 epithelial cell lines

The Beijing and KZN strains combined showed a higher rate of adhesion to the A549 cell line when compared with the unique strains ($P < 0.05$) (Fig. 3.1a). Differences were observed in the rate of adhesion between the two families of strains. The Beijing family adhered more than the XDR-KZN ($P<0.01$) and KZN ($P<0.001$) families (Table 3.1). The Beijing ($P<0.001$), XDR-KZN ($P<0.05$), KZN and unique isolates adhered more than the laboratory strains H37Rv and H37Ra (Table 3.1). Adhesion of virulent H37Rv was greater than that of the avirulent H37Ra ($P < 0.05$). No bacteria were observed on the glass coverslips without epithelial cells.

### 3.3.2 Invasion of the A549 epithelial cells by *M. tuberculosis* isolates

The Beijing and KZN strains together invaded the A549 cells more than the unique strains ($P<0.05$) (Fig. 3.1b). Differences were observed among the groups of isolates in their ability to invade the A549 cells. The Beijing, XDR-KZN and KZN strains had a higher rate of invasion of A549 cells when compared to the unique strains ($P<0.001$) and

the laboratory strains H37Rv (P<0.001) and H37Ra (P<0.001) (Table 3.2). The XDR-KZN strains had the highest invasion rate, followed by the Beijing strains and the KZN strains (Table 3.2). No growth was observed on the Middlebrook 7H11 agar plates that were inoculated with the discarded media, confirming that only internalized bacilli were reported.

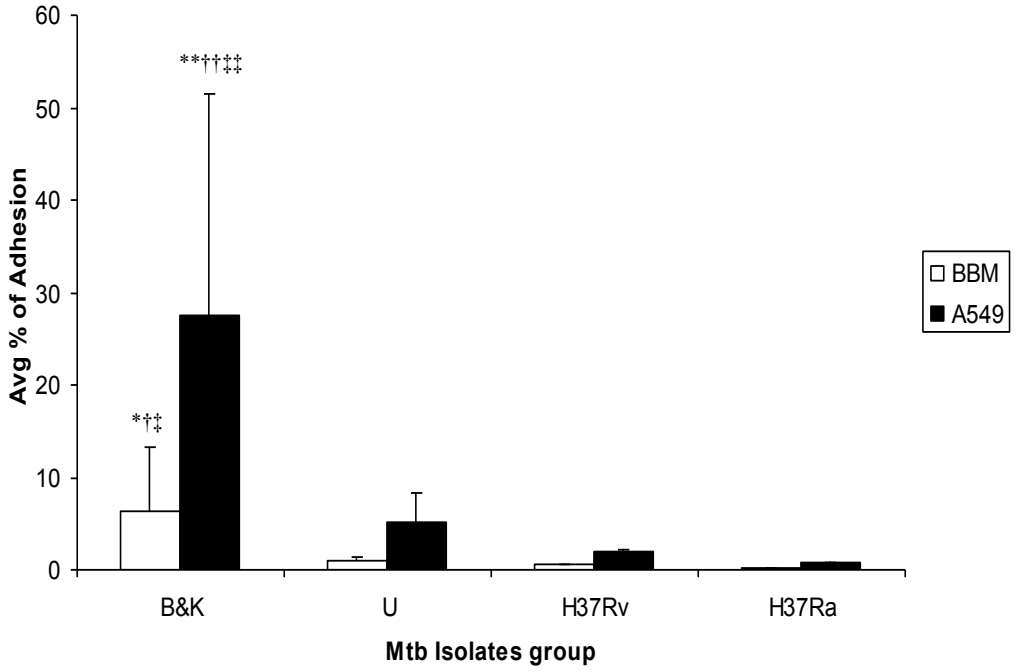


**Figure 3.1A. Adhesion after 1h**
**Comparison of the capacity of strains of *M. tuberculosis* to adhere to and invade A549 and BBM epithelial cell lines. B - Beijing; K - F15/LAM4/KZN isolates; U - Isolates with unique DNA fingerprint patterns; H37Rv - virulent laboratory strain (ATCC 27294 positive control) and H37Ra - avirulent laboratory strain (ATCC 25177 negative control). *P*<0.01(*), <0.001(**) as compared with H37Rv; *P*<0.01(†), <0.001(††) as compared with H37Ra; *P*<0.01(‡), <0.001(‡‡) as compared with U**

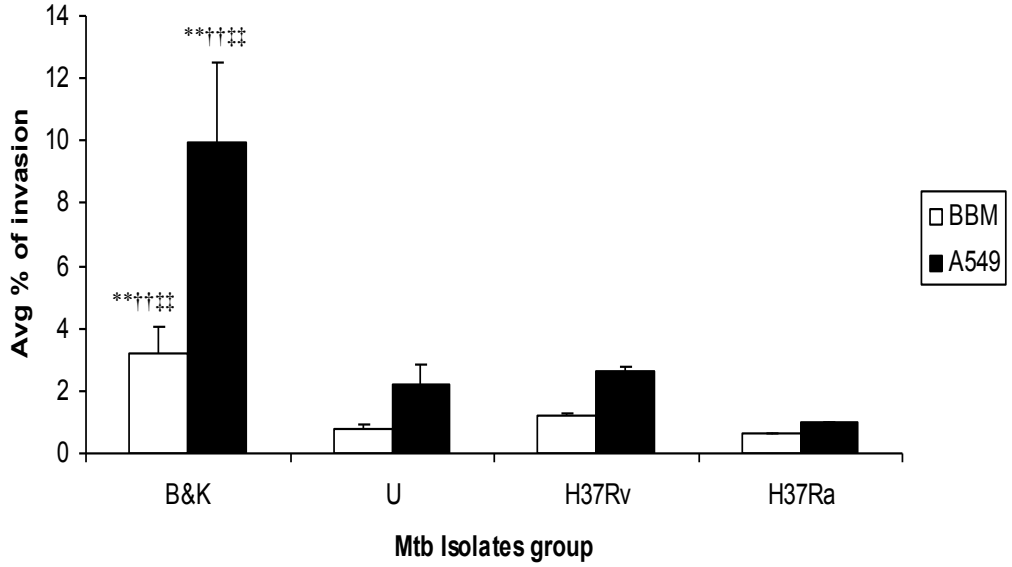


**Figure 3.1B. Invasion after 2h**
**Comparison of the capacity of strains of *M. tuberculosis* to adhere to and invade A549 and BBM epithelial cell lines. B - Beijing; K - F15/LAM4/KZN isolates; U - Isolates with unique DNA fingerprint patterns; H37Rv - virulent laboratory strain (ATCC 27294 positive control) and H37Ra - avirulent laboratory strain (ATCC 25177 negative control). *P*<0.01(*), <0.001(**) as compared with H37Rv; *P*<0.01(†), <0.001(††) as compared with H37Ra; *P*<0.01(‡), <0.001(‡‡) as compared with U**

Table 3.1. Adhesion to epithelial cells by *M. tuberculosis* strains

| | Average % of Adhesion | | | | | |
|---|---|---|---|---|---|---|
| *M. tuberculosis* | A549 | | | BBM | | |
| Strains | (mean ± SD) | $P^{\S}$ | CV (%) | (mean ± SD) | $P^{\S}$ | CV (%) |
| Beijing 1 | 62.44 ± 0.98 | *† | 1.18 | 7.11 ± 0.08 | *† | 1.17 |
| 2 | 64.37 ± 0.41 | *† | 0.13 | 21.91 ± 0.12 | *† | 0.55 |
| 3 | 6.89 ± 0.08 | *† | 0.59 | 1.93 ± 0.01 | *† | 0.30 |
| XDR-KZN 1 | 12.14 ± 0.06 | *† | 0.17 | 2.06 ± 0.04 | *† | 1.94 |
| 2 | 14.06 ± 0.06 | *† | 0.25 | 3.77 ± 0.11 | *† | 2.98 |
| 3 | 39.97 ± 0.08 | *† | 0.05 | 13.15 ± 0.03 | *† | 0.24 |
| KZN 1 | 5.49 ± 0.12 | *† | 1.29 | 1.24 ± 0.01 | *† | 0.47 |
| 2 | 7.17 ± 0.03 | *† | 0.16 | 2.35 ± 0.01 | *† | 0.25 |
| 3 | 35.76 ± 0.10 | *† | 0.08 | 3.19 ± 0.06 | *† | 1.71 |
| UNIQUE 1 | 4.64 ± 0.05 | *† | 0.33 | 0.74 ± 0.01 | † | 1.57 |
| 2 | 1.72 ± 0.07 | † | 4.41 | 0.63 ± 0.05 | † | 7.54 |
| 3 | 10.31 ± 0.31 | *† | 3.42 | 1.63 ± 0.01 | *† | 0.61 |
| 4 | 3.32 ± 0.05 | *† | 0.92 | 0.72 ± 0.01 | † | 1.39 |
| 5 | 3.25 ± 0.05 | *† | 0.47 | 0.71 ± 0.04 | † | 5.84 |
| 6 | 7.75 ± 0.06 | *† | 0.59 | 1.18 ± 0.04 | *† | 3.06 |
| H37Rv | 2.01 ± 0.03 | † | 0.84 | 0.66 ± 0.01 | † | 1.52 |
| H37Ra | 0.84 ± 0.03 | * | 3.81 | 0.26 ± 0.01 | * | 4.56 |

**Epithelial cells were exposed to the inoculum for 1h at 37°C. The results are means of three experiments performed in triplicate. CV, coefficient of variation; SD, standard deviation; $P < 0.05$ (*) compared with H37Rv; $P < 0.05$ (†) compared with H37Ra**

The coefficient of variation (CV) was low for the intra-assay variability for all the isolates in both the adhesion and invasion assays, while for the inter-assay variability the CV values of the three runs were close for each isolate (Supplementary Tables S1; S2; S3; S4 in JMM Online and Appendix B1; B2; B3; B4).

Although there is no consensus on the most appropriate method for enumerating attached bacilli and light microscopy may not be the optimal mechanism to quantify adhered bacteria, experiments were done three times in triplicate and the standard deviation was within an acceptable range (Appendix B1 and B2). The light microscopy could not differentiate between those bacteria that had invaded and those that adhered extracellularly. However, bacteria that invaded had first adhered to the epithelial cells.

The observation that adhesion and invasion of the avirulent H37Ra was minimal while adhesion and invasion of the virulent H37Rv was similar to that of some of the unique isolates suggests that these assays do reflect mycobacterial virulence. Once inhaled bacilli reach the alveoli, they interact with the alveolar macrophages as well as the epithelial cells lining the alveoli (Reddy and Hayworth, 2002). The infective dose is thought to be less than five bacilli (Small and Fujiwara, 2001).

While the role of the alveolar macrophages in the pathogenesis of TB has been extensively studied (Velasco-Velazquez *et al*. 2003; Aoki *et al*. 2004; Nguyen and Pieters, 2005; Xue *et al*. 2007), less attention has been paid to the interaction of *M. tuberculosis* with epithelial cells (Reddy and Hayworth, 2002; McDonough and Kress,

1995). Since there are only a small number of alveolar macrophages (between 50 and 100) but large numbers of epithelial cells (28 000 type I and 1400 type II) present in the alveolar space (Crandall and Kim, 1991), the bacilli have a greater chance of encountering an epithelial cell than a macrophage (Bermudez *et al*. 2002).

In addition, the chances of survival are much higher within epithelial cells than in the potential killing environment within the macrophage. Since invasion of non-professional phagocytes is usually microbe-initiated, the study of such interactions provides useful models to determine microbial determinants of infection (Glynn *et al*. 2002).

Our finding that all 17 *M. tuberculosis* isolates used in this study adhered to and invaded the alveolar cells (A549) more effectively than the bronchial cells (BBM) might be explained by the observation that bronchial epithelium resists infection by *M. tuberculosis* (Schluger and Rom, 1998), and that the organism needs to be inhaled right into the alveoli for infection to occur (Small and Fujiwara, 2001). Since bacterial adhesion to host cell surfaces is specific, our results suggest that *M. tuberculosis* possesses adhesins that interact with receptors present on the alveolar epithelial cell (Kohwiwattanagun *et al*. 2007).

The higher percentage of adhesion observed amongst the Beijing and KZN isolates as compared to the unique and the type culture strains indicates that these strains have an increased capacity to colonise the alveoli. Two of the unique isolates with greater

adhesion and invasion rates than some of the Beijing and KZN strains could have been involved in an undetected chain of transmission.

Table 3.2 Invasion of epithelial cells by *M. tuberculosis* strains

| | Average % of Invasion | | | | | |
|---|---|---|---|---|---|---|
| *M. tuberculosis* | A549 | | | BBM | | |
| strains | (mean ± SD) | $P^{\S}$ | CV (%) | (mean ± SD) | $P^{\S}$ | CV (%) |
| Beijing 1 | 8.52 ± 0.89 | *† | 10.42 | 2.38 ± <0.01 | *† | <0.01 |
| 2 | 13.17 ± 0.71 | *† | 5.38 | 2.96 ± 0.32 | *† | 10.72 |
| 3 | 6.46 ± 0.70 | *† | 10.81 | 2.30 ± 0.24 | *† | 10.31 |
| XDR-KZN 1 | 9.58 ± 0.72 | *† | 7.53 | 4.00 ± 0.27 | *† | 6.75 |
| 2 | 11.11 ± 1.02 | *† | 9.16 | 4.10 ± 0.02 | *† | 0.49 |
| 3 | 13.89 ± 0.68 | *† | 4.86 | 4.17 ± 0.14 | *† | 3.46 |
| KZN 1 | 7.04 ± 0.82 | *† | 11.58 | 2.06 ± 0.06 | *† | 2.91 |
| 2 | 8.94 ± 0.47 | *† | 5.29 | 3.43 ± 0.09 | *† | 2.67 |
| 3 | 11.11 ± 0.83 | *† | 7.50 | 3.48 ± 0.15 | *† | 4.41 |
| UNIQUE 1 | 2.55 ± 0.26 | | 10.08 | 0.74 ± 0.02 | * | 2.80 |
| 2 | 1.25 ± 0.18 | | 14.40 | 0.60 ± 0.05 | * | 8.60 |
| 3 | 2.94 ± 0.30 | * | 10.34 | 1.10 ± 0.01 | † | 8.67 |
| 4 | 2.78 ± 0.27 | * | 9.57 | 0.71 ± 0.07 | * | 9.33 |
| 5 | 1.85 ± 0.18 | | 9.47 | 0.60 ± 0.08 | * | 13.55 |
| 6 | 2.06 ± 0.26 | | 12.40 | 0.78 ± 0.10 | * | 12.13 |
| H37Rv | 2.61 ± 0.16 | | 6.08 | 1.23 ± 0.07 | † | 5.55 |

| | | | | | |
|---|---|---|---|---|---|
| H37Ra | 0.99 ± 0.01 | 1.16 | 0.65 ± <0.01 | * | <0.01 |

**Epithelial cells were exposed to the various inocula for 2h at 37$^{o}$C. The results are the mean of three experiments performed in triplicate. CV, coefficient of variation; SD, standard deviation; $P < 0.05$ (*) compared with H37Rv; $P < 0.05$ (†) compared with H37Ra**

However, it is also possible that the lack of correlation between the relative adhesion and invasion levels and the variation between isolates result from multiple pathways of adhesion. These may include pili, recently described by Alteri *et al*. (2007), as well as different surface proteins (Reddy and Hayworth, 2002) that use different cell surface receptors. In addition, invasion may be independent of adhesion (Garcia-Perez *et al*. 2003).

Our findings suggest that the higher adhesion to and invasion capacity of alveolar epithelial cells may contribute to the successful spread of the Beijing and KZN families of *M. tuberculosis*.

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# Chapter 4.

***Mycobacterium tuberculosis*** **isolates grown under oxygen deprivation invade pulmonary epithelial cells**

**Olubisi T. Ashiru, Manormoney Pillay, A. Willem Sturm**

*Anaerobe Journal* 18(4), 471-474 (2012)

**ABSTRACT**

*Mycobacterium tuberculosis* has the ability to adapt to and survive under different environmental conditions, including oxygen deprivation. To better understand the pathogenesis of *M. tuberculosis*, we studied the invasion of human alveolar (A549) and human bronchial (BBM) epithelial cell lines by *M. tuberculosis* isolates cultured under oxygen deprivation. We used isolates belonging to the Beijing and F15/LAM4/KZN families, isolates with unique DNA fingerprints and the laboratory strains H37Rv and H37Ra. We determined that: (1) *M. tuberculosis* bacilli grown under oxygen deprivation invade epithelial cells, (2) the invasion capacity of all 17 isolates differed, and (3) oxygen deprivation influenced the invasion capacity of these isolates. All isolates invaded the A549 more effectively than the BBM cells. Three of the F15/LAM4/KZN isolates, two of which had extensively drug resistance (XDR) profiles, were at least twice as invasive ($\geq$33%) as the most invasive Beijing isolate (15%) ($P<0.05$). We conclude that for a more comprehensive understanding of the pathogenesis of *M. tuberculosis,* studies should include isolates that have been cultured under oxygen deprivation.

## 4.1 INTRODUCTION

Tuberculosis (TB) remains a leading infectious cause of death among adults. The pathogenesis of *Mycobacterium tuberculosis* is, however, not well understood, and this in turn hampers research aimed at prevention of TB. One factor responsible for this may be that experimental research on this pathogen has been mostly performed with aerobically grown isolates, but some studies indicate that this organism adapts and survives in an oxygen deprived environment (Wayne and Lin, 1982; Wayne and Hayes, 1996; Li *et al*. 2002). A close *in vitro* simulation of the *in vivo* oxygen levels the organism is exposed to while in the host, would perhaps lead to a better understanding and facilitate further research on the pathogenesis of TB.

Previous studies have shown that *M. tuberculosis* isolates grown in the presence of oxygen are able to cause infection by adhering to and invading host cells (Bermudez and Goodman, 1996; Bermudez *et al*. 2002; Hall-Stoodley *et al*. 2006; Ashiru *et al*. 2010). *M. tuberculosis* is a lung pathogen that grows under aerobic conditions and it was previously believed to be an obligate aerobe (Todar, 2010). Therefore, most studies have focused on aerobically cultured organisms. However, studies on *M. tuberculosis* need to include bacilli grown in oxygen deprived environment. It has been shown that *M. tuberculosis* bacilli deprived of oxygen *in vitro* remain viable as well as virulent for several years (Corper and Cohn, 1933). Furthermore, *M. tuberculosis* bacilli adapt and survive (Wayne and Lin, 1982; Wayne and Hayes, 1996) in an oxygen depleted environment. In addition,

*M. tuberculosis* bacilli grown under oxygen deprivation invaded macrophages at a high rate (Li *et al*. 2002).

Oxygen deprived environments are common in the human body. One example is granuloma formation, a characteristic of latent TB infection. In granuloma, as in most parts of the body, the redox potential is low, creating a hypoxic (oxygen deprived) environment (McClane *et al*. 1999).

In a previous study, we showed that aerobically grown clinical isolates of *M. tuberculosis* displayed a greater capacity to adhere to and invade pulmonary epithelia than virulent (H37Rv) and avirulent (H37Ra) laboratory strains (Ashiru *et al*. 2010). In the present study, we investigated oxygen deprived *M. tuberculosis* using the same isolates to address the following questions: (1) Do *M. tuberculosis* bacilli grown under oxygen deprivation invade human alveolar (A549) and bronchial (BBM) epithelial cells *in vitro*? (2) If yes, is the invasion capacity the same for all the isolates used? And (3) if no, is there a difference between the invasion capacity of these isolates grown under oxygen deprivation and our previous results on the same isolates grown in the presence of oxygen?

## 4.2 Materials and methods

### 4.2.1 Strains

Fifteen clinical isolates of *M. tuberculosis* that were previously genotyped were obtained from the culture collection of the TB unit of the Department of Infection Prevention and Control, University of KwaZulu-Natal, Durban. Three of these belonged to the Beijing family, six belonged to the F15/LAM4/KZN family (including three extensively drug resistance - XDR) and six were strains with unique fingerprint patterns. *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177) were included as virulent and avirulent controls, respectively.

### 4.2.2 Growth conditions

The isolates were inoculated in triplicate in Middlebrook 7H9 broth (Difco) and incubated at 37$^{o}$C. The cultures were incubated in an upright position without agitation for a period of three months (to obtain an oxygen deprived environment) (Wayne, 1976). The concentration of amikacin that was used to kill extracellular bacteria was determined by first growing all isolates at 37$^{o}$C on Middlebrook 7H10 agar containing 50, 100, 150 and 200 mg/mL of the antibiotic.

### 4.2.3 Inocula preparation

Cultures were harvested and single cell suspensions were obtained as previously described (Ashiru *et al*. 2010). Briefly the vortex agitated suspensions were allowed to stand for 15 min, thereafter, the top 6 mL was forced 10 times up and down through a 26 gauge needle and then filtered using a 5 mm Millipore filter. Four 10-fold serial dilutions of the suspension (20 μL) were plated on Middlebrook 7H11 complete agar media (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma), in triplicate to determine the number of colony forming units (CFU) per mL.

### 4.2.4 Epithelial cell lines

The human type II alveolar epithelial cell line A549 (ATCC CCL 185) and the human bronchial epithelial cell line BBM (CRL 9482) were used for the invasion assay. The cells were maintained as previously described (Ashiru *et al*. 2010). The monolayers were detached from the flask by trypsinization using trypsin-versene (Cambrex Bio Science). Trypan blue exclusion was used to determine the number of viable cells using a hemocytometer and 1 mL of the cell suspensions containing 1 x $10^5$ cells/mL were seeded into each well of a 24-well tissue culture plate. These were incubated for $\pm$40 h at 37$^{o}$C in a 5% $CO_2$ atmosphere before being used for the experiment.

### 4.2.5 Invasion assay

The cells were washed thrice with phosphate buffered saline maintained at room temperature (PBS pH 7.3; Oxoid), after which the culture medium (maintained at room temperature) was added into each well. Following inoculation of the cells with the *M. tuberculosis* suspension at a multiplicity of infection (MOI) of 9-12 bacteria per epithelial cells, plates were incubated for 2 h at 37$^{o}$C in a 5% $CO_2$ atmosphere.

To kill extracellular bacteria, the cells were further incubated for 1 h with amikacin (200 mg/mL). The cells were washed thrice with PBS, pH 7.3 to remove the amikacin. Cells were exposed to 0.5 mL of 0.1% triton X-100 in distilled water for 20 min to release internalized bacilli. Thereafter, 0.5 mL of Middlebrook 7H9 broth was added into each well and pipetted 3 times up and down to resuspend the bacteria and 20 µL was plated on Middlebrook 7H11 agar. The media containing amikacin used to kill the extracellular bacteria was also plated out on Middlebrook 7H11 agar plates to verify that only internalized bacilli were reported in the assay. The plates were incubated for 3 weeks at 37$^{o}$C and the percentage of bacterial invasion was determined based on colony counts obtained.

We calculated the percentage of bacterial invasion by dividing the number of internalized bacilli by the number of bacilli in the inoculum per well (as obtained from the colony count) and then multiplied by a hundred.

### 4.2.6 Statistical analysis

All data were analysed initially by one-way analysis of variance (ANOVA) and thereafter Dunn's multiple comparisons test. All experiments were performed in triplicate and repeated three times.

### 4.2.7 Ethics approval

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no: H039/06).

## 4.3 Results

This is the first study describing the invasion of pulmonary epithelial cells by strains belonging to the Beijing and F15/LAM4/ KZN families of *M. tuberculosis*, grown under oxygen deprivation *in vitro*.

The amikacin killing experiments showed that all isolates were killed at a concentration of 100 mg/mL or higher. This was confirmed by the absence of growth from the discarded media for all 17 isolates. Therefore, only internalized bacteria were reported in this study.

All 15 clinical and the 2 laboratory isolates invaded the A549 cells more effectively than the BBM cells (Table 4.1). There was a distinct difference in the ability of the 15 clinical isolates to invade the A549 cells compared to the BBM cells (P< 0.0001) (Fig. 4.1). There were differences in the ability of the isolates to invade the A549 cells, with those belonging to both the Beijing and F15/LAM4/KZN families invading at a higher rate than strains with unique DNA fingerprints (P <0.01) (Fig. 4.1).

Table 4.1. Invasion of pulmonary epithelial cells by oxygen deprived ($O_2$) cultured strains of *M. tuberculosis*

| | Average % Invasion | | | | | |
|---|---|---|---|---|---|---|
| *M. tuberculosis* | A549 | | | BBM | | |
| Strains | (mean ± SD) | $P^{\S}$ | CV (%) | (mean ± SD) | $P^{\S}$ | CV (%) |
| Beijing 1 | 14.00 ± 0.26 | | 1.89 | 3.44 ± 0.18 | | 5.22 |
| 2 | 15.05 ± 0.27 | | 1.81 | 4.44 ± 0.06 | | 1.36 |
| 3 | 11.71 ± 0.13 | | 1.13 | 3.33 ± 0.10 | | 3.12 |
| XDR-KZN 1 | 19.33 ± 0.35 | ‡ | 1.82 | 5.33 ± 0.10 | | 1.95 |
| 2 | 33.90 ± 0.48 | **†‡‡‡ | 1.40 | 14.12 ± 0.15 | ***†††‡‡‡ | 1.08 |
| 3 | 35.09 ± 0.23 | ***†††‡‡‡ | 0.66 | 14.62 ± 0.09 | ***†††‡‡‡ | 0.59 |
| KZN 1 | 18.18 ± 0.70 | | 3.85 | 9.09 ± 0.10 | | 1.10 |
| 2 | 22.41 ± 0.56 | *‡‡ | 2.50 | 9.68 ± 0.08 | *‡‡ | 0.78 |
| 3 | 34.77 ± 0.59 | ***†††‡‡‡ | 1.71 | 13.33 ± 0.19 | ***†‡‡‡ | 1.42 |
| UNIQUE 1 | 7.58 ± 0.05 | | 0.66 | 2.77 ± 0.06 | | 2.09 |
| 2 | 6.58 ± 0.36 | | 5.40 | 2.65 ± 0.13 | | 4.74 |

| | | | | |
|---|---|---|---|---|
| 3 | 9.40 ± 0.32 | 3.36 | 2.87 ± 0.07 | 2.37 |
| 4 | 7.92 ± 0.17 | 2.18 | 2.78 ± 0.07 | 2.45 |
| 5 | 6.90 ± 0.28 | 3.99 | 2.70 ± 0.02 | 0.74 |
| 6 | 7.14 ± 0.24 | 3.39 | 2.74 ± 0.05 | 1.82 |
| H37Rv | 7.06 ± 0.16 | 2.21 | 2.78 ± 0.07 | 2.39 |
| H37Ra | 2.78 ± 0.23 | 8.10 | 1.11 ± 0.07 | 6.50 |

**Epithelial cells were exposed to the various inocula for 2h at 37°C. The results are the mean of three experiments performed in triplicate. SD, standard deviation; CV, coefficient of variation; *P*<0.05(*), *P*<0.01(**), *P*<0.001(***) as compared with B1; *P*<0.05(†), *P*<0.01(††), *P*<0.001(†††) as compared with B2; *P*<0.05(‡), *P*<0.01(‡‡), *P*<0.001(‡‡‡) as compared with B3. § Comparisons were performed initially by ANOVA and thereafter by Dunn's multiple comparisons test**

All 6 isolates belonging to the F15/LAM4/KZN family displayed the highest invasion rates (Table 4.1). Two of the three most invasive F15/LAM4/KZN isolates had the XDR resistance profile. The isolates belonging to the Beijing family were found to be less invasive than those belonging to the F15/LAM4/KZN family ($P < 0.01$) (Fig. 4.1). However, they were more invasive than the isolates with unique fingerprints. All, but two of the latter were found to be more invasive, when compared to the virulent laboratory strain (H37Rv) (Table 4.1).

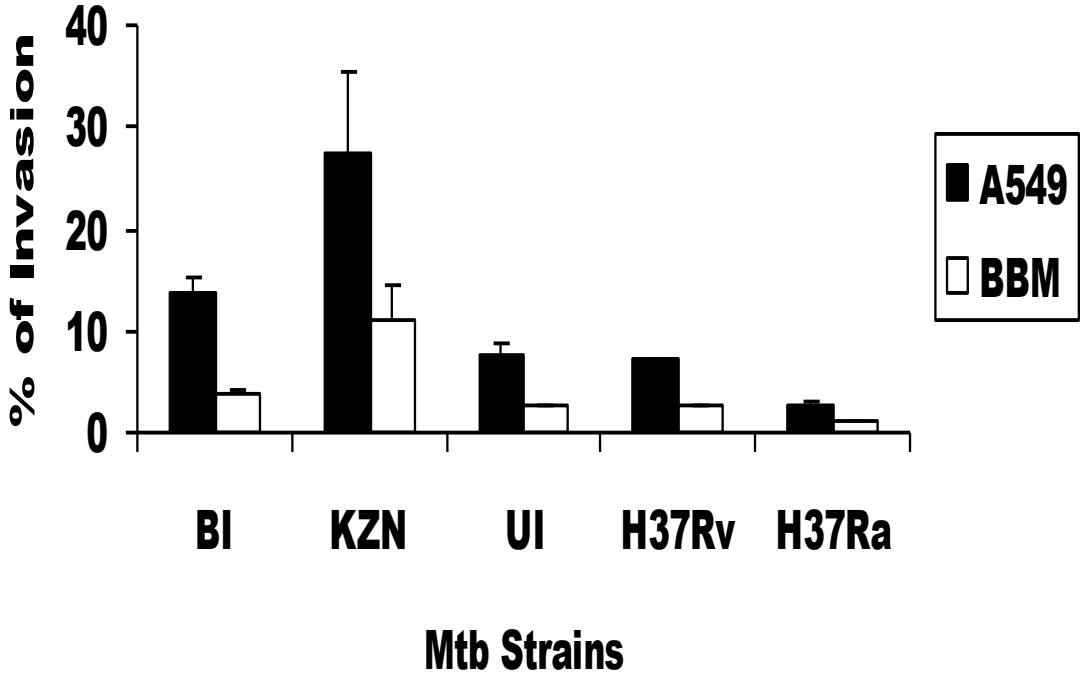


**Figure 4.1. Comparison of the capacity of *M. tuberculosis* strains grown under oxygen deprivation ($\dot{V}_{O_2}$) to invade A549 (black bars) and BBM (white bars) epithelial cell lines after 2h.**

A similar trend was observed in the invasion rates of all the isolates for the BBM cells, but with much lower values (Table 4.1). Differences were observed in the invasion rates of some of the isolates within each family (Table 4.1). Each experiment was done three times in triplicate. The coefficient of variation (CV) was within acceptable range (Supplementary Tables S1a and S1b in Anaerobe online; Appendix B5 and B6).

## 4.4 Discussion

Our results show that *M. tuberculosis* bacilli grown under oxygen deprivation invade epithelial cells *in vitro*. This is similar to the study of Li *et al.* (2002), who reported on macrophage invasion of anaerobically grown H37Rv and Erdman strains *in vitro*.

Following inhalation, *M. tuberculosis* enters into and survives in alveolar macrophages (Stokes *et al*. 1993; Gatfield and Pieters, 2000). Epithelial cells outnumber macrophages in the alveoli (Crandall and Kim, 1991), resulting in a much bigger surface area as compared to that represented by macrophages. It is therefore likely that these cells may be the first point of contact between host and microbe. However, not much attention has been paid to their possible role in the pathogenesis of tuberculosis.

Whole genome sequencing of the H37Rv strain of *M. tuberculosis* revealed the presence of a full set of genes involved in anaerobic metabolism (Cole *et al*. 1998), creating the possibility that this bacterial species is not a strict aerobe but a facultative organism. Indeed, active anaerobic metabolism has been shown in *M. tuberculosis* when grown under oxygen restriction (Ramchandra and Sturm, 2010). The use of different metabolic pathways may result in differences in pathophysiology. Although the alveoli are the point of gas exchange in the lungs and are therefore rich in oxygen, the low redox potential of tissue surfaces prevents the use of extracellular oxygen as an electron acceptor. We therefore investigated the interaction between *M. tuberculosis*, grown under oxygen deprivation, and alveolar epithelial cells.

The isolates we used did not have the same invasion capacity. This is in contrast to Li *et al* (2002), who reported a similar invasion capacity for the two *M. tuberculosis* strains they had used. We included 15 clinical isolates and this could explain the contrasting results. Difference in virulence of isolates belonging to the same family has been reported before (Palanisamy *et al*. 2009). The high percentage of invasion observed among the isolates belonging to the Beijing family is in keeping with other reports on their virulence (Palanisamy *et al*. 2009; Hanekom *et al*. 2007; Theus *et al*. 2007). Furthermore, our results suggest that isolates of F15/LAM4/KZN family have the highest percentage of invasion. This may reflect on the high virulence of this family that was reported to have been responsible for high mortality rates in the Tugela Ferry region of KwaZulu-Natal province, in South Africa (Gandhi *et al*. 2006), and supports the need for more research to be done using isolates belonging to this family.

Comparison of the results (Fig. 4.1) obtained in this study with that of our previous study (Ashiru *et al*. 2010) shows that there is a difference in the invasion capacity of isolates grown under oxygen deprivation and the same isolates grown in the presence of oxygen. The phenotype of *M. tuberculosis* bacilli grown under oxygen deprivation is more invasive than its counterpart grown in the presence of oxygen (Fig. 4.2). This is similar to the findings of Li *et al*. (2002) who observed an increase in the uptake of H37Rv and Erdman strain by macrophages *in vitro* when the organisms were grown anaerobically. As in our previous study (Ashiru *et al*. 2010), the avirulent H37Ra strain had lower levels

of invasion in both cell types than H37Rv, indicating that these assays reflect mycobacterial virulence.

An increased capacity of invasiveness of alveolar epithelial cells by both the Beijing and the F15/LAM4/KZN family is likely to play a major role in their successful spread. The higher level of invasion of the XDR isolates of the F15/LAM4/KZN family may be associated with the high mortality reported in patients infected with this variant (Gandhi *et al*. 2006).

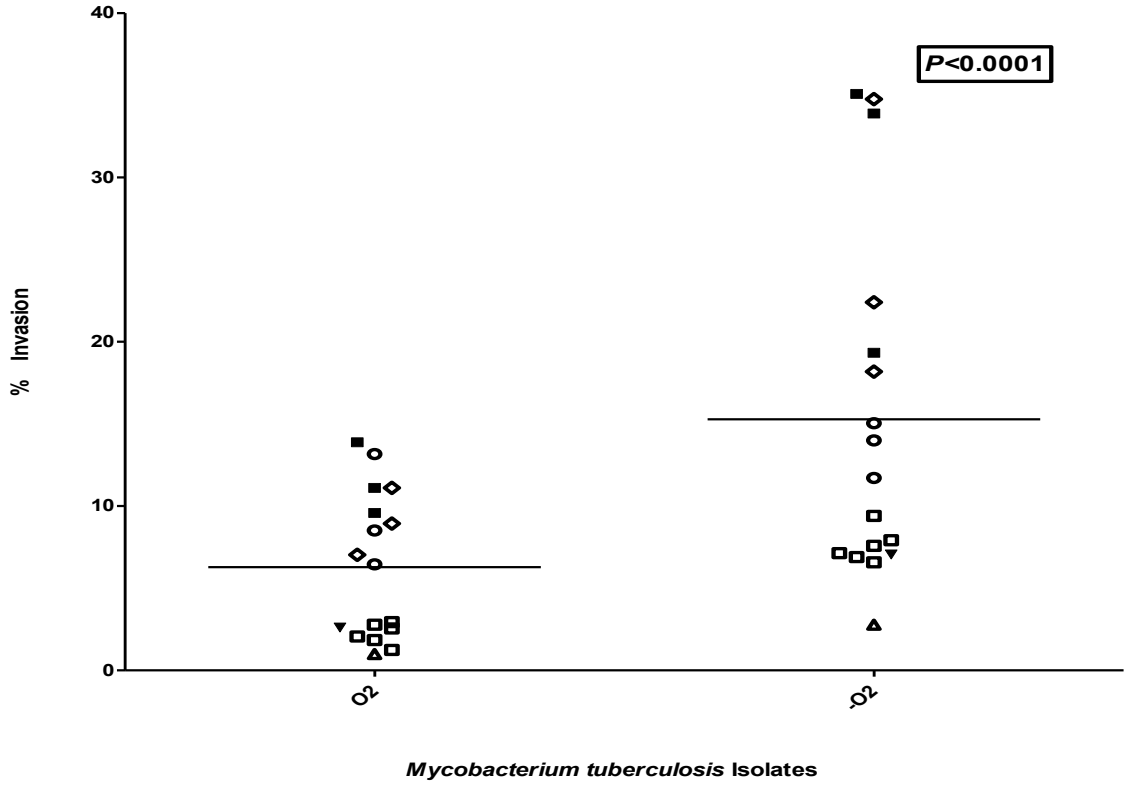


**Figure 4.2. Comparison of the invasion of A549 epithelial cells after 2h by *M. tuberculosis* isolates grown in the presence of oxygen ($O_2$) [Ashiru *et al.* 2010] and under oxygen deprivation ($O_2$). Each point represents the mean of the results obtained from three experiments performed in triplicate for each *M. tuberculosis* isolate. ○= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates;**

**◇= F15/LAM4/KZN isolates; □= Isolates with unique DNA fingerprint; ▼= H37Rv; △= H37Ra; *P*<0.0001**

We performed the invasion assay experiments under aerobic conditions. As postulated by others, the short duration of the experiment makes it unlikely that the bacilli grown under oxygen deprivation would have converted to the phenotype of bacilli grown in the presence of oxygen.

As there is yet no gold standard used to obtain an oxygen depleted environment in the *in vitro* growth of *M. tuberculosis*, we decided to use the Wayne approach: which allows for a gradual depletion of oxygen at the same time allowing the bacteria to gradually adjust to the change in the environment. There also would have been a gradual depletion in the nutrients available to the bacteria, but we did not investigate this aspect. However, the depletion in the available nutrients would have been encountered by all the isolates - as they were all subjected to the same condition.

From our results, we conclude that to better understand the pathogenesis of *M. tuberculosis*, studies should include isolates that have been cultured under oxygen deprivation.

**Funding**

The Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa funded this study.

**Supplementary material**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.anaerobe.2012.04.010 and appendix B5 and B6.

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# Chapter 5.

**Cytotoxicity induction in A549 alveolar epithelial cells by *Mycobacterium tuberculosis* isolates cultured in the presence and absence of oxygen**

**Olubisi T. Ashiru, Manormoney Pillay, A. Willem Sturm**

Microbial Pathogenesis: *In preparation*

**ABSTRACT**

The emergence of drug resistant (DR) *Mycobacterium tuberculosis* and our previous findings that clinical isolates of DR-*M. tuberculosis* adheres to and invades alveolar epithelial cells more effectively than the virulent laboratory strain (H37Rv); highlight the need to include isolates grown under oxygen deprivation in research on this organism. In this study, we report on clinical *M. tuberculosis* isolates induced cytotoxicity in A549 alveolar epithelial cells *in vitro*.

Cytotoxicity levels induced by the isolates varied. Growth in the presence of oxygen resulted in a lower level of cytotoxicity, when compared to the same isolate grown under oxygen deprivation. The highest cytotoxicity level (35.7%) was produced by an extensively drug resistant (XDR) isolate that belonged to the F15/LAM4/KZN family. The avirulent laboratory strain (H37Ra) induced the lowest level of cytotoxicity, under both growth conditions.

These results correlate well with our previous findings on the adherence and invasion rates of these isolates. The increased tissue destruction induced by the F15/LAM4/KZN (XDR) strain compared to the others attest to its virulence and may partly explain the high mortality rates of patients infected with this strain in KwaZulu-Natal in 2005.

## 5.1 INTRODUCTION

South Africa is responsible for almost half of the total TB caseload among the eleven countries of the Southern Africa sub-region. It currently has the highest TB burden in the whole of Africa, and is placed 3rd among the twenty-two high TB burden countries in the world (WHO, 2011). Historical neglect and poor management systems are contributing factors to the South African TB crisis. The progression of clinical isolates of *Mycobacterium tuberculosis* into multi-drug and extensively drug resistant (MDR and XDR) isolates as well as the high rate of HIV-TB co-infection also contribute to the high ranking of South Africa within the global TB epidemic (Gandhi *et al*. 2010).

Understanding of the pathogenesis of TB can assist in the prevention of spread of the infection as well as the development of new vaccines and drugs. For infection to occur there must be interaction between the host cell and the microbial pathogen. *M. tuberculosis* bacilli are thought to enter its host as droplet nuclei via the nasopharynx and the bronchial tree to reach the alveolar space (Dannenberg and Tomashefski, 1988; Nardell, 1993). Here, the bacilli are believed to make contact with the alveolar epithelium and colonise the alveolar tissue by adhering to and invading the epithelial cells. Previous reports show that *M. tuberculosis* bacilli adhere to and invade alveolar epithelial cells *in vitro* (McDonough and Kress, 1995; Bermudez and Goodman, 1996, Ashiru *et al*. 2010; Ashiru *et al*. 2012). It has been suggested that this colonisation is followed by the destruction of the alveolar epithelium, granuloma formation and subsequent caseation and liquefaction of the granulomatous tissue (Dannenberg, 1982).

Clarification of the virulence mechanism used by *M. tuberculosis* and the relationship between different virulence attributes will help in understanding the pathogenesis of TB. McDonough and Kress (1995) reported that the virulent laboratory strain H37Rv was toxic to both epithelial monolayers and macrophages. Their results were confirmed by Dobo *et al* (2000); who also reported that this cytotoxicity was caused by cellular necrosis. These studies were performed in the presence of oxygen. However, the low redox potential in granuloma is likely to prevent aerobic metabolism. It is therefore prudent to investigate the toxic effect of *M. tuberculosis* under anaerobic circumstances. There is also accumulating evidence that there are differences in virulence between different strains. Therefore, comparing successfully spreading strains with those that are less successful will result in additional valuable information.

In this study, we used clinical isolates of *M. tuberculosis* previously reported to adhere to and invade alveolar epithelial cells (Ashiru *et al*. 2010; Ashiru *et al*. 2012) to investigate whether clinical isolates of *M. tuberculosis* belonging to different strain families induce different levels of cytotoxicity in human alveolar epithelial cells (A549) *in vitro*. We also investigated whether there is a difference in the cytotoxicity levels induced by the isolates grown in the presence of oxygen and under oxygen deprivation. Finally, we also addressed the question whether the level of toxicity was related to the formerly observed differences in adhesion and invasion of A549 cells.

## 5.2. Materials and methods

### 5.2.1. Strains

Fifteen clinical isolates of *M. tuberculosis* with different restriction fragment length polymorphism (RFLP) signatures were obtained from the culture collection of the TB section of the Department of Infection Prevention and Control, University of KwaZulu-Natal, Durban. Three of these belonged to the Beijing family, six to the F15/LAM4/KZN family (including three XDR isolates) and six were strains with unique fingerprint patterns. *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177) were included as virulent and avirulent controls respectively.

### 5.2.2. Growth conditions

#### 5.2.2.1 Growth with oxygen

The isolates were inoculated in triplicate in Middlebrook 7H9 broth (Difco) and incubated with gentle agitation at 37$^{o}$C in a shaking incubator. The cultures were used when an optical density of 1 was reached (measured at 600nm) which took incubation periods between two to three weeks.

#### 5.2.2.2 Growth under oxygen deprivation

The isolates were inoculated in triplicate in Middlebrook 7H9 broth (Difco) in screw-capped 15 mL tubes with a diameter of 17 mm containing 9 mL of broth. To obtain an oxygen deprived environment the cultures were incubated at 37$^{o}$C in an upright position without agitation for a period of three months (Wayne, 1976; Ashiru *et al*. 2012).

### 5.2.3 Inocula preparation

The broths were centrifuged (3000 x g, 10 min) and the supernatant discarded. The bacteria containing pellets were resuspended in 5 mL Eagle's Minimum Essential Medium (Biowhittaker-Lonza), vortex agitated for 5 min, and sonicated (10 w for 10 sec) to reduce clumping. To determine the number of colony forming units (CFU) per mL, 20 μL of four 10-fold serial dilutions of the suspension were plated out in triplicate on Middlebrook 7H11 complete agar media (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma).

### 5.2.4 Epithelial cell line

The human type II alveolar epithelial cell line A549 (ATCC CCL 185) was used for the infection assay. The cells were maintained as previously described (Ashiru *et al*. 2012). The monolayers were detached from the flask by trypsinization using trypsin-versene (Cambrex Bio Science). The trypan blue exclusion test was used to determine the number

of viable cells. These were counted using a haemocytometer. One mL of suspension containing $10^5$ cells was seeded into each well of a 24-well tissue culture plate. These were incubated for $\pm$40 h at 37$^o$C in a 5% $CO_2$ atmosphere before being used for the experiment.

5.2.5. Infection Assay

The cells were washed thrice with phosphate buffered saline maintained at room temperature (PBS pH 7.3; Oxoid), after which EMEM (maintained at room temperature) was added into each well. Following inoculation of the cells with *M. tuberculosis* suspension at an m.o.i. (multiplicity of infection) of 9–12 bacteria per epithelial cell, the cells were incubated at 37$^o$C in a 5% $CO_2$ atmosphere for 4 h. Control wells were included following the manufacturers instruction. The plate layout is shown in Table 5.1

**Table 5.1. Layout of assay plate.**

| | | | | | |
|---|---|---|---|---|---|
| CMB | TSR | TMR | $ddH_2O$ | Experiment | ECSR |
| CMB | TSR | TMR | $ddH_2O$ | Experiment | ECSR |
| CMB | TSR | TMR | $ddH_2O$ | Experiment | ECSR |
| $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ |

**CMB – Culture medium (EMEM) background; TSR – Target spontaneous (uninfected A549 cells) release; TMR – Target maximum (lysed uninfected A549 cells) release; $ddH_2O$ – autoclaved distilled water; Experiment – infected A549 cells; ECSR – Effector cell (*M. tuberculosis* inoculum) spontaneous release.**

### 5.2.6. Measurement of lactate dehydrogenase release

The release of the cytosolic enzyme lactate dehydrogenase (LDH) from the infected A549 cells was measured using the CytoTox96 non-radioactive cytotoxicity assay (Promega) - a colorimetric kit and the manufacturer's instructions were followed with a slight modification. Briefly, 50µL supernatant from each well (*M. tuberculosis* inoculum - effector spontaneous; uninfected A549 cell – target spontaneous; uninfected lysed A549 cells – target maximum and infected A549 cells - experimental) was transferred into designated wells of a 96 well tissue culture plate and the CytoTox 96 kit components were added. LDH activity resulted in the conversion of a tetrazolium salt (INT) into a red formazan product. The intensity of the colour formed was directly proportional to the number of cells lysed. The absorbance of each designated well was measured at 450nm (Glox Max). The average values of the different readings were each normalized by subtracting from each, the average absorbance value of the culture medium (EMEM) background. The percentage cytotoxicity was then calculated using the formula in the assay protocol stated below:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

### 5.2.7. Statistical analysis

All data were analysed initially by one-way analysis of variance (ANOVA) and thereafter Dunn's multiple comparisons test with statistical significance (*P*) determined. Spearman's correlation coefficient ($r_2$) was calculated to determine the correlation between pairs of previous and present studied virulence mechanisms. A correlation was considered: perfect if $r_2$ is 1, strong as $r_2$ approaches 1, intermediate if $r_2$ was close to 0.5 and weak if $r_2$ was close to zero. All experiments were performed in triplicate and repeated three times.

### 5.2.8. Ethics approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no: H039/06).

## 5.3 RESULTS

All the 15 clinical and the 2 laboratory isolates showed cytotoxicity in the A549 cells, but at different levels (Fig. 5.1). The growth conditions under which the isolates were cultured before the infection assay influenced the level of cytotoxicity they induced in the A549 cells. The isolates grown under oxygen deprivation induced a higher percent of

cytotoxicity, when compared with the same isolate that was grown in the presence of oxygen ($P$<0.0001).

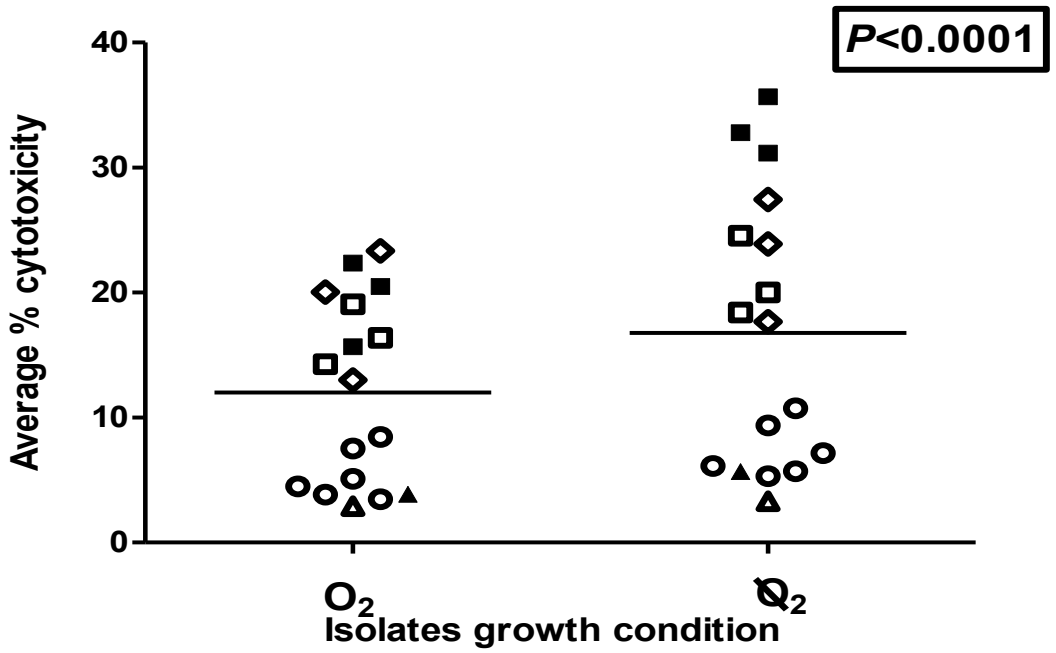


**Figure 5.1. Comparison of induced cytotoxicity in A549 cells by *M. tuberculosis* isolates grown in the presence of oxygen ($O_2$) and under oxygen deprivation ($\not{O}_2$) after 4h. Each point represents the average of the results obtained from three experiments performed in triplicate for each *M. tuberculosis* isolate. ◇= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates; □= F15/LAM4/KZN isolates; O= Isolates with unique DNA fingerprint; ▲= H37Rv; △= H37Ra; $P$<0.0001**

### 5.3.1 Isolates cultured in the presence of oxygen

The level of cytotoxicity of organisms grown in the presence of oxygen ranged from 14.3% - 22.4% for the F15/LAM4/KZN strains, and 13% - 23.3% for the Beijing strains (Fig. 5.1). The isolates with unique DNA fingerprint induced between 3.5% - 8.5%

toxicity, while for the laboratory strains these values were 3.9% (H37Rv) and 2.9% (H37Ra) (Fig. 5.1). Two of the Beijing and four of the F15/LAM4/KZN family members showed a level of cytotoxicity significantly higher than that of the virulent laboratory strain (H37Rv) ($P<0.05$). Two of the three Beijing and all the six F15/LAM4/KZN isolates also showed a level of cytotoxicity that was significantly higher than that of the avirulent laboratory strain (H37Ra) ($P<0.05$), which showed the lowest level of cytotoxicity. Each experiment was performed three times in triplicate and the coefficient of variation (CV) was within acceptable range (Appendix B7).

### 5.3.2 Isolates cultured under oxygen deprivation

The Beijing and F15/LAM4/KZN isolates that were grown under oxygen deprivation induced cytotoxicity in A549 cells ranging from 17.7% to 27.4% and from18.4% to 35.7% respectively (Fig. 5.1). The percent cytotoxicity induced by isolates with unique DNA fingerprint ranged from 5.3% to 10.7% while that of laboratory strain H37Rv was 5.7% and of H37Rv 3.3% (Fig. 5.1). The highest cytotoxicity level (35.7%) was induced by an XDR isolate of the F15/LAM4/KZN strain family (Fig. 5.1). The mean cytotoxicity induced by the three Beijing and the XDR-F15/LAM4/KZN isolates were higher than the H37Rv ($P<0.05$ and $P<0.01$ respectively). There was a significant difference in the percent cytotoxicity induced by the XDR-F15/LAM4/KZN isolates when compared to the isolates with unique DNA fingerprint ($P<0.05$). The avirulent strain (H37Ra) was observed to have induced the lowest level of cytotoxicity among the isolates and the difference was significant when compared to two of the Beijing ($P<0.05$) and all of the

F15/LAM4/KZN ($P$<0.05) family members. Each experiment was done three times in triplicate and the coefficient of variation (CV) was within acceptable range (Appendix B8).

### 5.3.3 Comparison between isolates grown with and without oxygen present

Isolates that had been grown in the presence of oxygen induced a lower level of cytotoxicity after exposure to A549 cells when compared to the isolates grown under oxygen deprivation (Fig. 5.1) ($P$<0.0001). We observed that the level of cytotoxicity of isolates with unique DNA fingerprints clustered with the two laboratory controls (H37Rv and H37Ra) under both growth environments.

**Table 5.2. Spearman's correlation coefficient ($r_2$) values between different pairs of virulent mechanisms under different isolate growth environment**

| Growth Environment | Virulent Mechanism | |
|---|---|---|
| | Cytotoxicity vs adherence | Cytotoxicity vs invasion |
| Oxygen present | 0.939 | 0.956 |
| Oxygen deprived | ND | 0.939 |

### 5.3.4 Relationship between adherence, invasion and cytotoxicity of the isolates

To determine whether a relation existed between adherence and invasion and toxicity we compared the toxicity results obtained from this study with the adherence and invasion results from our previous studies (Ashiru *et al*. 2010; Ashiru *et al*. 2012) using the Spearman's correlation coefficient ($r_2$). A strong and significant positive correlation was observed between cytotoxicity and adherence ($r_2 = 0.939$) as well as cytotoxicity and invasion ($r_2 = 0.956$ – oxygen present; $r_2 = 0.939$ – oxygen deprived) (Table 5.2).

## 5.4 Discussion

The hallmark of pulmonary tuberculosis is damage to alveolar tissue. Several research groups have studied the toxic effect of *M. tuberculosis* on alveolar macrophages and alveolar epithelial cells (McDonough and kress, 1995; Rojas *et al*. 1997; Dobos *et al*. 2000; Danelishvili *et al*. 2003). We previously reported on the ability of strains belonging to the Beijing and F15/LAM4/KZN families of *M.* tuberculosis grown in the presence of oxygen and under oxygen deprivation to adhere to and invade A549 alveolar cells (Ashiru *et al*. 2010; Ashiru *et al*. 2012). We report different levels of cytotoxicity in alveolar epithelial cells by clinical isolates of *M. tuberculosis* that, based on their RFLP signature, belong to different strain families.

The high level of cytotoxicity that we observed in the A549 cells induced by the isolates belonging to the Beijing and F15/LAM4/KZN in comparison to H37Rv and isolates with unique fingerprints, confirms the high virulence capacity of these successful families of strains. This is in keeping with earlier reports (Hanekom *et al*. 2007; Theus *et al*. 2007; Palanisamy *et al*. 2009). This also agrees with the report by McDonough *et al* (1995), that cytotoxicity is specific to virulent mycobacteria.

Our observation that the XDR isolates of the F15/LAM4/KZN family had the highest level of cytotoxicity suggests that XDR variants of this family may cause increased tissue destruction compared to the other isolates. This attest to its virulence and may have contributed to the high mortality rates of patients infected with this strain in KwaZulu-Natal province of South Africa as reported by Gandhi *et al* (2006)

A number of studies have been done on the effect of *M. tuberculosis* on alveolar macrophages (Keane *et al*. 1997; Danelishvili *et al*. 2003). These studies reported that infection of macrophages with *M. tuberculosis* triggered apoptosis. It was further suggested that this is accompanied by antiapoptotic mechanisms (Sly *et al*. 2003). Most of these studies used one virulent laboratory strain of *M. tuberculosis* (H37Rv)

Reports have also been published on the effect of *M. tuberculosis* on alveolar epithelial cells following infection with one of the virulent laboratory strains. These all suggested

that in contrast to apoptosis that had been observed in alveolar macrophages, necrosis was observed in alveolar epithelial cells (Dobos *et al*. 2000; Danelishvili *et al*. 2003).

That the isolates grown under oxygen deprivation had a more virulent phenotype by inducing higher levels of necrotic cytotoxicity in comparison to the same isolates grown in the presence of oxygen is in line with our previous report on the increased invasive abilities of isolates grown under oxygen deprivation (Ashiru *et al*. 2012).

We show a significant positive correlation between cytotoxicity and adherence, as well as cytotoxicity and invasion. From this, we conclude that invasion is a post adherence event, that leads to cytotoxicity and that adherence of the bacteria to the host cell is a prerequisite for invasion to occur as reported for other bacteria (Hensen *et al*. 2000; El-Housseiny *et al*. 2010).

In conclusion, we show for the first time that Beijing and F15/LAM4/KZN isolates of *M. tuberculosis* are cytotoxic to alveolar epithelial cells *in vitro* and that this cytotoxicity is upregulated when the organisms are grown under oxygen deprivation. Further studies are required to determine the virulence of these isolates at a molecular level.

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# Chapter 6.

**Clinical isolates of *Mycobacterium tuberculosis* induce morphological changes in A549 alveolar epithelial cells**

**Olubisi T. Ashiru, Manormoney Pillay, James Wesley-Smith, A. Willem Sturm**

Journal of Medical Microbiology: *In preparation*

**ABSTRACT**

*Mycobacterium tuberculosis* primarily infects the lungs. In former chapters, we reported differences of isolates within and across *M. tuberculosis* families of strains (Beijing and F15/LAM4/KZN) in adhesion, invasion and toxicity to alveolar epithelial cells. Here we report on clinical *M. tuberculosis* isolates induced changes in the morphology of A549 alveolar epithelial cells *in vitro*.

Projections were observed on the surface of infected but not on non-infected A549 cells. Scanning electron microscopy revealed bleb-like structures on the surface of bacilli that were cultured under oxygen deprivation. Isolates grown under oxygen deprivation had a higher ratio of induced projections to bacilli, when compared to the same isolate grown in the presence of oxygen. The Beijing and extensively drug resistant (XDR) F15/LAM4/KZN isolates both recorded the highest ratio of induced projections per bacillus (16±5).

The morphological changes observed in the infected A549 cells correlate well with previous results on adherence, invasion and cytotoxicity of these isolates. The results suggest that the Beijing and F15/LAM4/KZN isolates of *M. tuberculosis* may be more virulent than the virulent laboratory strain H37Rv and the isolates with unique fingerprints, and may explain the more aggressive spread of these families.

## 6.1 INTRODUCTION

Tuberculosis (TB) is a major health challenge in many parts of under resourced parts of the world. The gravity of the problem of TB and HIV infection in South Africa cannot be overstated. Worldwide TB is the leading cause of death in the HIV infected (CDC, 2011). South Africa harbours less than 1% of the world population, yet it contributes approximately 25% to the total global burden of HIV-TB co-infection (WHO, 2009). The incidence of new TB infections in South Africa rose by 400% over the past 15 year to 970 per 100 000 people in 2009 (WHO, 2010).

*Mycobacterium tuberculosis* primarily infects the lung, with 75% of active TB cases being pulmonary. The bacilli have been reported to adhere, invade and multiply in the cells that line the alveolar space (Ashiru *et al*. 2010; Hall-Stoodley *et al*. 2006; Bermudez *et al*. 2002; Castro-Garza *et al*. 2002; Bermudez and Goodman, 1996). This includes alveolar macrophages and alveolar epithelium. The effect of *M. tuberculosis* on these host cells has been the focus of different research groups (Wickremasinghe *et al*. 1999; Lin *et al*. 1998; Zhang *et al*. 1997). The virulent laboratory strain (H37Rv) has mainly been used to investigate the bacilli ability to induce cytotoxicity in its host cell (Danelishvili *et al*. 2003; Dobos *et al*. 2000; McDonough and Kress, 1995). Differences in virulence, adherence and invasion properties of isolates from the *M. tuberculosis* Beijing and F15/LAM4/KZN families of strains have recently been reported (Ashiru *et al*. 2010; Palanisamy *et al*. 2009).

Most studies on the virulence of *M. tuberculosis* have been performed with bacterial cells grown in the presence of oxygen (Sanjay *et al*. 2006; Birkness *et al*. 1999; Bermudez and Goodman, 1996). As early as 1933, it was reported that *M. tuberculosis* deprived of oxygen *in vitro* remains viable and virulent for several years (Corper and Cohn, 1933). *In vivo*, several niches for example granuloma and cavities have been identified where oxygen deprivation prevails (McClane *et al*. 1999). Bacilli grown under oxygen deprivation have been reported to also invade alveolar epithelial cells and macrophages *in vitro* (Ashiru *et al*. 2012; Li *et al*. 2002).

The ultrastructural details of the interaction between the bacterial cell and alveolar epithelial cells have not been clearly elucidated. We used scanning electron microscopy (SEM) to investigate whether: 1) infection with clinical isolates of *M. tuberculosis* induce changes in the morphology of A549 alveolar epithelial cells and whether isolates belonging to different strain families induced similar responses; 2) there is a difference in the response induced by *M. tuberculosis* isolates grown under oxygen deprivation or in the presence of oxygen, and 3) a relationship exists between the response induced in the infected A549 cells and the adherence, invasion and cytotoxicity properties of these isolates reported previously (Ashiru *et al*. 2010; Ashiru *et al*. 2012).

## 6.2 Materials and methods

### 6.2.1 Strains

Five *M. tuberculosis* isolates were selected out of the group of fifteen of which adherence, invasion and cytotoxicity to alveolar epithelial cell line A549 have been described in chapters 3, 4 and 5. This group of fifteen isolates included three from the Beijing family, six from the F15/LAM4/KZN family of which three had the extensively drug resistance (XDR) resistance profile, and six isolates with unique fingerprint patterns. One of the Beijing isolates, one of the F15/LAM4/KZN isolates with the XDR profile, one other F15/LAM4/KZN and two isolates with unique DNA fingerprint patterns were randomly selected from their respective groups. Laboratory strain H37Rv (ATCC 27294) was included for comparison.

### 6.2.2 Growth conditions

#### 6.2.2.1 Growth in the presence of oxygen

Each isolate was inoculated in triplicate in Middlebrook 7H9 broth (Difco) and incubated with gentle agitation in a shaking incubator at a temperature of 37$^{o}$C. The cultures were harvested at an optical density of 1 (measured at 600nm wavelength), which was between two to three weeks of incubation.

#### 6.2.2.2 Growth under oxygen deprivation

Each isolate was inoculated in 9 mL of Middlebrook 7H9 broth (Difco) in screw-capped 15 mL tubes with a diameter of 17 mm and incubated at 37$^{o}$C. In order to achieve an oxygen deprived environment, the cultures were maintained in an upright position without agitation for the duration of three months (Ashiru *et al*. 2012; Wayne, 1976).

### 6.2.3 Preparation of inocula

Bacterial cells of each isolate were harvested by centrifugation (3000 x g, 10 min). The supernatant was discarded and the pellet was resuspended in Eagle's Minimum Essential Medium (Biowhittaker-Lonza), vortex agitated, and sonicated (10 w for 10 sec) to break the clumps. The number of colony forming units (CFU) per mL was determined by plating out four 10-fold serial dilutions of the suspension (20µl) on Middlebrook 7H11 complete agar (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma). Counts were performed in triplicate.

### 6.2.4 Epithelial cell line

The human type II alveolar epithelial cell line A549 (ATCC CCL 185) was used in the assay. We used the same protocol as previously reported by Ashiru *et al* (2010). Briefly, detachment of A549 confluent cell monolayer from the flask was achieved by trypsinization using trypsin-versene (Cambrex Bio Science). The number of viable cells

was determined by trypan blue exclusion. The cells were counted using a haemocytometer. One mL of the suspension containing $10^5$ viable cells was seeded onto glass coverslips (12 mm diameter) placed in a 24-well tissue culture plate. The seeded tissue culture plates were incubated for $\pm$40 hours in a $CO_2$ incubator with 5% $CO_2$ at 37$^{o}$C before being used for the experiment.

### 6.2.5 Infection Assay

Phosphate buffered saline maintained at room temperature (PBS pH 7.3; Oxoid) was used to wash the cells thrice, and culture medium (maintained at room temperature) was added into each well. *M. tuberculosis* suspension was used to inoculate the cells at an m.o.i. (multiplicity of infection) of 9-12 bacteria per cell and the cells were reincubated for 2 hours in the $CO_2$ incubator (6.2.4).

### 6.2.6 Scanning electron microscopy (SEM)

After the incubation period, the cells were washed with PBS (pH 7.3) to remove extracellular bacilli, followed by fixation with 2.5% glutaraldehyde overnight (18 – 24 h). The fixed cells were washed with PBS thrice (5 min per wash) and then post-fixed with 1% osmium tetroxide for 1 hour. Thereafter, the cells were washed thrice with PBS (5 min per wash), followed by dehydration with increasing concentrations of ethanol (25%, 50%, 75%, 100%). The dehydrated cells were critical point dried with liquid $CO_2$. The dehydrated cells on the glass coverslips were mounted on stubs, and thereafter, sputter

coated with gold. The gold-coated cells on the glass coverslips were observed through a Leo 1450 scanning electron microscope.

The images obtained were analysed using SIS iTEM software. *M. tuberculosis* bacilli and induced projections seen on the infected A549 cells were counted in five fields (constant area of 100$\mu m^2$) per image and four images per isolate. The diameter of the induced projections was measured and the ratio of induced projections to bacilli was obtained by dividing the number of projections by the number of bacilli within an area (100 $\mu m^2$).

### 6.2.7 Statistical analysis

Microsoft Excel and Graphpad Prism 5 software were used to analyse our results. Fisher's test was used to determine whether there were significant differences in the ratio of induced projections to bacilli among the isolates, as well as between oxygen deprived and oxygen present grown isolates. Spearman's correlation coefficient ($r_2$) was calculated to determine the correlation between SEM results and adherence, invasion and cytotoxicity of the same isolates (Chapters 3, 4 and 5). A correlation was considered perfect if $r_2$ is 1, strong as $r_2$ approaches 1, intermediate if $r_2$ was close to 0.5 and weak if $r_2$ was close to zero. All experiments were performed in triplicate and repeated two times.

### 6.2.8 Ethics approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no: H039/06).

## 6.3 RESULTS

This is the first study reporting on induced morphological changes in A549 cells by members of the *M. tuberculosis* Beijing and F15/LAM4/KZN families of strains cultured in the presence of oxygen as well as under oxygen deprived conditions *in vitro*.

All five clinical isolates induced morphological changes in the A549 cells and so did H37Rv. Projections were observed on the surface of the infected A549 cells with all isolates and H37Rv (Fig. 6.1) but not on the non-infected A549 cells (Fig. 6.2).

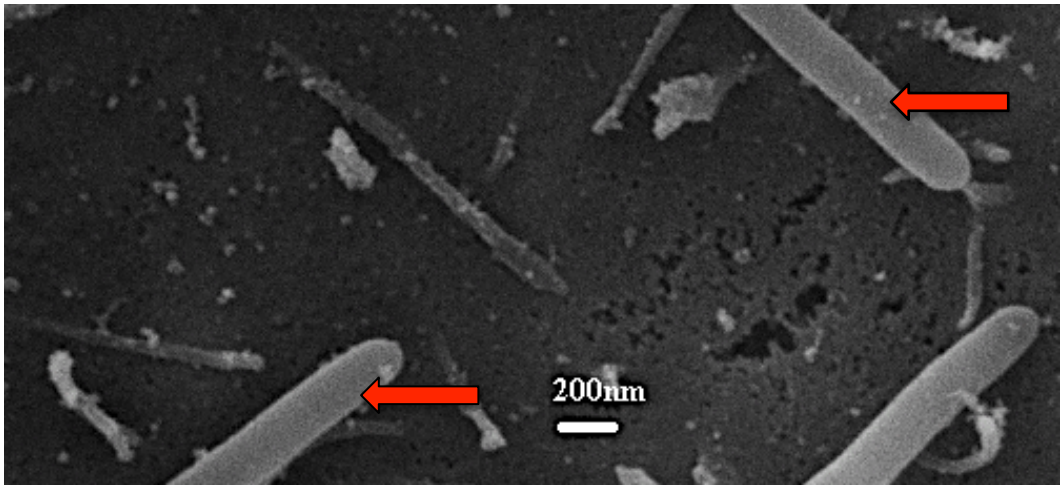


**Figure 6.1. Micrograph of infected A549 cell exposed to *M. tuberculosis* isolate (red arrow) (F15/LAM4/KZN) grown in the presence of oxygen ($O_2$) (Mag x 30000)**

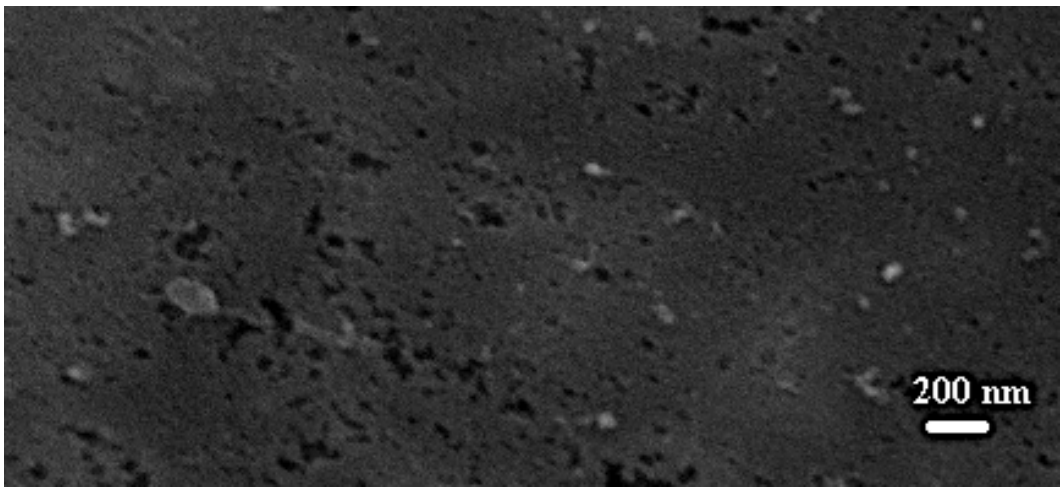


**Figure 6.2. Micrograph showing non-infected A549 cell (Mag x 30000)**

Bleb-like structures were observed on the surface of the *M. tuberculosis* bacilli grown under oxygen deprivation (Fig. 6.3) but not on the bacilli grown in the presence of oxygen (Fig. 6.1).

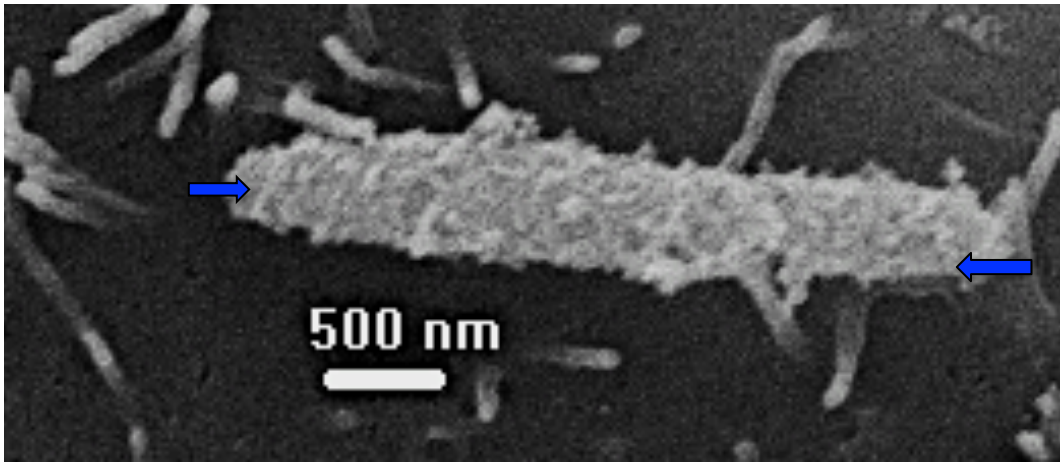


**Figure 6.3. Micrograph of infected A549 cell with *M. tuberculosis* isolate grown under oxygen deprivation ($O_2$) and bleb-like structures (blue arrow) on the surface of the bacillus (Mag x 40000).**

It was not possible to measure accurately the length of the projections induced on the surface of the infected A549 cells, but these appeared to vary across isolates, with diameters ranging between 0.09 µm and 0.14 µm (Fig. 6.4). The ratio of projections on the infected A549 cells to bacilli varied significantly (*P*=0.031) across isolates (Appendix B9 and B10).

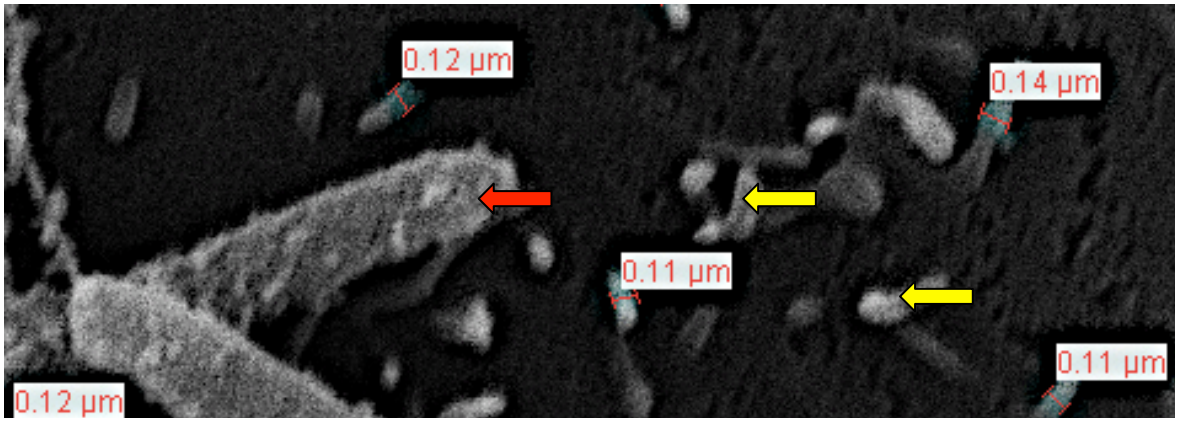


**Figure 6.4. Micrograph showing diameter of projections (yellow arrow) on infected A549 cell with *M. tuberculosis* isolate (red arrow) grown under oxygen deprivation ($O_2$), and bleb-like structures on the surface of the bacilli (Mag x 35000)**

We obtained similar ratios for both the Beijing and the XDR-F15/LAM4/KZN isolates under each of the two growth conditions (Table 1). The projections/bacilli ratio was significantly greater (*P*=0.035) in the isolates grown under oxygen deprivation.

**Table 6.1. Mean ratio of induced projection to *M. tuberculosis* bacilli on A549 cells**

| *M. tuberculosis* isolate | Number of isolates | Aerobic Incubation Mean ratio ± SEM | Anaerobic incubation Mean ratio ± SEM |
|---|---|---|---|
| Beijing | 1 | 13 ± 3 | 16 ± 10 |
| XDR-KZN | 1 | 13 ± 4 | 16 ± 5 |
| KZN | 1 | 4 ± 2 | 9 ± 4 |
| UNIQUE | 2 | 6 ± 3 | 8 ± 5 |
| | | 4 ± 1 | 5 ± 2 |
| H37Rv | 1 | 4 ± 1 | 6 ± 2 |

**A549 cells were exposed to the inocula for 2h at 37$^{o}$C. The results are mean ratios of triplicate experiments repeated two times. *M. tuberculosis* bacilli and projections seen on the infected A549 cells were counted in five different fields (areas =100 µm$^2$) per image and four images per experiments for each isolate. SEM, standard error of mean; there was a significant difference between the isolates grown in the presence of oxygen and those grown under oxygen deprivation (*P*=0.035).**

Previous results of these isolates on adherence (Ashiru *et al*. 2010), invasion (Ashiru *et al*. 2012) and cytotoxicity were paired with the results from this present study to determine whether a relation existed between these different observations.

**Table 6.2. Spearman's correlation coefficient ($r_2$) values between different pairs of observations under different isolate growth environment**

| Growth Environment | Virulence mechanisms | | |
|---|---|---|---|
| | SEM vs adherence | SEM vs invasion | SEM vs cytotoxicity |
| Oxygen present | 0.949 | 0.919 | 0.897 |
| Oxygen deprived | ND | 0.768 | 0.961 |

Table 6.2 show Spearman's correlation coefficient ($r_2$) value of the analysed paired virulence mechanisms. There was a strong and positive correlation observed between SEM and adherence ($r_2 = 0.949$); SEM and invasion ($r_2 = 0.919$ – oxygen present; $r_2 = 0.768$ – oxygen deprived) as well as SEM and cytotoxicity ($r_2 = 0.897$ – oxygen present; $r_2 = 0.961$ – oxygen deprived) (Table 6.2).

## 6.4 DISCUSSION

There have been several studies on the interaction between *M. tuberculosis* and alveolar epithelial cells, mostly using the H37Rv virulent laboratory strain (Garcia-Perez *et al*. 2003; Bermudez and Goodman, 1996; Mehta, *et al*. 1996).

In this study we were able to demonstrate that infection of A549 cells with clinical isolates of *M. tuberculosis* induces changes in the morphology of the epithelial cells *in vitro* (Fig. 6.1). We observed projections on the infected A549 cells, but not on the non-infected A549 cells (Fig. 6.2).

We were able to quantify the level of morphological changes by counting the projections and the bacilli within a constant area (100 $\mu m^2$) in different fields on the infected A549 cells. This allowed us to calculate the ratio of projections to bacilli. Using this parameter, we were able to demonstrate that the different isolates did not induce similar responses,

with the exception of the Beijing and XDR-F15/LAM4/KZN isolates which were very similar ($P$<0.05). Isolates of these two families induced the highest ratio of projections to bacilli under both growth conditions (Table 6.1), but with both family representatives the ratios were significantly higher with organisms grown under anaerobic circumstances.

While we were also able to measure the diameter of the induced projections (Fig. 6.4), we were not able to measure the length due to various degrees of bending (Figs. 6.1, 6.3, 6.4, 6.5).

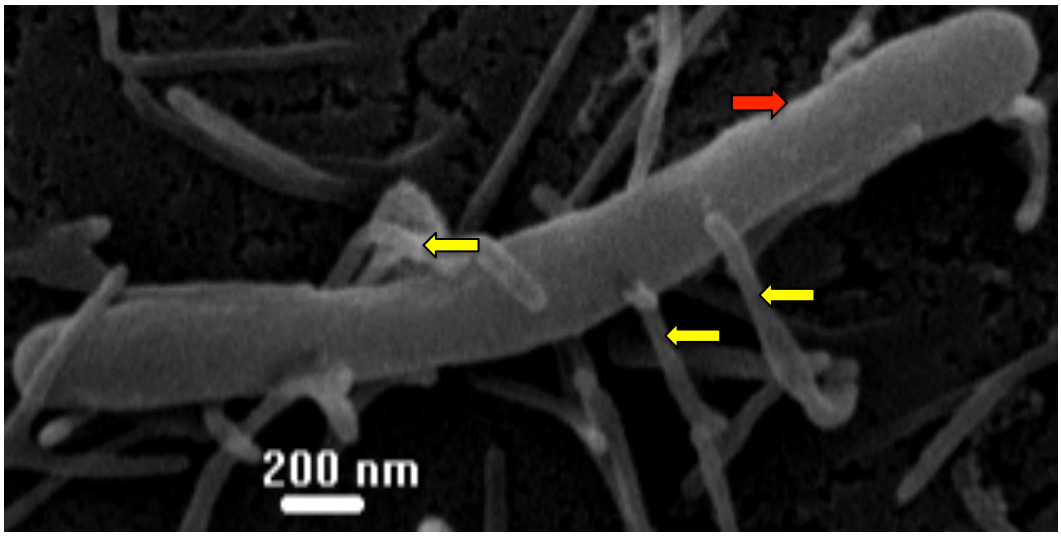


**Figure 6.5. Micrograph showing induced projections (yellow arrow) on infected A549 cell surrounding *M. tuberculosis* bacillus (red arrow) grown under aerobic conditions ($O_2$)**
**(Mag x 100000)**

We also observed that there was a difference in the response induced by the isolates based on their growth conditions. Isolates grown in an oxygen deprived environment induced a higher ratio of projections to bacillus. This supports our former observations that the phenotype of the bacillus grown under oxygen deprivation invades alveolar

epithelium more effectively as compared to aerobic incubated bacteria (Ashiru, *et al*. 2012).

We also observed the presence of bleb-like structures on the surface of the bacilli of all the isolates that were grown under oxygen deprivation (Fig. 6.3 and Fig. 6.4). These bleb-like structures might be as a result of the stress the bacilli are subjected to under this growth condition. Dahl (2005) reported the presence of bleb-like structures accumulation as the *M. tuberculosis* culture aged. The exposure of *M. tuberculosis* to eosinophil peroxidase also resulted in the formation of surface blebs on the bacilli (Borelli *et al*. 2003). However, our observations that the formation of blebs is associated with a higher projection/bacilli ratio could also be interpreted as that these blebs play a role in the interaction between host cell and bacterial cell inducing invasion. This needs further investigation.

Our results show that a strong positive correlation exists between the induction of projections on the epithelial cells and adherence, invasion and cytotoxicity of these isolates.

The findings strengthen previous suggestions on the need to include organisms cultured under oxygen deprivation in studies on the effect of *M. tuberculosis* on host cells as this may shed valuable information on the organism interaction with the host cell. Which may be useful in the development of new drug targets in treatment therapy.

Our results suggest that Beijing and F15/LAM4/KZN isolates may be more virulent than H37Rv virulent strain. The isolates with unique fingerprints, which are thought to result from reactivation of dormant infection rather than newly acquired infection, showed projection/bacteria ratios similar to H37Rv. This indicates that such isolates are less virulent than those belonging to the Beijing, F15/LAM4/KZN and other successfully spreading families of strains.

In conclusion, this work adds to the accumulating evidence that the XDR variant of the F15/LAM4/KZN strain expresses increased virulence towards alveolar epithelial cells. The anaerobic environment as found in tuberculous granuloma, results in increased expression of virulence. If this also happens *in vivo*, these observations might explain more effective spread of this organism due to increased damage to lung tissue and improved adhesion to the alveolar surface.

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# Chapter 7.

**Expression of genes coding for genes potentially associated with virulence in oxygen deprived Beijing and F15/LAM4/KZN isolates of *Mycobacterium tuberculosis***

**Olubisi T. Ashiru, Manormoney Pillay, A. Willem Sturm**

Journal of Clinical Microbiology: *In preparation*

Infection Prevention and Control, College of Health Sciences,

University of KwaZulu-Natal, South Africa.

**ABSTRACT**

Little is known about differences in gene potentially associated with virulence expression in *Mycobacterium tuberculosis* isolates with different restriction fragment length polymorphism (RFLP) signature and exogenous factors that influence this.

We report on the expression of four genes by fifteen *M. tuberculosis* clinical isolates belonging to the Beijing or F15/LAM4/KZN families and with unique DNA fingerprints. Laboratory strains H37Rv and H37Ra were included for comparison. Gene expression was measured when grown in the presence of oxygen and under oxygen deprivation, as well as before and after exposure to A549 cells. Real-time quantitative PCR was performed. Results were normalized against *16S* rRNA and *ftsZ* gene transcription and reported as fold difference as compared with strain H37Rv.

In aerobically grown isolates *hbhA* (iron-regulated heparin binding hemagglutinin) expression was increased in all, while *mdp1* (mycobacterial DNA-binding protein) expression was higher in unique isolates. Under oxygen deprivation *hbhA* expression was higher in F15/LAM4/KZN isolates (1.312 – 1.838); *mdp1* expression was higher in Beijing and F15/LAM4/KZN isolates; *fdxA* (ferredoxin) and *hspX* (heat shock protein) expressions were higher in F15/LAM4/KZN isolates (1.003 – 1.833; 1.033 -1.999).

In aerobically grown isolates, exposed to A549 cells, *hbhA* expression was higher in Beijing and F15/LAM4/KZN isolates (1.526 - 2.673), while *mdp1* expression was higher in unique isolates. Under oxygen deprivation, expression of all four genes was higher in Beijing and F15/LAM4/KZN isolates.

These results indicate that the production of potential virulence attributes is up regulated in successfully spreading strains when exposed to alveolar cells after bacilli initial growth under oxygen depriving conditions.

## 7.1 INTRODUCTION

WHO's global report (2011), states that South Africa is rated 3rd after India and China, among the 22 high-burden countries in the world with tuberculosis (TB) and 1st in Africa. This shows the enormous challenge posed by TB in South Africa. This is in KwaZulu-Natal further complicated by the high prevalence of HIV-TB co-infection and the levels of multi-drug resistance. TB is a multifactorial disease and a better understanding of all its aspects is important in the quest to prevent, treat, control and eventually eradicate this disease.

A good understanding of TB pathogenesis will be of benefit in the quest to eventually stop this disease. There have been numerous reports dealing with this organism (Brennan and Nikaido, 1995; Shinnick and Good, 1994; Lessel, 1960, Corper and Cohn, 1933), and its interaction with host cells (Hall-Stoodley *et al*. 2006; Glickman and Jacobs, 2001; Ernst, 1998; Bermudez and Goodman, 1996).

Research on *M. tuberculosis* at the molecular level contributes to information on new drug targets and vaccine candidates. Over a decade ago, Cole *et al* (1998) published the full genome sequence of the virulent laboratory strain H37Rv. Other researchers have also sequenced and published full genome sequences of some *M. tuberculosis* strains: CDC 1551 (Fleischmann *et al*. 2002); Erdman (Miyoshi-Akiyama *et al*. 2012); Haarlem (Broad institute); F15/LAM4/KZN (Broad institute, Ioerger *et al*. 2009).

Information obtained from sequenced genomes is vital and have been applied in studies on various aspect of *M. tuberculosis* (Ramchandra and Sturm, 2010; Mostowy and Behr, 2005; Hawkey *et al.* 2003; Brosch *et al*. 2002). Yet, much is still unknown about genes potentially associated with virulence expression in *M. tuberculosis*.

The *hbhA* gene (Rv0457) that codes for the iron-regulated heparin binding hemagglutin protein (HBH-A) was examined in this study. This protein is required for extrapulmonary dissemination (Pethe *et al*. 2001) and mediates adherence to epithelial cells (Menozzi *et al*. 1998). This protein is also responsible for hemagglutination *in vitro* (Pethe *et al*. 2002) and also induces mycobacterial aggregation (Mueller-Ortiz *et al*. 2001).

The *mdp1* gene (Rv2986c) also referred to as *hupB* gene that codes for the mycobacterial DNA-binding protein (MDP1) was also examined. This protein belongs to the histone like family of prokaryotic DNA-binding proteins (Kumar *et al*. 2010; Furugen *et al*. 2001). These proteins stabilize the DNA by wrapping and prevent denaturation of the DNA under extreme environmental conditions (TubercuList, 2012).

The *fdxA* gene (Rv2007c) that codes for ferredoxin (FDXA) was examined as well. This protein is involved in electron transfer under oxygen deprivation (TubercuList, 2012). Lastly, expression of the *hspX* gene (Rv2031c) that codes for heat shock protein (HSPX) was also included. This is a stress protein that is induced by anoxia. It is proposed to be involved in long-term viability maintenance during asymptomatic (latent) infection (TubercuList, 2012).

Previously, we reported on the interaction of clinical isolates of *M. tuberculosis* with different restriction fragment length polymorphism (RFLP) signature and pulmonary epithelial cells *in vitro* (Ashiru *et al*. 2012; Ashiru *et al*. 2010). All the isolates previously investigated adhered to and invaded specifically alveolar cells. We also measured the cytotoxic effect of these same isolates (chapter 5) as well as their ability to induce morphological changes (chapter 6) in the A549 alveolar cells. In this chapter, we report on the expression of the above mentioned four genes potentially associated with virulence by these same *M. tuberculosis* isolates grown in the presence and absence of oxygen as well as before and after exposure to A549 cells.

## 7.2 Materials and methods

### 7.2.1 Strains

All the isolates of *M. tuberculosis* were obtained from the culture collection of the TB unit of the Department of Infection Prevention and Control, University of KwaZulu-Natal, Durban. These included three members of the Beijing family and six members of the F15/LAM4/KZN family, of which three had the XDR resistance profile. Six had unique DNA fingerprint patterns. The virulent H37Rv (ATCC 27294) and avirulent H37Ra (ATCC 25177) laboratory strains of *M. tuberculosis* were included for comparison.

### 7.2.2 Growth conditions

Isolates were grown in the presence and absence of oxygen. For aerobic growth, each isolate was inoculated in Middlebrook 7H9 broths (Difco) incubated at 37$^{o}$C in a shaking incubator with gentle agitation. The cultures were harvested when an optical density of 1 (measured at 600nm wavelength) was reached. This usually took two to three weeks.
To achieve anaerobic growth conditions, each isolate was inoculated in Middlebrook 7H9 broth (Difco) in screw-capped 15 mL tubes with a diameter of 17 mm containing 9 mL of broth and incubated at 37$^{o}$C. The cultures were incubated in an upright position without agitation for a three month incubation period (Ashiru *et al*. 2012; Wayne, 1976).

### 7.2.3 Inocula preparation

The bacteria were harvested by centrifugation (3000 x g 10 min). The supernatant was discarded, and the pellet resuspended in Eagle's Minimum Essential Medium (Biowhittaker-Lonza). This suspension was then vortex agitated and sonicated (10 w 10 sec). Thereafter, four 10-fold serial dilutions were plated out on Middlebrook 7H11 complete agar media (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma), to determine the number of colony forming units (CFU) per mL. Each isolate was processed in triplicate.

### 7.2.4 Pulmonary epithelial cell line

The A549 human alveolar epithelial cell line (ATCC CCL 185) was used for the infection assay. The protocol previously reported by Ashiru *et al* (2010) was used with slight modifications. The A549 confluent cell monolayer was detached from the tissue culture flask by trypsinization with trypsin-versene (Cambrex Bio Science). The number of viable cells was determined in a hemocytometer using the trypan blue exclusion test. The cell suspension was diluted as required and 20 mL of this containing 1 x $10^5$ cells/mL was seeded into 75cc tissue culture flasks. The seeded flasks were incubated for $\pm$40h in 5% $CO_2$ at 37$^o$C before being used for the experiment.

### 7.2.5 Exposure of *M. tuberculosis* to A549 cells

The A549 cells were washed three times with phosphate buffered saline maintained at room temperature (PBS pH 7.3; Oxoid), and thereafter EMEM culture medium (maintained at room temperature) was added to each flask. Following inoculation of the cells with *M. tuberculosis* suspension (7.2.4) at a multiplicity of infection (m.o.i) of 9 – 12 bacteria per epithelial cell, the cells were incubated at 37$^o$C in a 5% $CO_2$ atmosphere for 2h.

### 7.2.6 Molecular processing

#### 7.2.6.1 Pulmonary epithelial cell lysing

A modified version of the method reported by Butcher *et al* (1998) and Mangan *et al* (2002) was used. After the exposure period (7.2.5), the A549 cells were lysed with guanidinum thiocyanate (GTC) lysing solution (containing 4M GTC with 0.7% βmercapethanol), vortex agitated for 2 min and then centrifuged at 3000 x g for 20 min at 4$^{o}$C. The supernatant was discarded and the pellet resuspended in the GTC lysing solution in screw-capped microcentrifuge tube. This was centrifuged again but at 15000 x g for 20 seconds, after which the supernatant was discarded and trizol reagent (Invitrogen) along with sterile glass microbeads was added to resuspend the pellet. The microcentrifuge tubes containing the trizol, glass beads and exposed bacilli (obtained from the lysed A549 cells) were placed in the MagNa lyser (Roche) to disrupt the bacterial cells and facilitate the isolation of the total RNA.

#### 7.2.6.2 Total RNA isolation

The trizol reagent was used according to the manufacturer's protocol with slight modifications. The suspension in the trizol reagent was centrifuged at 12000 x g for 10 min at 4$^{o}$C. The supernatant (containing the RNA) was transferred to a new microcentrifuge tube and incubated at room temperature for 10 min. This was followed by the addition of cold chloroform (20% of the initial volume of trizol). The

microcentrifuge tubes were vigorously shaken for 30 seconds and re-incubated at room temperature for another 10 min. The centrifugation step was repeated at the same speed and temperature, but for 15 min. The total RNA was precipitated with cold isopropanol (50% of the initial volume of trizol) and then washed with 75% ethanol (made-up with diethylpyrocarbonate (DEPC) treated water). The RNA was resuspended in RNASecure (Ambion) resuspension solution. The RNA suspension was treated with DNase 1, RNase-free enzyme (Fermentas) following manufacturer's instructions. The nanodrop spectrophotometer was used to estimate the quantity and purity of the isolated RNA, while its integrity and quality was analysed by denaturing 3-(N-morpholino) propanesulfonic acid (MOPS) gel electrophoresis.

7.2.6.3 Complementary DNA (cDNA) synthesis

A two-step PCR was performed. First, the high capacity cDNA reverse transcription kit with RNAse inhibitor (ABI) was used to quantitatively convert 2 μg of the purified RNA in a 20μL reaction into cDNA following manufacturer's instruction.

7.2.6.4 Bioinformatics

The sequences of all the genes under investigation were retrieved from the Tuberculist webserver accessible at http://genolist.pasteur.fr/TubercuList/. A set of primer pairs (forward and reverse) and a minor groove binding (MGB) TaqMan probe was designed

for each gene and synthesized by Applied biosystems (ABI) for custom TaqMan gene expression assays. Table 7.1 shows the primers and FAM-labelled probe sequences.

**Table 7.1. Sequences of primers and probes**

| Gene | Annotation | Forward primer | Reverse Primer | Probe |
|---|---|---|---|---|
| *16S rRNA* | Rv2907c | CAGGGCTTCACACAT GCTACAAT | GTATTCACCGCAGCGTTGCT | TCTGCGATTACTAGCGACTCCG ACTTCACG |
| *ftsZ* | Rv2150c | CGGTATCGCTGATGG ATGCTTT | CGGACATGATGCCCTTGACG | CGCCGACGAGGTGCTGCT CAACG |
| *hbhA* | Rv0475 | GCAGAGCTTCGAGGA AGTGTC | AACGCCTCCTGGGTCAACTC | CGCCTGGTCCACGTAGCC TTCGG |
| *mdp1* | Rv2986c | GCGTCTCCCGG CAGAA | TGCCTTCTTCGCTACCT TCTTG | CCCACACCACGCTTAA |
| *fdxA* | Rv2007c | CGACGAGTGCGTGG ATTGT | GGTAGATCGCCTTCCCAG TAGAT | CCGGTTTGCACGCACC |
| *hspX* | Rv2031c | GTCCGCGATGGT CAGCT | CGAAGGAACCGTACG CGAAT | ACCGTCGAAGTCCTTC |

7.2.6.5 Polymerase chain reaction (PCR) Amplification

The ABI 7500 Real-Time PCR System was used. For each gene experimental runs were performed in triplicate and repeated three times to generate a standard curve. The PCR cycling conditions were in 3 stages: stage 1 was for 2 min at 50$^{o}$C; stage 2 was for 10 min at 95$^{o}$C; and stage 3 was 40 PCR cycles with initial melting for 15 sec at 95$^{o}$C followed by annealing/extension for 1 min at 60$^{o}$C.

#### 7.2.6.6 Relative comparative threshold cycle ($C_T$) assay

The assay was performed with the ABI 7500 Real-Time PCR System. The primer pairs and probes listed in Table 7.1 as well as the TaqMan gene expression master mix was used. The 3-staged PCR cycling conditions as described in section: 7.2.6.5 were applied. The relative mRNA transcript expression levels of the *hbhA* gene (Rv0475), the *mdp1* gene (Rv2986c), the *fdxA* gene (Rv2007c) and the *hspX* gene (Rv2031c) were normalized against the level of 16*S* rRNA (Rv2907c) and *ftsZ* gene (Rv2150c) transcripts. The results are given as fold difference compared with the normalised expression levels of laboratory strain H37Rv.

### 7.2.7 Statistical analysis

All data were analysed initially using the one-way analysis of variance (ANOVA) and thereafter by means of Dunn's multiple comparisons test. All experiments were done in triplicate and repeated three times.

### 7.2.8 Ethics approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no: H039/06).

## 7.3 RESULTS

The $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios of the isolated total RNA ranged between 1.87 and 2.0 for all. Two distinct bands were visible on the denaturing MOPS gel (Fig. 7.1). The standard curves for all four gene transcripts had similar amplification efficiencies, as the graphs had $R^2$ values between 0.992 to 0.998 and slopes between -3.116 and -3.319 (Figs. 7.2, 7.3, 7.4, 7.5, 7.6, 7.7).

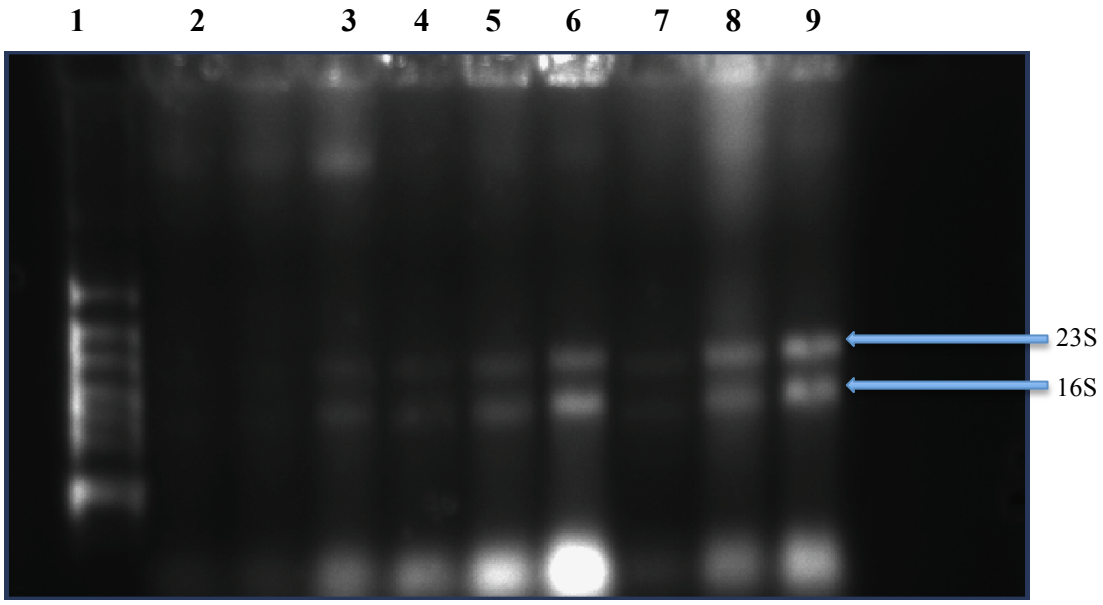


**Figure 7.1. MOPS gel showing two distinct bands of total RNA. Lane 1 (marker), lane 2 (water), lanes 3, 4, 5 & 6 (*M. tuberculosis* RNA dilutions), lanes 7, 8 & 9 (*M. tuberculosis* RNA dilutions)**

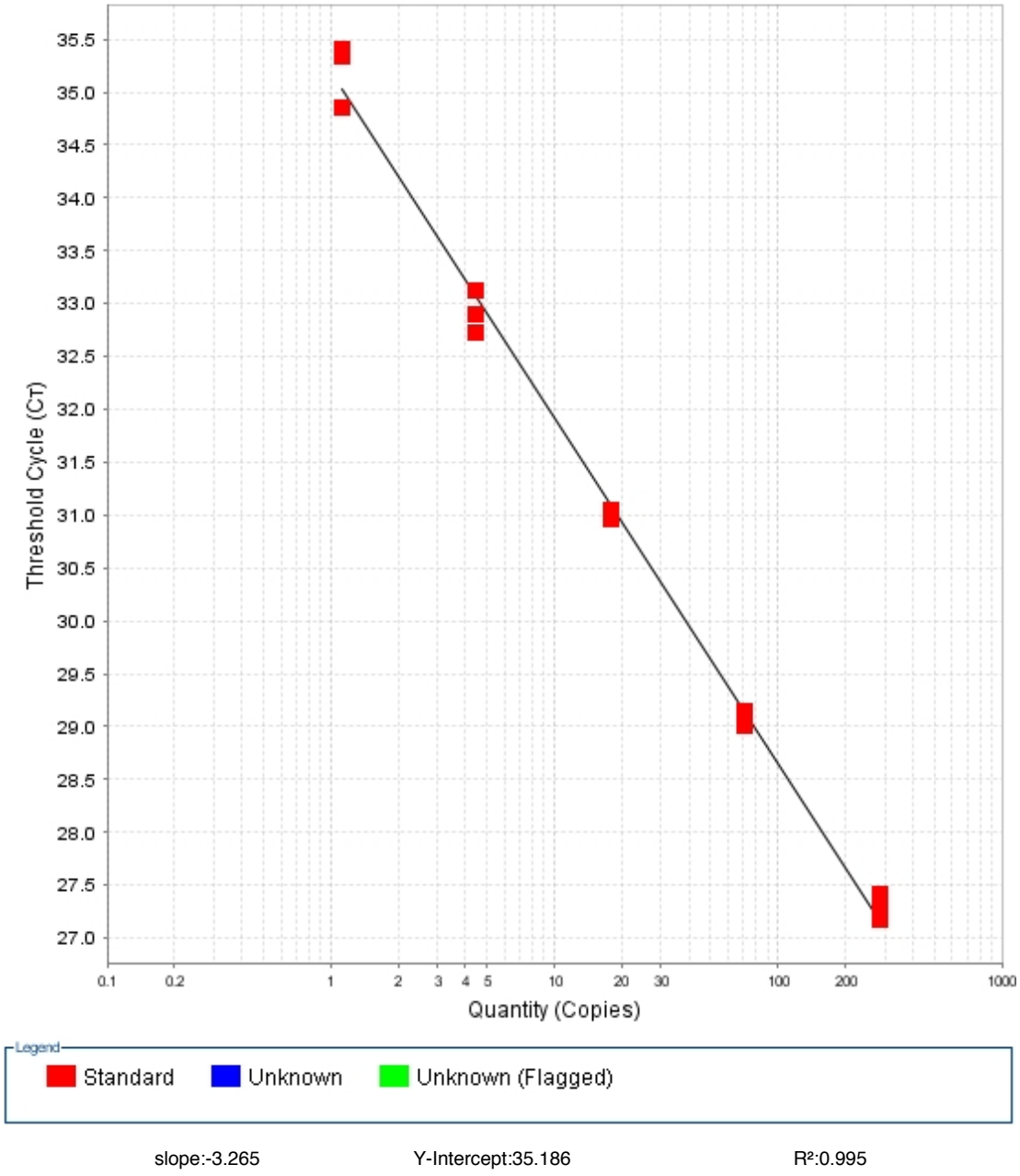


**Figure 7.2. Standard curve for *hbhA* gene expression**

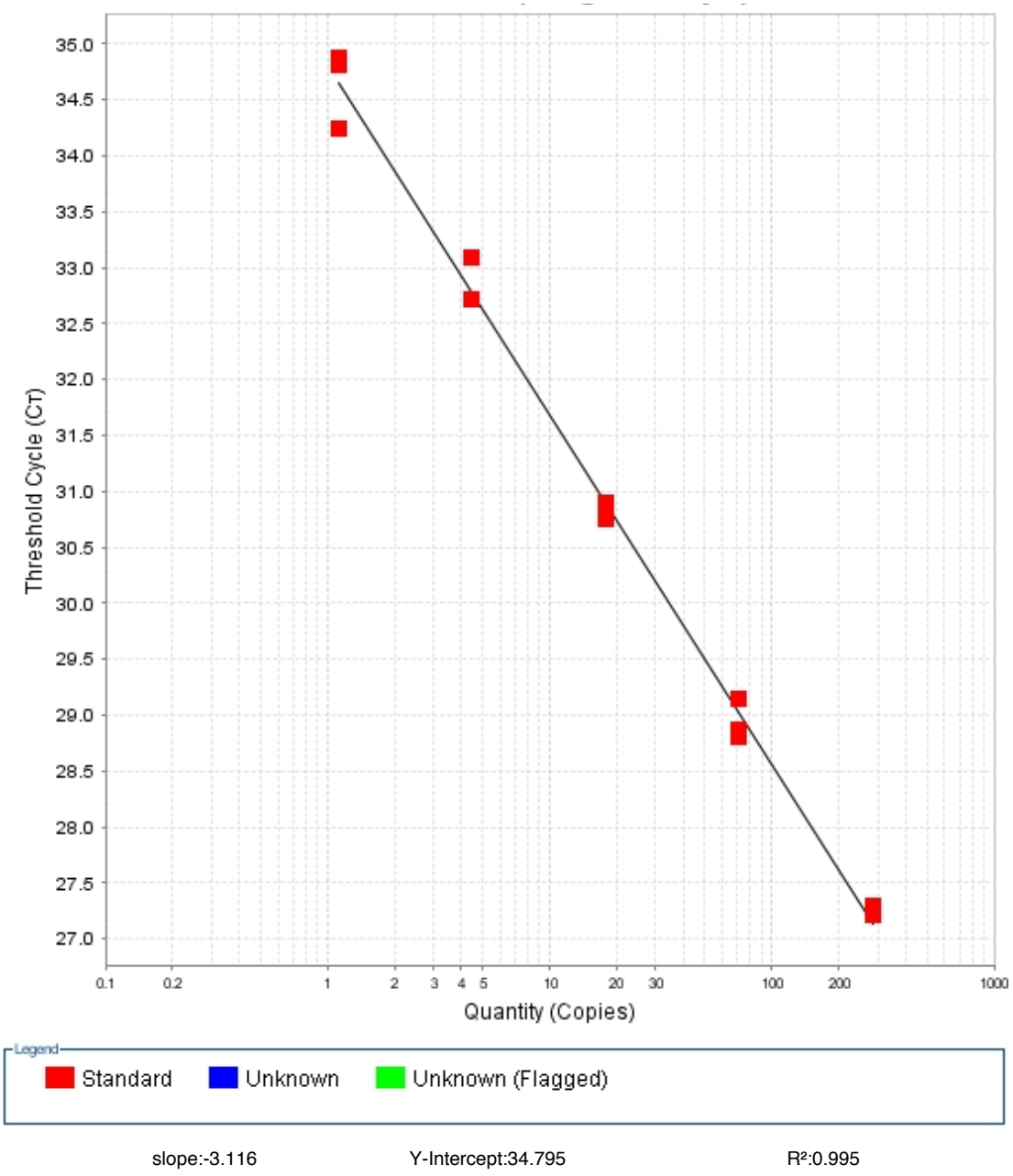


**Figure 7.3. Standard curve for *mdp1* gene expression**

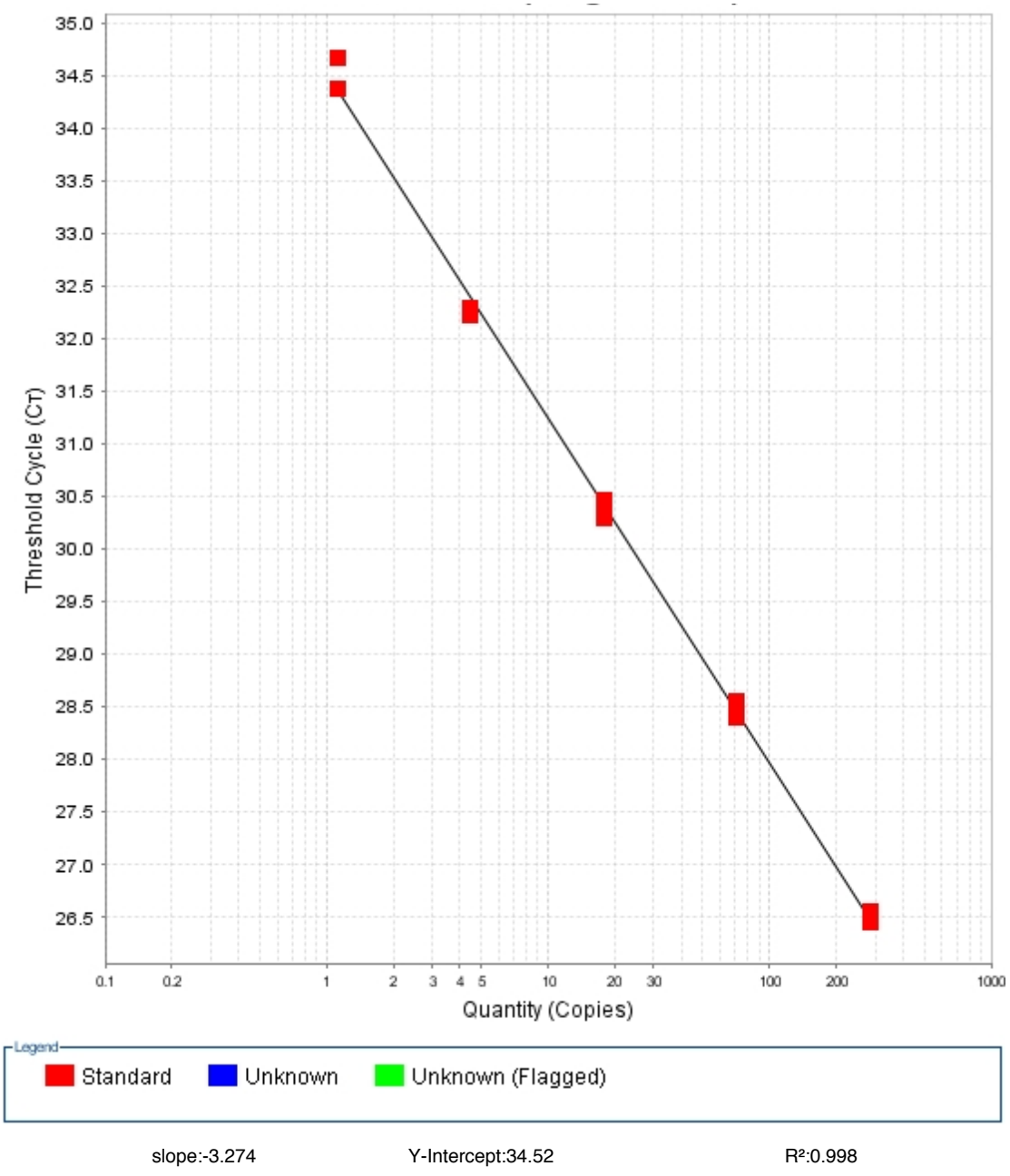


**Figure 7.4. Standard curve for *fdxA* gene expression**

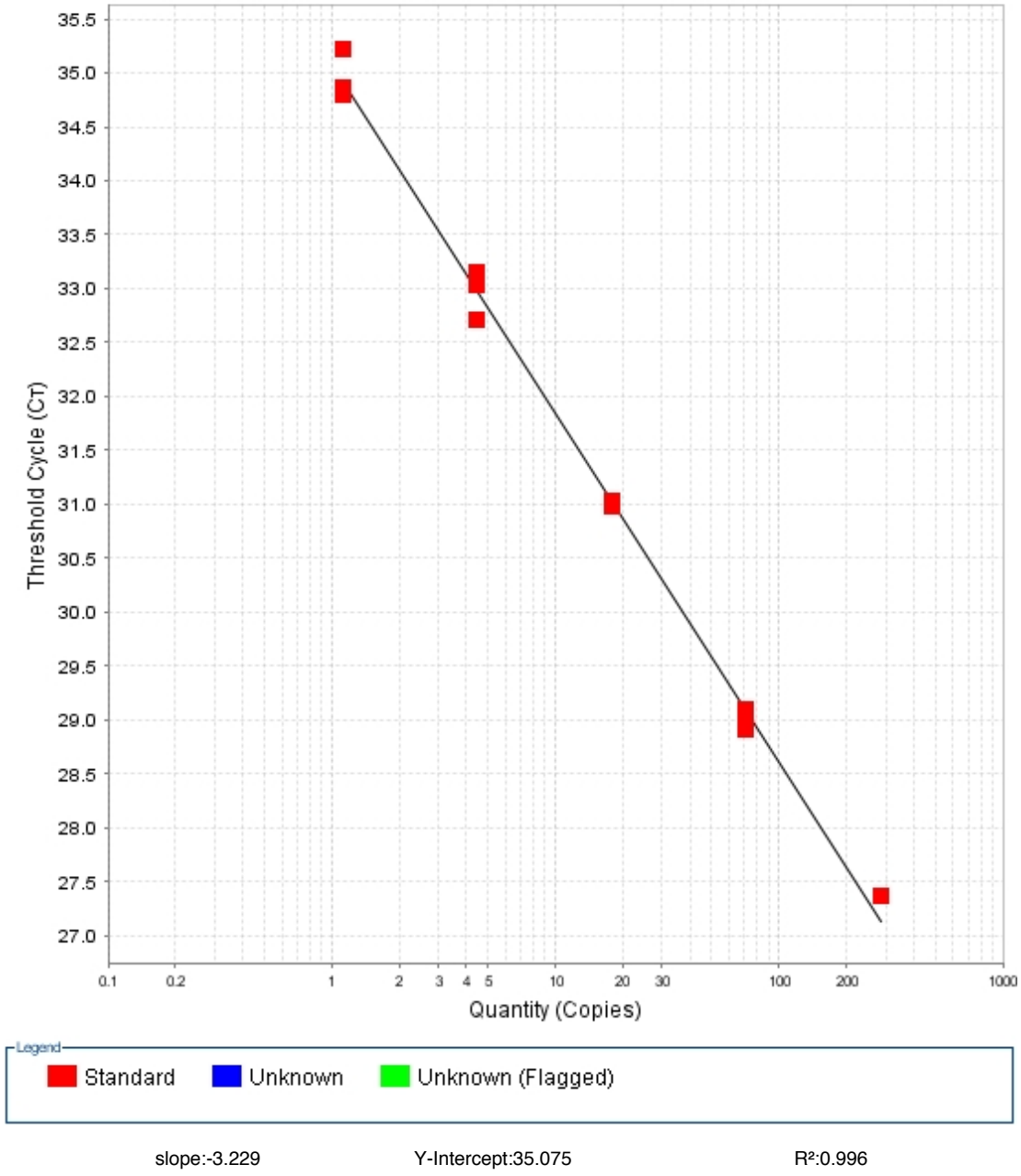


**Figure 7.5. Standard curve for *hspX* gene expression**

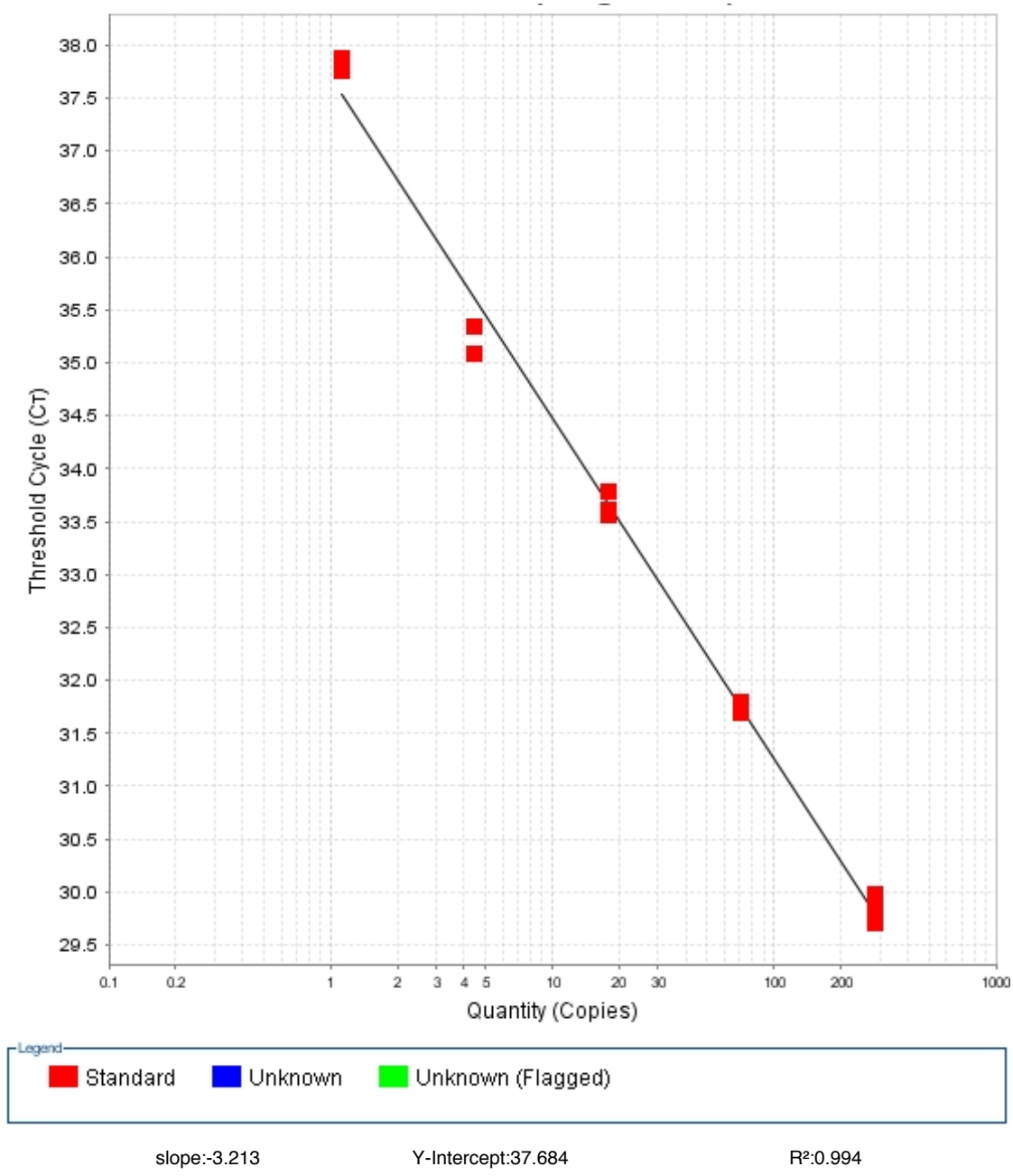


**Figure 7.6. Standard curve for *16S rRNA* gene expression**

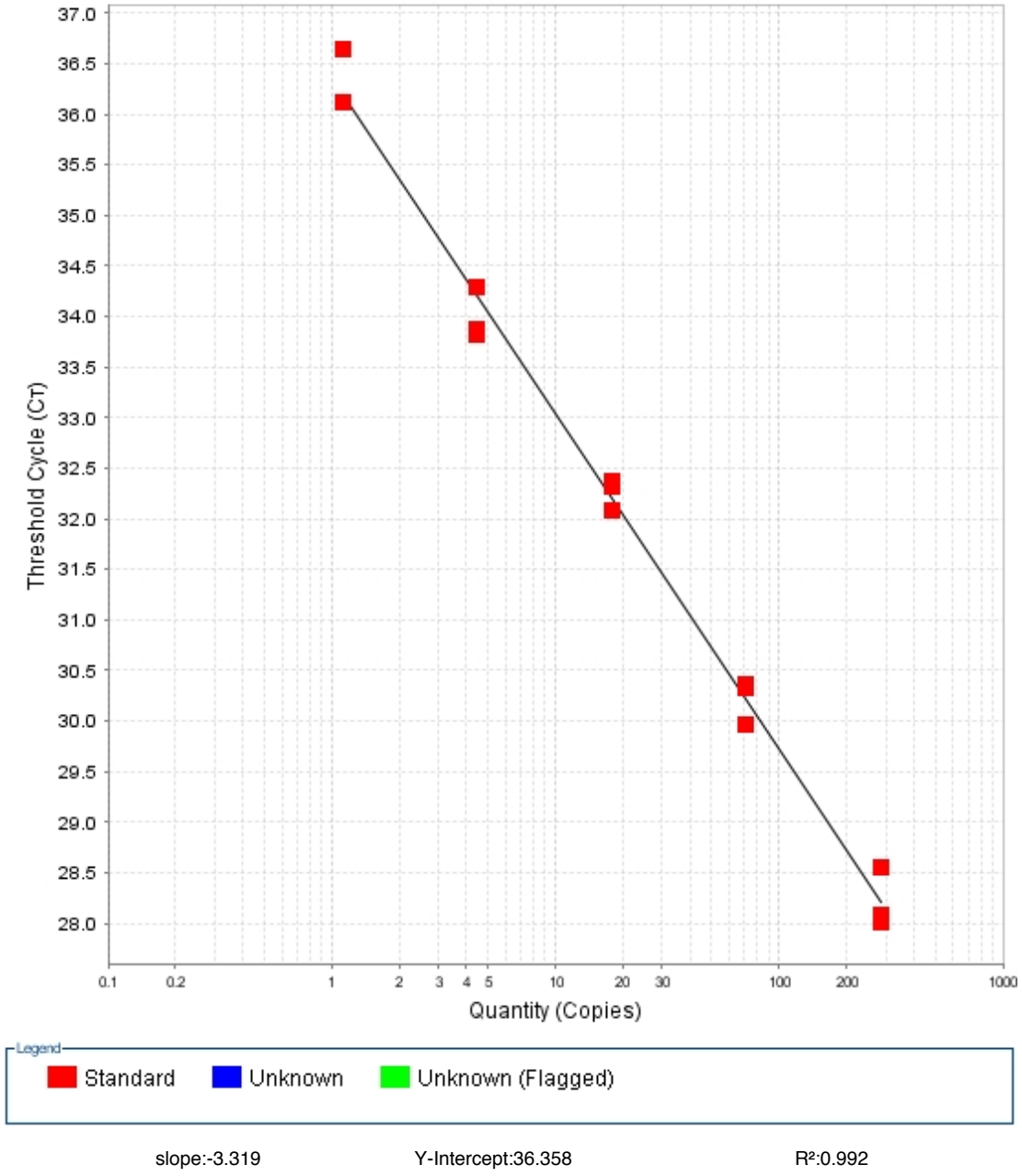


**Figure 7.7. Standard curve for *ftsZ* gene expression**

The 16*S* rRNA ratios were higher than the *ftsZ* ratios for all the isolates in all four genes both before and after exposure to A549 cells, but this did not affect the final results (Tables 7.2; 7.3; 7.4; 7.5; 7.6; 7.7; 7.8; 7.9).

### 7.3.1 Expression of *hbhA* gene

The isolates grown in the presence of oxygen, before and after exposure to A549 cells had a similar average expression level of *hbhA* (Fig. 7.8). However, there was a difference in the distribution of the isolates before and after exposure to A549 cells (Fig. 7.8; Tables 7.2; 7.3).

The expression level of *hbhA* was up regulated in all three XDR-F15/LAM4/KZN, two of the three F15/LAM4/KZN, one Beijing and one of the isolates with unique fingerprints grown in the presence of oxygen and before exposure to A549 cells (Fig. 7.8; Table 7.2). While the expression level of *hbhA* in the remaining two Beijing, one F15/LAM4/KZN and four of the five isolates with unique fingerprints clustered with the H37Rv strain (Fig. 7.8; Table 7.2). The exception to this was the H37Ra strain and one of the isolates with unique fingerprints, in which the expression level of *hbhA* was down regulated (Fig. 7.8; Table 7.2).

The expression level of *hbhA* in isolates grown in the presence of oxygen after exposure to A549 cells were up regulated in all six members of the F15/LAM4/KZN and all three members of the Beijing family of strains (Fig. 7.8; Table 7.3). Expression was down regulated in the H37Ra strain and all six isolates with unique fingerprints, three of which clustered with the H37Rv strain (Fig. 7.8; Table 7.3).

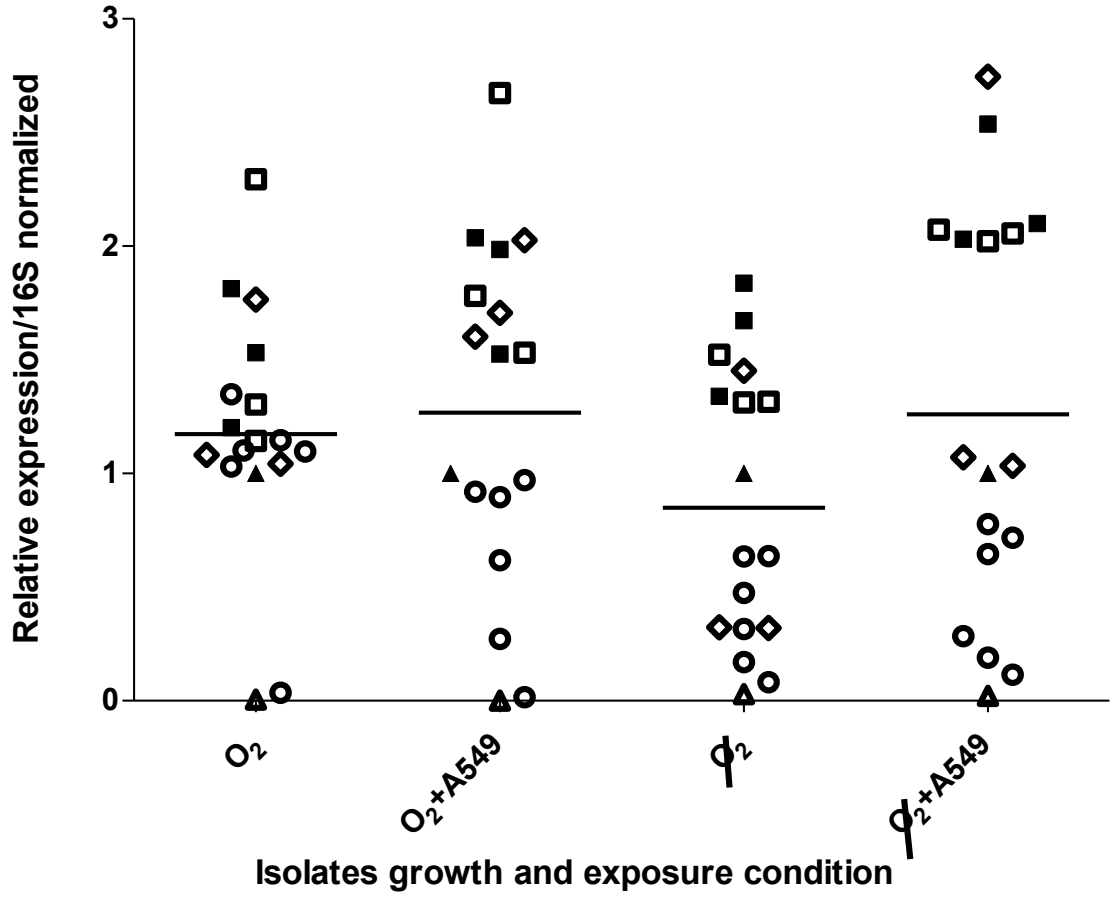


**Figure 7.8. Comparison of the expression of *hbhA* gene in *M. tuberculosis* isolates under different conditions. ◇= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates; □= F15/LAM4/KZN isolates; ○= Isolates with unique DNA fingerprint pattern; ▲= H37Rv; △= H37Ra**

**Table 7.2. Expression level of *hbhA* gene in *M. tuberculosis* isolates before exposure to A549 cells**

| Mean relative expression of *hbhA* gene before exposure to A549 | | | | | |
|---|---|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen present | | Oxygen deprived | |
| | | $^{hbhA}/_{16S}$ | $^{hbhA}/_{ftsZ}$ | $^{hbhA}/_{16S}$ | $^{hbhA}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 1.081±0.0002 | 1.041±0.0001 | 0.321±0.0001 | 0.21±0.0001 |
| | 2 | 1.766±0.0002 | 1.49±0.0002 | 1.452±0.0001 | 1.244±0.003 |
| | 3 | 1.043±0.0002 | 1.021±0.0001 | 0.324±0.0002 | 0.219±0.0003 |
| XDR-F15/LAM4/KZN | 1 | 1.202±0.0002 | 1.155±0.0003 | 1.673±0.0001 | 1.455±0.0003 |
| | 2 | 1.531±0.0002 | 1.325±0.0002 | 1.339±0.0002 | 1.154±0.0004 |
| | 3 | 1.813±0.0003 | 1.699±0.0002 | 1.838±0.0002 | 1.659±0.0004 |
| F15/LAM4/KZN | 1 | 1.143±0.0002 | 1.121±0.0001 | 1.522±0.0001 | 1.367±0.0004 |
| | 2 | 1.304±0.0003 | 1.191±0.0003 | 1.316±0.0002 | 1.133±0.0003 |
| | 3 | 2.294±0.0002 | 1.858±0.0003 | 1.312±0.0002 | 1.113±0.0001 |
| Unique DNA fingerprint | 1 | 1.097±0.0002 | 1.075±0.0001 | 0.637±0.0002 | 0.548±0.0004 |
| | 2 | 1.349±0.0001 | 1.257±0.0003 | 0.475±0.0001 | 0.313±0.0002 |
| | 3 | 1.146±0.0003 | 1.125±0.0002 | 0.316±0.0001 | 0.208±0.0003 |
| | 4 | 1.101±0.0003 | 1.095±0.0001 | 0.635±0.0001 | 0.513±0.0001 |
| | 5 | 0.036±0.0004 | 1.019±0.0002 | 0.082±0.0002 | 0.067±0.0002 |
| | 6 | 1.031±0.0002 | 0.076±0.0002 | 0.17±0.0002 | 0.093±0.0003 |
| H37Rv | 1 | 1 | 1 | 1 | 1 |
| H37Ra | 1 | 0.005±0.0002 | 0.003±0.00003 | 0.029±0.0002 | 0.019±0.0001 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

For the isolates that were grown under oxygen deprivation, the average expression level of the *hbhA* was higher after exposure to A549 cells (Fig. 7.8). The expression level of *hbhA* was up regulated in all the members of the F15/LAM4/KZN family of strains as well as in one Beijing isolate grown under oxygen deprivation and before exposure to A549 cells (Fig. 7.8; Table 7.2). Under the same circumstances, the expression of *hbhA* was down regulated in the remaining two Beijing isolates, all the isolates with unique fingerprints and the H37Ra strain (Fig. 7.8; Table 7.2).

**Table 7.3. Expression level of *hbhA* gene in *M. tuberculosis* isolates after exposure to A549 cells**

| Mean relative expression of *hbhA* gene after exposure to A549 | | | | | |
|---|---|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen present | | Oxygen deprived | |
| | | $^{hbhA}/_{16S}$ | $^{hbhA}/_{ftsZ}$ | $^{hbhA}/_{16S}$ | $^{hbhA}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 1.707±0.0001 | 1.339±0.0002 | 1.072±0.0008 | 1.057±0.0003 |
| | 2 | 2.026±0.0002 | 1.765±0.0002 | 2.746±0.0004 | 2.557±0.0003 |
| | 3 | 1.602±0.0002 | 1.319±0.0001 | 1.034±0.0002 | 1.012±0.0003 |
| XDR-F15/LAM4/KZN | 1 | 1.526±0.0004 | 1.072±0.0002 | 2.538±0.0003 | 2.325±0.0002 |
| | 2 | 1.985±0.0002 | 1.601±0.0003 | 2.03±0.0002 | 1.784±0.0002 |

| | | | | | |
|---|---|---|---|---|---|
| | 3 | 2.037±0.0002 | 2.01±0.0002 | 2.099±0.0002 | 1.975±0.0004 |
| F15/LAM4/KZN | 1 | 1.781±0.0001 | 1.511±0.0001 | 2.056±0.0003 | 1.826±0.0006 |
| | 2 | 1.531±0.0002 | 1.284±0.0002 | 2.073±0.0002 | 1.867±0.0003 |
| | 3 | 2.673±0.0003 | 2.21±0.0003 | 2.021±0.0003 | 1.747±0.0003 |
| Unique DNA fingerprint | 1 | 0.272±0.0001 | 0.149±0.0002 | 0.778±0.0003 | 0.541±0.0001 |
| | 2 | 0.92±0.0004 | 0.882±0.0002 | 0.718±0.0004 | 0.519±0.0001 |
| | 3 | 0.97±0.0002 | 0.899±0.0002 | 0.645±0.0003 | 0.505±0.0007 |
| | 4 | 0.895±0.0002 | 0.763±0.0003 | 0.19±0.0002 | 0.092±0.0002 |
| | 5 | 0.016±0.0002 | 0.164±0.0003 | 0.115±0.0002 | 0.064±0.0003 |
| | 6 | 0.619±0.0003 | 0.556±0.0002 | 0.283±0.0001 | 0.116±0.0003 |
| H37Rv | 1 | 1 | 1 | 1 | 1 |
| H37Ra | 1 | 0.001±0.0001 | 0.0004±0.0001 | 0.022±0.0004 | 0.011±0.0002 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

The expression level of *hbhA* in the isolates grown under oxygen deprivation and after exposure to A549 cells was up regulated in all six members of the F15/LAM4/KZN family as well as in one of the Beijing isolates (Fig. 7.8; Table 7.3). Expression was down regulated in the H37Ra strain, in the remaining two Beijing isolates as well as in all isolates with unique fingerprints (Fig. 7.8; Table 7.3).

On average, there was a significant down regulation of the expression level of *hbhA* when the isolates were grown under oxygen deprivation ($P = 0.01$). Exposure of the isolates grown under oxygen deprivation to A549 cells resulted in return of the average expression level to that observed under aerobic conditions (Fig. 7.8). There was also a significant difference in the average expression level of *hbhA* in the isolates grown under oxygen deprivation before and after exposure to A549 cells ($P = 0.001$) (Fig. 7.8). The H37Ra strain showed the lowest expression level of *hbhA* under all the different conditions (Fig. 7.8; Tables 7.2; 7.3).

### 7.3.2 Expression of *mdp1* gene

The isolates grown in the presence of oxygen, before and after exposure to A549 cells had a similar average expression level of *mdp1* (Fig. 7.9). However, there was a difference in the distribution of the isolates before and after exposure to A549 cells (Fig. 7.9; Tables 7.4; 7.5).

The expression level of *mdp1* in all the isolates grown in the presence of oxygen and before exposure to A549 cells clustered with the H37Rv strain, except for one F15/LAM4/KZN (Fig. 7.9). Four of the six F15/LAM4/KZN, one Beijing and four isolates with unique fingerprints were up regulated. In isolates grown in the presence of oxygen and after exposure to A549 cells expression levels were up regulated in five of the six isolates with unique fingerprints, the H37Ra strain and one Beijing isolate (Fig.

7.9; Table 7.5) while it was down regulated in all the six members of the F15/LAM4/KZN family of strains, the two remaining Beijing isolates and one isolate with unique fingerprint. All isolates in the down regulated group clustered with H37Rv (Fig. 7.9; Table 7.5).

When grown under oxygen deprivation, the average expression level of the *mdp1* was lower before exposure to A549 cells when compared to after exposure to A549 cells (Fig. 7.9). However, this did not reach statistical significance.

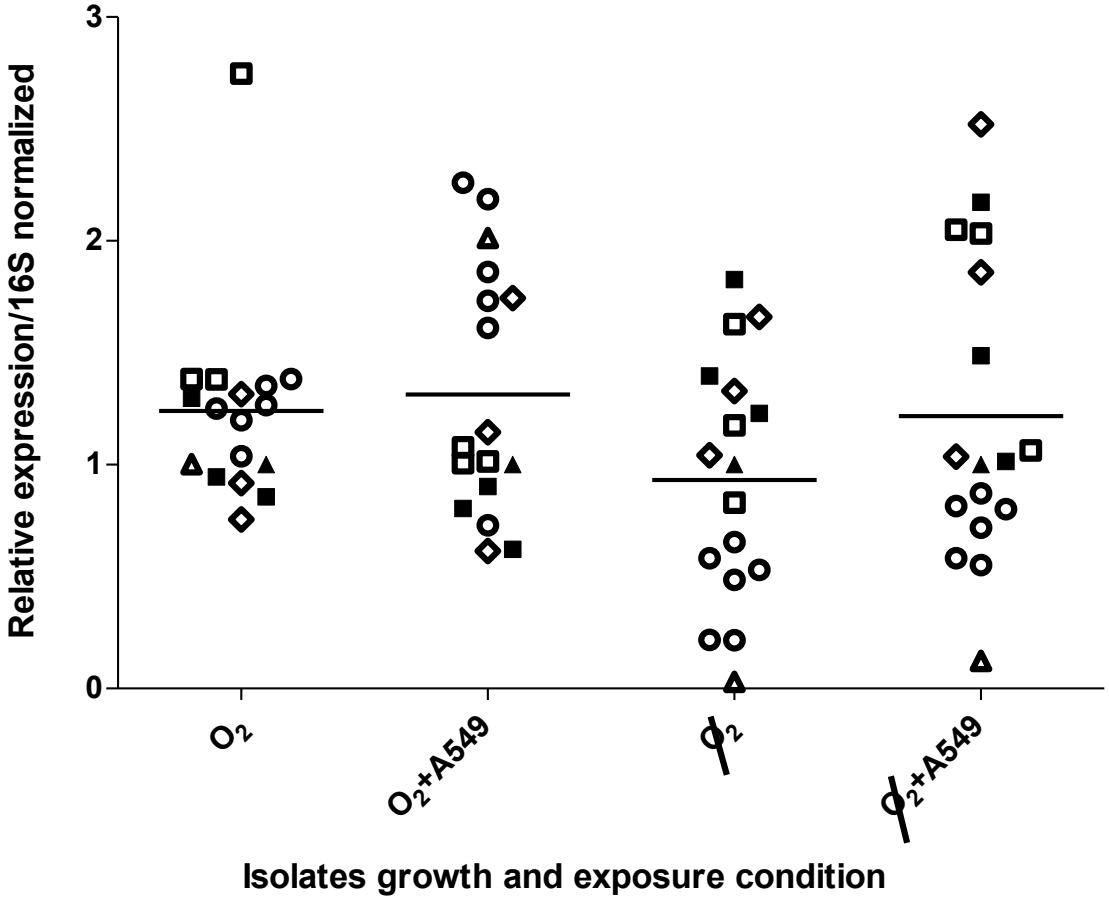

**Figure 7.9. Comparison of the expression of *mdp1* gene in *M. tuberculosis* isolates under different conditions. ◇= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates; □= F15/LAM4/KZN isolates; O= Isolates with unique DNA fingerprint pattern; ▲= H37Rv; △= H37Ra**

**Table 7.4. Expression level of *mdp1* gene in *M. tuberculosis* isolates before exposure to A549 cells**

| Mean relative expression of *mdp1* gene before exposure to A549 | | | | | |
|---|---|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen present | | Oxygen deprived | |
| | | $^{mdp1}/_{16S}$ | $^{mdp1}/_{ftsZ}$ | $^{mdp1}/_{16S}$ | $^{mdp1}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 0.918±0.0004 | 0.671±0.0003 | 1.329±0.0001 | 1.119±0.0002 |
| | 2 | 1.315±0.0004 | 1.115±0.0002 | 1.66±0.0001 | 1.428±0.0003 |
| | 3 | 0.755±0.0003 | 0.541±0.0002 | 1.043±0.0003 | 1.001±0.0002 |
| XDR-F15/LAM4/KZN | 1 | 0.946±0.0002 | 0.767±0.0004 | 1.396±0.0001 | 1.195±0.0005 |
| | 2 | 0.857±0.0003 | 0.657±0.0004 | 1.229±0.0002 | 1.009±0.0001 |
| | 3 | 1.297±0.0003 | 1.085±0.0003 | 1.827±0.0001 | 1.658±0.0003 |
| F15/LAM4/KZN | 1 | 2.748±0.0001 | 2.471±0.0003 | 1.176±0.0002 | 1.003±0.0002 |
| | 2 | 1.381±0.0003 | 1.148±0.0003 | 0.831±0.0002 | 0.684±0.0003 |
| | 3 | 1.383±0.0002 | 1.334±0.0002 | 1.628±0.0002 | 1.444±0.0003 |
| Unique DNA fingerprint | 1 | 1.352±0.0002 | 1.124±0.0003 | 0.654±0.0002 | 0.465±0.000 |
| | 2 | 1.382±0.0006 | 1.161±0.0001 | 0.582±0.0001 | 0.341±0.0001 |
| | 3 | 1.251±0.0004 | 1.024±0.0002 | 0.216±0.0002 | 0.084±0.0004 |

| | | | | | |
|---|---|---|---|---|---|
| | 4 | 1.266±0.033 | 1.025±0.0003 | 0.53±0.0002 | 0.4±0.0002 |
| | 5 | 1.038±0.0003 | 1.019±0.0002 | 0.486±0.0001 | 0.239±0.0002 |
| | 6 | 1.199±0.0002 | 1.015±0.0001 | 0.217±0.0002 | 0.093±0.0003 |
| H37Rv | 1 | 1 | 1 | 1 | 1 |
| H37Ra | 1 | 1.002±0.0002 | 1.001±0.0001 | 0.03±0.0001 | 0.009±0.0001 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

The expression level of *mdp1* was up regulated in five of the six members of the F15/LAM4/KZN family of strains and all three Beijing isolates when grown under oxygen deprivation and before exposure to A549 cells (Fig. 7.9; Table 7.4) while the expression level was down regulated in all the isolates with unique fingerprints, the remaining F15/LAM4/KZN isolate and H37Ra (Fig. 7.9; Table 7.4).

**Table 7.5. Expression level of *mdp1* gene in *M. tuberculosis* isolates after exposure to A549 cells**

| Mean relative expression of *mdp1* gene after exposure to A549 | | | | | |
|---|---|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen present | | Oxygen deprived | |
| | | $mdp1/_{16S}$ | $mdp1/_{ftsZ}$ | $mdp1/_{16S}$ | $mdp1/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 1.145±0.0003 | 1.09±0.0001 | 1.86±0.0002 | 1.657±0.0003 |
| | 2 | 0.614±0.0004 | 0.326±0.0002 | 2.52±0.0003 | 2.247±0.0002 |

| | | | | | |
|---|---|---|---|---|---|
| | 3 | 1.745±0.0002 | 1.674±0.0002 | 1.035±0.0001 | 1.016±0.0002 |
| XDR-F15/LAM4/KZN | 1 | 0.805±0.0001 | 0.597±0.0002 | 1.488±0.0004 | 1.264±0.0002 |
| | 2 | 0.623±0.0002 | 0.398±0.0002 | 1.014±0.0002 | 1.01±0.0002 |
| | 3 | 0.903±0.0002 | 0.762±0.0003 | 2.172±0.0003 | 1.948±0.0004 |
| F15/LAM4/KZN | 1 | 1.014±0.0003 | 1.009±0.0002 | 2.05±0.0002 | 1.889±0.0002 |
| | 2 | 1.077±0.0004 | 1.052±0.0001 | 1.063±0.0003 | 1.048±0.0002 |
| | 3 | 1.007±0.0001 | 1.002±0.00003 | 2.033±0.0001 | 1.784±0.0002 |
| Unique DNA fingerprint | 1 | 2.26±0.0003 | 1.998±0.0002 | 0.801±0.0002 | 0.602±0.0002 |
| | 2 | 2.187±0.0002 | 1.981±0.0003 | 0.718±0.0003 | 0.585±0.0002 |
| | 3 | 0.729±0.0003 | 0.519±0.0002 | 0.551±0.0003 | 0.315±0.0003 |
| | 4 | 1.733±0.0002 | 1.513±0.0003 | 0.872±0.0003 | 0.651±0.0002 |
| | 5 | 1.861±0.0003 | 1.724±0.0002 | 0.816±0.0004 | 0.614±0.0003 |
| | 6 | 1.612±0.0002 | 1.405±0.0004 | 0.582±0.0003 | 0.372±0.0002 |
| H37Rv | 1 | 1 | 1 | 1 | 1 |
| H37Ra | 1 | 2.013±0.0003 | 1.948±0.0002 | 0.124±0.0002 | 0.103±0.0002 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

After exposure to A549 cells, the expression level of *mdp1* in two of the Beijing and four of the F15/LAM4/KZN isolates grown under oxygen deprivation was up regulated (Fig. 7.9; Table 7.5). In all six isolates with unique fingerprints, the remaining one Beijing isolate and two members of the F15/LAM4/KZN family expression was down regulated. Those with down regulated *mdp1*expression clustered with H37Rv (Fig. 7.9; Table 7.5).

When grown under oxygen deprivation, the expression levels of *mdp1* before and after exposure to A549 cells differed significantly ($P = 0.0008$) (Fig. 7.9). Exposure to A549 cells did not affect expression levels when isolates were grown in the presence of oxygen or under oxygen deprivation (Fig. 7.9).

### 7.3.3 Expression of *fdxA* gene

In the isolates that were grown under oxygen deprivation, the average expression level of the *fdxA* was lower before exposure to A549 cells when compared to after exposure to A549 cells (Fig. 7.10). There was also a difference in the distribution of these isolates before and after exposure to A549 cells (Fig. 7.10; Tables 7.6; 7.7).

Before exposure to A549 cells, the expression level of *fdxA* was up regulated in all the six members of the F15/LAM4/KZN family of strains and two of the three Beijing isolates grown under oxygen deprivation (Fig. 7.10; Table 7.6). Under the same circumstances, the expression level of *fdxA* was down regulated in all the isolates with unique fingerprints, one Beijing isolate and the H37Ra strain (Fig. 7.10; Table 7.6).

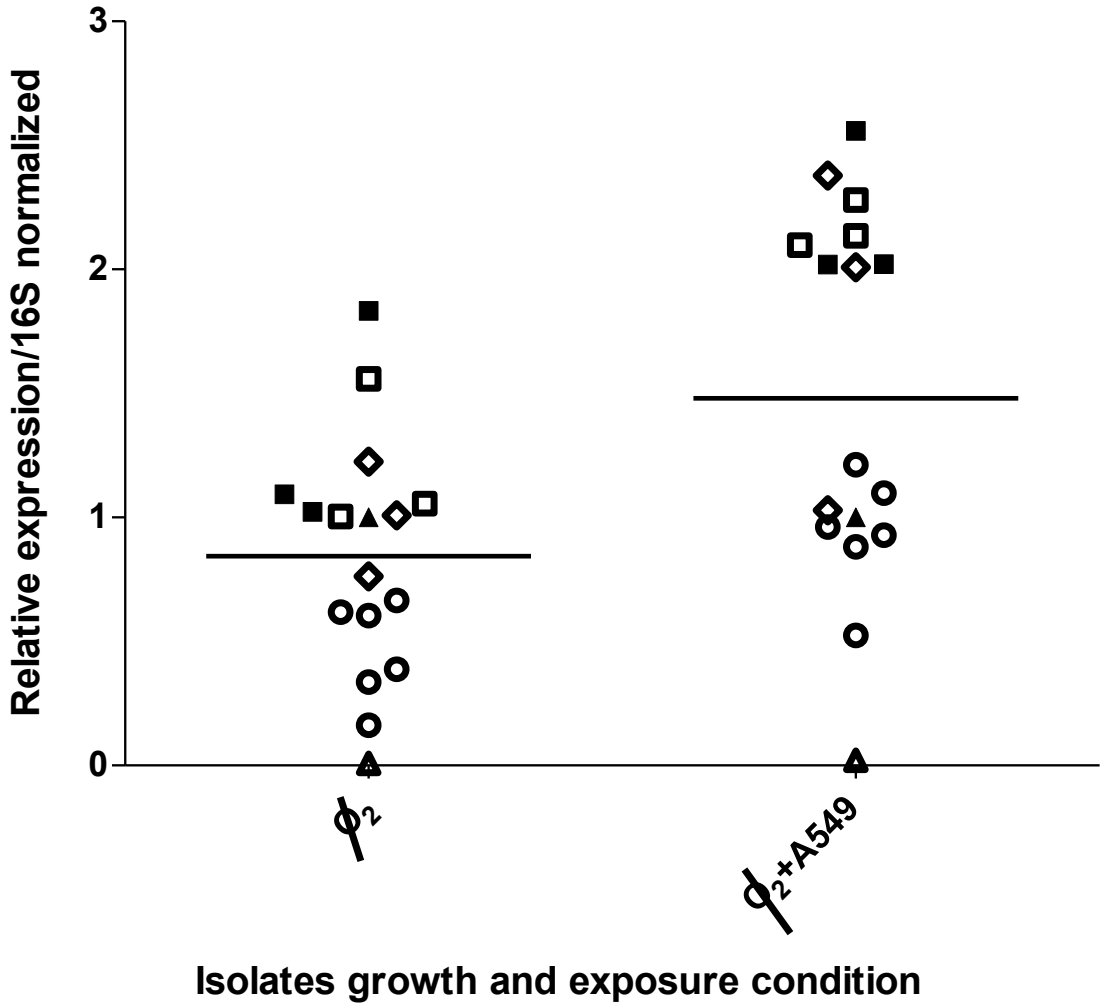


**Figure 7.10. Comparison of the expression of *fdxA* gene in *M. tuberculosis* isolates under different conditions. ◇= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates; □= F15/LAM4/KZN isolates; O= Isolates with unique DNA fingerprint pattern; ▲= H37Rv; △= H37Ra**

**Table 7.6. Expression level of *fdxA* gene in *M. tuberculosis* isolates before exposure to A549 cells**

| Mean relative expression of *fdxA* gene before exposure to A549 | | | |
|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen deprived | |
| | | $^{fdxA}/_{16S}$ | $^{fdxA}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 1.008 ± 0.0003 | 1.005±0.0002 |
| | 2 | 1.225 ± 0.0004 | 1.1±0.0001 |
| | 3 | 0.763 ± 0.0002 | 0.519±0.0003 |
| XDR-F15/LAM4/KZN | 1 | 1.093 ± 0.0006 | 1.063±0.0002 |
| | 2 | 1.022 ± 0.0002 | 1.01±0.0003 |
| | 3 | 1.833 ± 0.0006 | 1.64±0.0003 |
| F15/LAM4/KZN | 1 | 1.559 ± 0.0003 | 1.38±0.0006 |
| | 2 | 1.056 ± 0.0003 | 1.032±0.0003 |
| | 3 | 1.003 ± 0.0005 | 1.001±0.0002 |
| Unique DNA fingerprint | 1 | 0.665 ± 0.0004 | 0.49±0.0001 |
| | 2 | 0.387 ± 0.0006 | 0.155±0.0003 |
| | 3 | 0.336 ± 0.0003 | 0.123±0.0004 |
| | 4 | 0.618 ± 0.0004 | 0.452±0.0002 |
| | 5 | 0.162 ± 0.0003 | 0.099±0.0006 |
| | 6 | 0.605 ± 0.0004 | 0.392±0.0004 |
| H37Rv | 1 | 1 | 1 |
| H37Ra | 1 | 0.009 ± 0.0003 | 0.002±0.0001 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

When grown under oxygen deprivation and exposed to A549 cells, the expression level of *fdxA* in all the members of F15/LAM4/KZN family of strains and two of the three Beijing isolates was up regulated (Fig. 7.10; Table 7.7) while it was down regulated in one Beijing isolate, all the six isolates with unique fingerprints and H37Ra (Fig. 7.10; Table 7.7). Five of the six isolates with unique fingerprints and the single Beijing isolate clustered with the H37Rv strain (Fig. 7.10; Table 7.7). The H37Ra strain showed the lowest expression level of *fdxA* gene (Fig. 7.10; Tables 7.6; 7.7).

**Table 7.7. Expression level of *fdxA* gene in *M. tuberculosis* isolates after exposure to A549 cells**

| Mean relative expression of *fdxA* gene after exposure to A549 | | | |
|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen deprived | |
| | | $^{fdxA}/_{16S}$ | $^{fdxA}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 2.009 ± 0.0025 | 1.874±0.0002 |
| | 2 | 2.379 ± 0.0004 | 2.123±0.0002 |
| | 3 | 1.029 ± 0.0002 | 1.01±0.0002 |
| XDR-F15/LAM4/KZN | 1 | 2.02 ± 0.0002 | 2.01±0.0001 |
| | 2 | 2.02 ± 0.0002 | 2.009±0.0001 |

| | | | |
|---|---|---|---|
| | 3 | 2.559 ± 0.0003 | 2.381±0.0002 |
| F15/LAM4/KZN | 1 | 2.28 ± 0.0002 | 2.064±0.0002 |
| | 2 | 2.099 ± 0.0001 | 2.071±0.0002 |
| | 3 | 2.136 ± 0.0002 | 2.092±0.0003 |
| Unique DNA fingerprint | 1 | 1.212 ± 0.0001 | 1.097±0.0003 |
| | 2 | 1.097 ± 0.0002 | 1.071±0.0002 |
| | 3 | 0.882 ± 0.0003 | 0.696±0.0003 |
| | 4 | 0.962 ± 0.0002 | 0.773±0.0003 |
| | 5 | 0.525 ± 0.0003 | 0.36±0.0003 |
| | 6 | 0.928 ± 0.0001 | 0.718±0.0001 |
| H37Rv | 1 | 1 | 1 |
| H37Ra | 1 | 0.021 ± 0.0003 | 0.008±0.0003 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

There was a significant difference between the expression level of *fdxA* before and after exposure to A549 cells ($P < 0.0001$) (Fig. 7.10). The H37Ra strain showed the lowest expression level of *fdxA* under both conditions (Fig. 7.10; Tables 7.6; 7.7).

### 7.3.4 Expression of *hspX* gene

In the isolates that were grown under oxygen deprivation, the average expression level of the *hspX* was lower before exposure to A549 cells as compared to after exposure to A549 cells (Fig. 7.11). There was also a difference in the distribution of these isolates before and after exposure to A549 cells (Fig. 7.11; Tables 7.8; 7.9).

The expression level of *hspX* was up regulated in all the six members of the F15/LAM4/KZN family of strains and two of the three Beijing isolates grown under oxygen deprivation and before exposure to A549 cells (Fig. 7.11; Table 7.8). The expression level of this gene was down regulated in five of the six isolates with unique fingerprints, one Beijing isolate and the H37Ra (Fig. 7.11; Table 7.8). The H37Rv and the remaining isolate with unique fingerprint were on the average line.

When grown under oxygen deprivation and exposed to A549 cells, the expression level of *hspX* in all the members of F15/LAM4/KZN family of strains and two of the three Beijing isolates was up regulated (Fig. 7.11; Table 7.9) while it was down regulated in the H37Ra, one Beijing isolate and all the six isolates with unique fingerprints (Fig. 7.11; Table 7.9). The expression level of *hspX* in three isolates with unique fingerprints and one Beijing isolate clustered with the H37Rv strain (Fig. 7.11, Table 7.9).

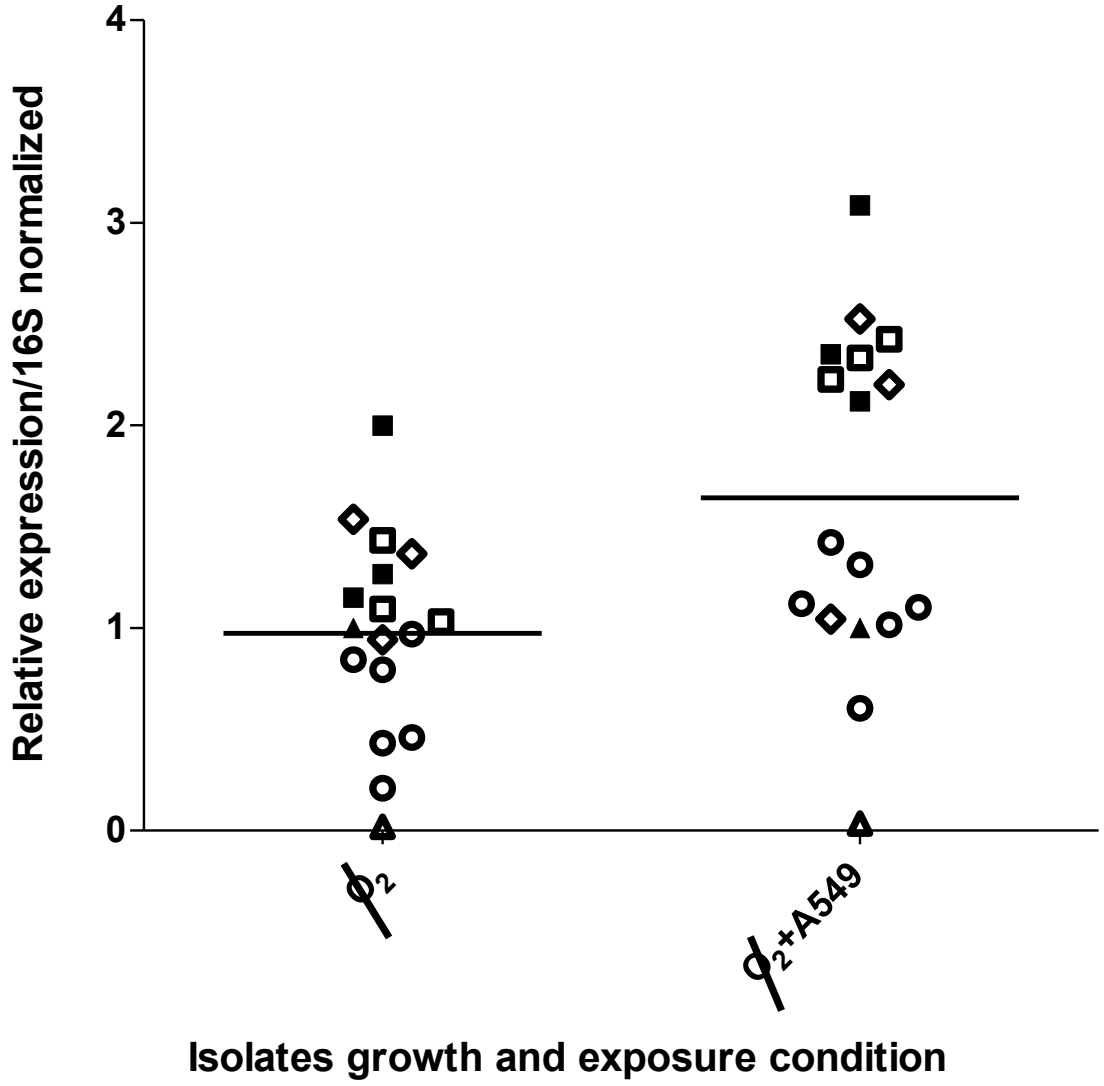


**Figure 7.11. Comparison of the expression of *hspX* gene in *M. tuberculosis* isolates under different condition. ◇= Beijing isolates; ■= XDR-F15/LAM4/KZN isolates; □= F15/LAM4/KZN isolates; O= Isolates with unique DNA fingerprint pattern; ▲= H37Rv; △= H37Ra**

**Table 7.8. Expression level of *hspX* gene in *M. tuberculosis* isolates before exposure to A549 cells**

| Mean relative expression of *hspX* gene before exposure to A549 | | | |
|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen deprived | |
| | | $^{hspX}/_{16S}$ | $^{hspX}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 1.366 ± 0.0005 | 1.197±0.0002 |
| | 2 | 1.536 ± 0.0004 | 1.339±0.0001 |
| | 3 | 0.942 ± 0.0003 | 0.728±0.0002 |
| XDR-F15/LAM4/KZN | 1 | 1.149 ± 0.0004 | 1.115±0.0002 |
| | 2 | 1.266 ± 0.0004 | 1.124±0.0003 |
| | 3 | 1.999 ± 0.0006 | 1.732±0.0003 |
| F15/LAM4/KZN | 1 | 1.433 ± 0.0006 | 1.259±0.001 |
| | 2 | 1.095 ± 0.0006 | 1.069±0.0002 |
| | 3 | 1.033 ± 0.0003 | 1.014±0.0002 |
| Unique DNA fingerprint | 1 | 0.971 ± 0.0004 | 0.769±0.0003 |
| | 2 | 0.432 ± 0.0003 | 0.221±0.0003 |
| | 3 | 0.46 ± 0.0001 | 0.271±0.0003 |
| | 4 | 0.843 ± 0.0006 | 0.678±0.0002 |
| | 5 | 0.209 ± 0.0006 | 0.117±0.0002 |
| | 6 | 0.794 ± 0.0009 | 0.582±0.0002 |
| H37Rv | 1 | 1 | 1 |
| H37Ra | 1 | 0.017 ± 0.0003 | 0.01±0.0003 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

**Table 7.9. Expression level of *hspX* gene in *M. tuberculosis* isolates after exposure to A549 cells**

| Mean relative expression of *hspX* gene after exposure to A549 | | | |
|---|---|---|---|
| *M. tuberculosis* strains | | Oxygen deprived | |
| | | $^{hspX}/_{16S}$ | $^{hspX}/_{ftsZ}$ |
| | | Mean ± SEM | Mean ± SEM |
| Beijing | 1 | 2.202 ± 0.0003 | 2.003±0.0003 |
| | 2 | 2.527 ± 0.0002 | 2.35±0.0002 |
| | 3 | 1.045 ± 0.0003 | 1.013±0.0003 |
| XDR-F15/LAM4/KZN | 1 | 2.352 ± 0.0003 | 2.197±0.0002 |
| | 2 | 2.12 ± 0.0002 | 1.93±0.0002 |
| | 3 | 3.086 ± 0.0003 | 2.972±0.0002 |
| F15/LAM4/KZN | 1 | 2.333 ± 0.0002 | 2.169±0.0003 |
| | 2 | 2.227 ±0.0002 | 2.005±0.0003 |
| | 3 | 2.426 ± 0.0002 | 2.285±0.0004 |
| Unique DNA fingerprint | 1 | 1.422 ± 0.0002 | 1.258±0.0001 |
| | 2 | 1.121 ± 0.0006 | 1.09±0.0002 |
| | 3 | 1.015 ± 0.0002 | 1.008±0.0003 |
| | 4 | 1.102 ± 0.0001 | 1.075±0.0002 |

| | | | |
|---|---|---|---|
| | 5 | 0.603 ± 0.0001 | 0.462±0.0002 |
| | 6 | 1.313 ± 0.0003 | 1.153±0.0003 |
| H37Rv | 1 | 1 | 1 |
| H37Ra | 1 | 0.036 ± 0.0003 | 0.013±0.0001 |

**The results are means of three experiments performed in triplicate. SEM, standard error of mean**

When grown under oxygen deprivation the expression level of *hspX* in the isolates before and after exposure to A549 cells differed significantly ($P < 0.0001$) (Fig. 7.11). H37Ra had the lowest expression level under both conditions (Fig. 7.11; Tables 7.8; 7.9).

## 7.4 DISCUSSION

This is the first study investigating the expression of genes potentially associated with virulence in F15/LAM4/KZN isolates of *M. tuberculosis* grown in the presence of oxygen and under oxygen deprivation, before and after exposure to A549 alveolar cells. The results are compared with the expression levels in isolates belonging to the Beijing family. Different studies have investigated the expression of genes potentially associated with virulence in laboratory strains (Badillo-Lopez *et al*. 2010; Delogu *et al*. 2006) and clinical isolates (Lam *et al*. 2008; Gao *et al*. 2005) of *M. tuberculosis*.

Our results show that expression levels of genes potentially associated with virulence vary between laboratory and clinical isolates grown under the same conditions. We also observed that there was variation in the expression levels of the same gene among clinical isolates of *M. tuberculosis* with different RFLP signature when grown under identical conditions.

This is similar to the observations by Gao *et al* (2005), who reported diversity among ten clinical isolates and laboratory strains of *M. tuberculosis*. They concluded that strains grown under the same condition have different expression levels of genes with important functions. They proposed that genes of interest should be examined individually for gene expression variability among strains. They also proposed that this individual examination of gene expression should be performed under relevant conditions. We measured expression of individual genes in a group of clinical isolates *in vitro*. The anaerobic growth provides the low redox environment as found in tissue. This in combination with exposure to alveolar epithelial cells mimics *in vivo* conditions.

We observed an up regulation in the expression levels of three of the four genes potentially associated with virulence after exposure to A549 cells, compared with the expression level before exposure to A549 cells. In general, the highest levels of expression were found if oxygen depletion was combined with exposure to A549 cells. The *hbhA* gene did not show any effect of A549 exposure when the organisms were grown aerobically. When deprived of oxygen, *hbhA* expression was down regulated but exposure to A549 increased the expression levels again. Delogu *et al* (2006) also reported

on the up regulation of expression of the *hbhA* gene in lungs of mice infected with the Erdman strain. The up regulation of the expression of this gene after exposure to A549 cells suggests that the gene product may play a role in the interaction between the bacilli and the alveolar epithelial cells. Knock out of this gene in the bacteria, followed by the exposure of the bacilli to the cells of the lung *in vitro* and in mouse inhalation experiments can provide further information.

The expression levels of the investigated genes in the isolates grown under oxygen deprivation were up regulated after exposure to the A549 cells, compared with the expression level in the same isolates that were grown in the presence of oxygen and thereafter exposed to A549 cells, although the averages showed the same pattern in both *hbhA* and *mdp1* genes. This suggests that oxygen deprivation might instigate expression of virulence attributes. We had previously observed that the phenotypes of these same isolates grown under oxygen deprivation were more invasive (Ashiru *et al*. 2012) and cytotoxic to the A549 cells *in vitro*.

We did perform statistical analyses on our results, none was found to be significantly different when compared with the H37Rv strain (Tables 7.2; 7.3; 7.4; 7.5; 7.6; 7.7; 7.8; 7.9) although some were statistically significant against H37Ra. Expression levels were consistently lowest in H37Ra under all conditions except for the *mdp1* gene, when the bacteria were grown in the presence of oxygen before as well as after exposure to A549 cells. We also observed that the expression level of *mdp1* was up regulated under these

conditions in the isolates with unique fingerprints. This suggests that the gene product of *mdp1* is not involved in the transmission of *M. tuberculosis*.

No significant difference was observed between the XDR isolates and other F15/LAM4/KZN isolates. All six isolates behaved similarly. This was not so in the case of the three Beijing isolates. One of the Beijing isolates consistently behaved differently.

It was observed that the expression level of *hbhA* in the members of the F15/LAM4/KZN grown in the presence of oxygen before exposure to A549 cells was on or above the average line. After the exposure to A549 cells the expression level of *hbhA* in the members of the F15/LAM4/KZN and Beijing family of strains were above average, while the isolates with unique fingerprints were below the average line. This observation indicates that exposure to A549 cells resulted in higher expression levels of *hbhA* in isolates belonging to successful strain.

We also observed that the average expression level of *hbhA* in isolates grown under oxygen deprivation before exposure to A549 cells, decreased because of decreased expression in isolates with unique fingerprints and the Beijing isolates, while the F15/LAM4/KZN isolates remained the same as observed in the circumstance of the isolates grown in the presence of oxygen. After exposure to A549 cells the difference between the F15/LAM4/KZN isolates and the isolates with unique fingerprints became more pronounced. Although the Beijing isolates had a higher level of *hbhA* expression than the isolates with unique fingerprints, two of the three were below the average line.

Exposure to A549 cells favoured the expression of *hbhA* in F15/LAM4/KZN isolates grown under oxygen deprivation. If indeed; this is related to the observation in mice by Delogu *et al* (2006), we can conclude that the F15/LAM4/KZN strain is more virulent than the Beijing strain.

Compared with *hbhA*, the expression of *mdp1* follows a different pattern. When grown in the presence of oxygen before exposure to A549 cells, all the members of the F15/LAM4/KZN and Beijing family of strains, as well as the isolates with unique fingerprints clustered together around the average line. After exposure to A549 cells the expression level of *mdp1* in the unsuccessful strains rose above the average line while the successful strains showed the opposite.

When the isolates were grown under oxygen deprivation before exposure to A549 cells, a reverse pattern of *mdp1* expression was seen as compared to the isolates grown in the presence of oxygen after exposure to A549 cells. The *mdp1* expression of the successful strains is above the average line while that of the unsuccessful strains is below average. After exposure to A549 cells the difference between the successful and unsuccessful strains is more pronounced. This observation confirms the role of the gene product that the *mdp1* gene codes for, which is to prevent the denaturation of the DNA under extreme environmental condition (Katsube *et al*. 2007; Tuberculist, 2012). The growth of the isolates under oxygen deprivation and subsequent exposure to A549 cells might constitute an extreme environmental condition under which successful strains of *M. tuberculosis* are better adapted.

The expression of *fdxA* in the isolates grown under oxygen deprivation was expected as previously reported by Ramchandra and Sturm, 2010. After exposure to A549 cells, the expression of *fdxA* in all the isolates increased to significantly higher levels in the successful group of isolates as compared to the isolates with unique fingerprints. The oxygen deprived environment provides a low redox potential, which is further lowered when exposed to A549 cells. This indicates that the successful isolates are better adapted to oxygen deprived environment.

The expression of *hspX* gene followed the same pattern as reported above for the expression of *fdxA* gene both before and after exposure to A549 cells. This observation further confirms that successful strains of *M. tuberculosis* are better equipped to deal with stressful circumstances.

All four investigated genes had higher expression levels in the Beijing and F15/LAM4/KZN isolates initially cultured under oxygen deprivation and thereafter exposed to the A549 cells. We propose that this suggests a role of these gene products in the virulence of these successful strains of *M. tuberculosis*.

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# Chapter 8.

**SUMMARY**

## 8.1 Adhesion of *M. tuberculosis* isolates to pulmonary epithelial cells

Fifteen clinical isolates and two laboratory strains were used in adhesion experiments to lung epithelial cells. All adhered to the alveolar epithelial (A549) cell-line much more effectively than to the bronchial epithelial (BBM) cell-line and this supports the observation by others that bronchial epithelium resists infection by *M. tuberculosis* (Schluger and Rom, 1998). This also suggests that *M. tuberculosis* possesses adhesins that interact with receptors present on the alveolar epithelial cell (Kohwiwattanagun *et al*. 2007). The Beijing and F15/LAM4/KZN isolates both showed a higher rate of adhesion to the A549 cells when compared to the isolates with unique DNA fingerprints and laboratory strains H37Rv and H37Ra. The H37Ra strain had the lowest rate of adhesion among the isolates used in this study, suggesting that these assays do reflect mycobacterial virulence.

The higher adhesion to the alveolar epithelial cells by the Beijing and F15/LAM4/KZN families of *M. tuberculosis* may contribute to their successful spread. Previous studies also reported that *M. tuberculosis* bacilli adhered to pulmonary epithelial cells (Bermudez and Goodman, 1996; Bermudez *et al.* 2002; Hall-Stoodley *et al*. 2006).

The use of the light microscopy may not be the optimal mechanism to quantify adhered bacteria, as it could not differentiate between bacilli that have invaded and those that adhered only. Invasion assays were then preformed to address this limitation.

## 8.2 Invasion of pulmonary epithelial cells by *M. tuberculosis* isolates

All the investigated isolates invaded the A549 cells much more effectively than the BBM cells. However, there was a difference in the invasion capacity of isolates grown under oxygen deprivation and the same isolates grown in the presence of oxygen. *M. tuberculosis* bacilli grown under oxygen deprivation were more invasive than the same isolates grown in the presence of oxygen. This is similar to the report by Li *et al.* (2002), on macrophage invasion by anaerobically grown H37Rv and Erdman strains *in vitro*. Others have also reported on the ability of *M. tuberculosis* bacilli to invade pulmonary epithelial cells (Bermudez and Goodman, 1996; Bermudez *et al.* 2002; Hall-Stoodley *et al*. 2006).

The Beijing and F15/LAM4/KZN isolates both invaded the A549 cells more than the isolates with unique fingerprints. Differences in invasion capacities were also observed among the *M. tuberculosis* isolates grown under the same condition that belonged to the same family. A similar observation was also made by Palanisamy *et al*. (2009) who reported on differences in virulence of isolates belonging to the same family. Our result that members of the F15/LAM4/KZN family had the highest percentage of invasion supports the reported high virulence of this family *in vivo* by Gandhi *et al.* (2006).

All the invasion assay experiments in this study were performed under aerobic conditions. As postulated by others, the short duration of the experiment makes it unlikely that the bacilli grown under oxygen deprivation would have converted to the phenotype of bacilli grown in the presence of oxygen. In this study the Wayne approach (Wayne and Lin, 1982; Wayne and Hayes, 1996) was used to achieve anaerobiosis. Currently there is no gold standard to achieve this condition.

The higher capacity to invade alveolar epithelial cells by members of the Beijing and F15/LAM4/KZN families of *M. tuberculosis* indicate that these strains have an increased capacity to colonise the alveoli and this may contribute to their successful spread. This led to our decision to investigate the cytotoxic abilities of these isolates in A549 cells. As we observed a similar trend but low rates of adhesion and invasion in the BBM cells, we decided to proceed our investigations using A549 cells only.

### 8.3 Cytotoxicity of *M. tuberculosis* isolates in A549 cells

All clinical isolates of *M. tuberculosis* independent on their restriction fragment length polymorphism (RFLP) signature induced cytotoxicity in the A549 cells *in vitro*. This agrees with the report by McDonough and Kress (1995) that cytotoxicity is specific to virulent mycobacteria. The high level of cytotoxicity that was observed in the A549 cells induced by members of the Beijing and F5/LAM4/KZN families in comparison to the H37Rv strain attests to the virulence of these two families of strains. This confirms reports by others on virulence of these isolates (Hanekom *et al*. 2007; Theus *et al*. 2007; Palanisamy *et al*. 2009).

Isolates grown under oxygen deprivation had a more virulent phenotype, as they induced higher levels of cytotoxicity in comparison to the same isolates grown in the presence of oxygen. Isolates of the XDR variant of F15/LAM4/KZN had the highest level of cytotoxicity, suggesting that these isolates might cause increased tissue destruction. This might also explain the high mortality rates of patients in Tugela Ferry (Gandhi *et al*. 2006).

The positive correlation between adhesion and invasion confirms that invasion is a post-adhesion event (Hensen *et al*. 2000; El-Housseiny *et al*. 2010). The correlation between cytotoxicity and invasion suggests that cytotoxicity follows invasion.

The Cytotox 96 non-radioactive cytotoxicity assay kit (Promega) was used to establish necrotic cell death. The protocol recommended that the absorbance to be measured at a wavelength of 490 nm. However, the GloMax multi-detection system (Promega) that we used to measure the absorbance did not allow measurement at 490 nm but at 450 nm and 560 nm only. Hence, we used a wavelength of 450 nm to measure the absorbance.

To understand the damage to alveolar macrophages and alveolar epithelial cells following infection with *M. tuberculosis* (McDonough *et al*. 1993; Rojas *et al*. 1997; Dobos *et al*. 2000; Danelishvili *et al*. 2003) we decided to examine the morphological changes in A549 cells after exposure to *M. tuberculosis* bacilli using the scanning electron

microscopy (SEM). As it was not possible to process all the isolates cultured under the two growth conditions for SEM, a decision was reached in agreement with the statistician to select isolates based on previous experimental results. The experimental results obtained from the adhesion, invasion and cytotoxicity assays were stratified into three strata (high, middle and low), from the highest to the lowest values (Fig. 8.1). Two isolates were then randomly selected from each stratum. Six isolates were selected and used for the SEM investigation.

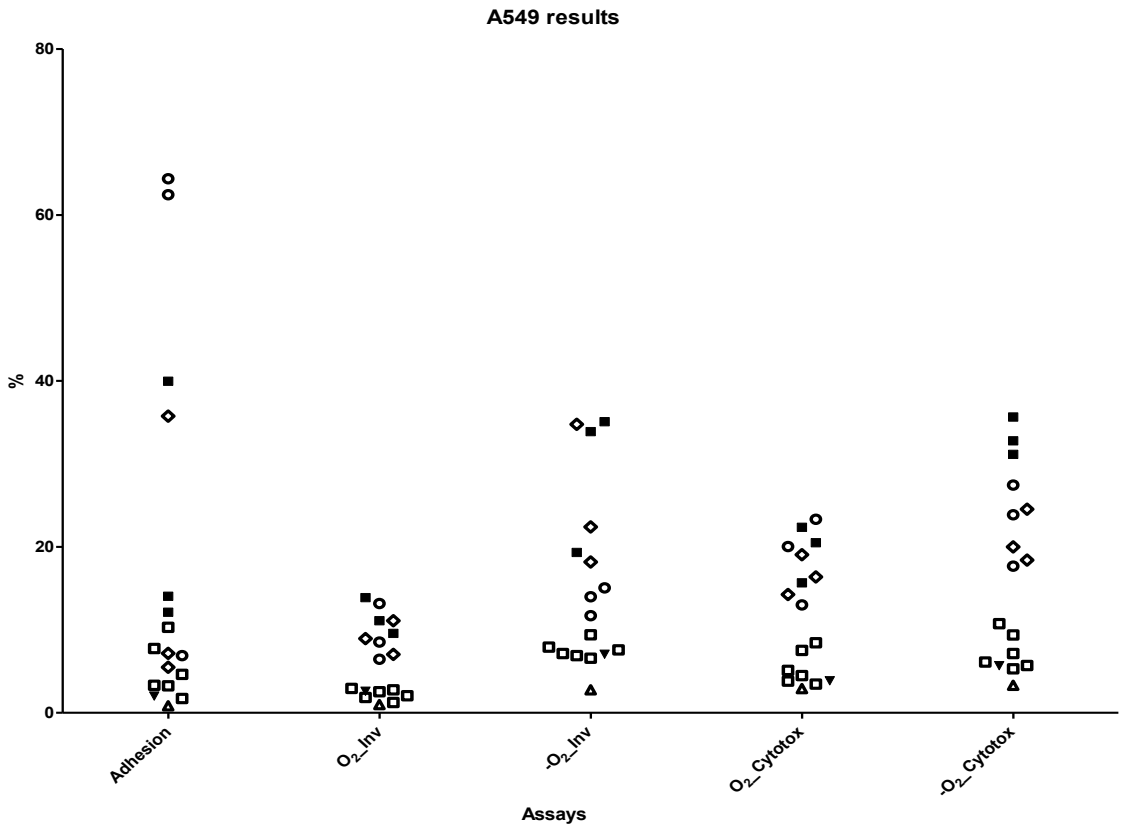


**Figure 8.1. Shows all the previously obtained results from the adhesion, invasion and cytotoxicity assays. O = Beijing isolates; ■ = XDR-F15/LAM4/KZN isolates; ◇ = F15/LAM4/KZN isolates; □ = Isolates with unique DNA fingerprint pattern; ▼ = H37Rv; △ = H37Ra**

## 8.4 Induced morphological changes in A549 cells by *M. tuberculosis* isolates

All six isolates that were semi-randomly selected induced morphological changes in the infected A549 cells but no morphological change was observed in non-infected A549 cells. Projections with a diameter between 0.09μm and 0.14μm were visible on the infected A549 cells. The different isolates differed in their responses, with the exception of the Beijing and XDR-F15/LAM4/KZN isolates that induced a similar response.

Both the Beijing and XDR-F15/LAM4/KZN isolates induced the highest ratio of projections to bacilli under the two growth conditions. There were differences in response induced by the isolates based on their growth conditions. Isolates grown under oxygen deprivation induced higher ratio of projections to bacilli. This suggests that the phenotype of the bacillus grown under oxygen deprivation is more virulent (Ashiru, *et al*. 2012).

Bleb-like structures were visible on the surface of the bacilli of all isolates that were cultured under oxygen deprivation. This might have been due to the stress the bacilli encountered under this growth condition. Dahl (2005) also reported the presence of bleb-like structures on *M. tuberculosis* bacilli in aged cultures. Borelli *et al* (2003) observed that surface blebs formed on *M. tuberculosis* bacilli after exposure to eosinophil

peroxidase.

A strong positive correlation was found between the morphological changes induced by these isolates in A549 cells and results on adhesion, invasion and cytotoxicity. One limitation was the fact that we were not able to include all organisms tested in these assays for SEM. We then proceeded to investigate expression of genes potentially associated with virulence in all fifteen clinical isolates and the two laboratory strains that were cultured in the presence of oxygen and under oxygen deprivation, before and after exposure to A549 cells.

## 8.5 Expression of genes potentially associated with virulence in *M. tuberculosis* isolates

There were variations in the expression of genes potentially associated with virulence among all fifteen clinical isolates and laboratory strains before and after exposure to A549 cells. Gao *et al* (2005) reported similar observations on gene expression diversity among *M. tuberculosis* isolates grown under identical condition. There was also variation in the expression of these genes based on the growth environment of the isolates.

It was observed that the expression levels of the genes potentially associated with

virulence were upregulated after exposure to A549 cells. This suggests that the protein product that these genes code for might play a role in the interaction between the *M. tuberculosis* bacilli and the alveolar epithelial cells. Delogu *et al* (2006) reported a similar observation that mRNA levels of *hbhA* gene transcripts were upregulated in the lung of mice infected with *M. tuberculosis*.

Higher levels of these genes potentially associated with virulence were expressed in the Beijing and F15/LAM4/KZN isolates that were exposure to A549 cells after initial growth under oxygen deprivation. This suggests that growth of the isolates in an oxygen deprived environment might instigate expression of virulence attributes. This observation was also evident in previous investigations on these isolates (Ashiru *et al*. 2012).

We did not knockout these genes neither did we re-complement the genes, which would have given better information on their roles.

Publications on similar observations by other authors was only available for the Beijing strain isolates (Hanekom *et al*. 2007; Theus *et al*. 2007; Palanisamy *et al*. 2009), which allowed us to compare some of our results but this was not possible for the F15/LAM4/KZN isolates.

Since only *in vitro* experiments were performed, *in vivo* investigations in laboratory animals using these same isolates would be an appropriate next step. We conclude this investigation with the suggestion that the Beijing and F15/LAM4/KZN isolates' ability to colonize the lung is a quantitative virulent attribute that increases pathogenicity. This may explain the more aggressive spread of these families of strains.

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## APPENDIX A – REAGENTS AND MEDIA

A549 cell freezing fluid

42.75ml EMEM with L-glutamine and HEPES

4.75ml FBS

2.5ml dimethyl sulphoxide (DMSO) (Fluka, Steinheim, Germany)

4.75ml of FBS and 2.5ml of DMSO were added to 42.75ml EMEM and the solution filter-sterilized through a 0.22µm filter into a sterile container.

BBM cell freezing fluid

40.75ml Leibovitz's L-15 medium with L-glutamine and HEPES

5ml FBS

0.5ml polyvinylprrolidone (PVP)

3.75ml DMSO (Fluka)

Five millilitres of FBS, 0.5ml of PVP and 3.75ml of DMSO were added to 40.75ml of Leibovitz's L-15 and the solution filter-sterilized through a 0.22µm filter into a sterile container.

Diethylpyrocarbonate (DEPC) treated water

1ml 0.1% DEPC

Distilled water

Add 1ml of 0.1% DEPC to 1L of distilled water. Mix thoroughly and leave at room temperature for 1h. Autoclave at 121$^{o}$C for 15 minutes and allow to cool before use.

Glycerol, 50% (w/v)

50g glycerol

100ml distilled water

Fifty grams of glycerol was dissolved in approximately 80ml of autoclaved distilled water and the volume was brought up to 100ml. The solution was sterilized by filtration through a 0.22μm membrane into a sterile container.

GTC lysing solution

60g / 100ml GTC

0.5g / 100ml sodium-*N*-lauryl sarcosine

1g / 100ml tri-sodium citrate

0.7ml / 100ml β-mercaptoethanol (BME)

Fifty grams of GTC, 0.5g of sodium-*N*-lauryl sarcosine and 1g of tri-sodium citrate were dissolved in approximately 80ml of autoclaved distilled water and the volume brought up to 99.3ml. 0.7ml of BME should be added just before using the GTC solution.

Middlebrook 7H9 broth

4.7g Middlebrook 7H9 powder (Difco)

100ml OADC (BD)

10ml 50% (w/v) glycerol

2.5ml 20% Tween 80

4.7g of Middlebrook 7H9 powder was dissolved in approximately 800ml of autoclaved distilled water. 10ml of 50% (w/v) glycerol, 2.5ml of 20% Tween 80 and 100ml of OADC were added. The volume was brought up to 1000ml and the broth was sterilized by filtration through a 0.22µm membrane.

Middlebrook 7Hll solid agar

21g Middlebrook 7H11 powder (Difco)

100ml OADC (BD)

10ml 50% (w/v) glycerol

Twenty-one grams of Middlebrook 7H11 powder was dissolved in 900ml of triple distilled water and autoclaved at 121$^{o}$C for 15 minutes. The solution was placed in pre-heated waterbath set to 50$^{o}$C to cool with gentle swirling for approximately 30 minutes. 100ml of OADC and 10ml of 50% (w/v) of glycerol were added and decanted into sterile petri dishes.

10x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer

41.9g MOPS (200 mM)

4.1g Sodium acetate (80mM)

3.7g Ethylenediaminetetraacetic acid (EDTA)

DEPC treated water

800ml of DEPC treated water is added to 41.9g of MOPS, 4.1g of sodium acetate and 3.7g of EDTA to dissolve. Volume was made up to 1L and pH adjusted to 7.0.

MOPS 1% agarose gel

0.5g agarose

5ml 10x MOPS buffer

37.5ml DEPC treated water

3.5ml Formaldehyde (37%)

Add 5ml of 10x MOPS and 37.5ml of DEPC treated water to 0.5g of agarose and swirl to mix. Heat in microwave oven to dissolve (do not allow to boil) and cool to hand-warm. Then add 3.5ml of formaldehyde (37%) in a fume hood.

Phosphate buffered saline (PBS)

10PBS tablets (Oxoid)

1000ml distilled water

Ten PBS tablets were dissolved in 1000ml autoclaved distilled water. The PBS was autoclaved at 121$^{o}$C for 15 minutes, thereafter decanted into 20ml aliquots and refrigerated at 4$^{o}$C until use.

Tween 80, 20%

20ml Tween 80 (Fisher)

80ml distilled water

20ml of Tween 80 (Fisher) was added to 80ml of autoclaved distilled water. The solution was placed in a pre-heated waterbath set to 56$^{o}$C and then sterilized by filtration through a 0.22µm membrane.

## APPENDIX B – RAW DATA

## Adhesion assay

B1. Adhesion to A594 cells by *M. tuberculosis* grown in the presence of $O_2$

| | Rate of Adhesion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 Well1 | Run1 Well2 | Run1 Well3 | Run1 Mean | Run2 Well1 | Run2 Well2 | Run2 Well3 | Run2 Mean | Run3 Well1 | Run3 Well2 | Run3 Well3 | Run3 Mean | All3 Mean | Run1 SD | Run1 %CV | Run2 SD | Run2 %CV | Run3 SD | Run3 %CV |
| B1 | 60.94 | 62.51 | 62.58 | 62.01 | 63.02 | 61.84 | 61.17 | 62.01 | 62.65 | 64.01 | 63.21 | 63.29 | 62.44 | 0.93 | 1.49 | 0.93 | 1.50 | 0.48 | 0.76 |
| 2 | 63.96 | 64.79 | 64.63 | 64.46 | 64.77 | 64.57 | 63.62 | 64.32 | 64.49 | 63.98 | 64.49 | 64.32 | 64.37 | 0.44 | 0.68 | 0.62 | 0.96 | 0.21 | 0.32 |
| 3 | 6.85 | 6.98 | 6.86 | 6.90 | 6.97 | 6.75 | 6.82 | 6.85 | 6.96 | 6.90 | 6.94 | 6.93 | 6.89 | 0.07 | 1.03 | 0.11 | 1.61 | 0.02 | 0.30 |
| X1 | 12.01 | 12.21 | 12.13 | 12.12 | 12.20 | 12.16 | 12.12 | 12.16 | 12.12 | 12.19 | 12.13 | 12.15 | 12.14 | 0.10 | 0.81 | 0.04 | 0.32 | 0.03 | 0.21 |
| 2 | 14.04 | 14.21 | 14.06 | 14.10 | 14.04 | 14.03 | 14.03 | 14.03 | 14.05 | 14.07 | 14.05 | 14.06 | 14.06 | 0.09 | 0.67 | 0.01 | 0.04 | 0.01 | 0.07 |
| 3 | 39.90 | 40.00 | 40.06 | 39.99 | 39.99 | 39.98 | 39.93 | 39.97 | 39.88 | 40.10 | 39.89 | 39.95 | 39.97 | 0.08 | 0.21 | 0.03 | 0.07 | 0.10 | 0.24 |
| K1 | 5.39 | 5.39 | 5.49 | 5.43 | 5.59 | 5.73 | 5.40 | 5.57 | 5.39 | 5.50 | 5.57 | 5.48 | 5.49 | 0.06 | 1.09 | 0.17 | 2.99 | 0.06 | 1.18 |
| 2 | 7.20 | 7.21 | 7.14 | 7.18 | 7.19 | 7.20 | 7.16 | 7.18 | 7.20 | 7.14 | 7.13 | 7.16 | 7.17 | 0.04 | 0.50 | 0.02 | 0.24 | 0.03 | 0.39 |
| 3 | 35.70 | 35.80 | 35.84 | 35.78 | 35.92 | 35.71 | 35.70 | 35.78 | 35.90 | 35.64 | 35.66 | 35.73 | 35.76 | 0.07 | 0.21 | 0.13 | 0.35 | 0.10 | 0.28 |
| U1 | 4.71 | 4.60 | 4.61 | 4.64 | 4.62 | 4.60 | 4.62 | 4.62 | 4.64 | 4.60 | 4.72 | 4.65 | 4.64 | 0.06 | 1.35 | 0.01 | 0.24 | 0.04 | 0.90 |
| 2 | 1.60 | 1.63 | 1.67 | 1.63 | 1.76 | 1.72 | 1.84 | 1.77 | 1.75 | 1.75 | 1.76 | 1.75 | 1.72 | 0.04 | 2.24 | 0.06 | 3.28 | <0.00 | 0.26 |
| 3 | 10.00 | 9.98 | 10.03 | 10.00 | 10.20 | 10.16 | 10.30 | 10.22 | 10.72 | 10.60 | 10.76 | 10.69 | 10.31 | 0.02 | 0.25 | 0.07 | 0.73 | 0.06 | 0.57 |
| 4 | 3.30 | 3.29 | 3.45 | 3.35 | 3.30 | 3.32 | 3.25 | 3.29 | 3.31 | 3.30 | 3.33 | 3.31 | 3.32 | 0.09 | 2.79 | 0.03 | 1.05 | 0.01 | 0.36 |
| 5 | 3.28 | 3.21 | 3.31 | 3.27 | 3.21 | 3.29 | 3.24 | 3.25 | 3.19 | 3.21 | 3.30 | 3.24 | 3.25 | 0.05 | 1.58 | 0.04 | 1.24 | 0.04 | 1.25 |
| 6 | 7.86 | 7.70 | 7.83 | 7.80 | 7.80 | 7.70 | 7.72 | 7.74 | 7.70 | 7.72 | 7.72 | 7.71 | 7.75 | 0.09 | 1.12 | 0.05 | 0.69 | 0.01 | 0.10 |
| H37Rv | 2.10 | 2.10 | 2.02 | 2.07 | 2.02 | 2.05 | 2.05 | 2.04 | 2.09 | 2.06 | 2.06 | 2.07 | 2.06 | 0.05 | 2.31 | 0.02 | 0.79 | 0.01 | 0.51 |
| H37Ra | 0.81 | 0.85 | 0.84 | 0.83 | 0.87 | 0.88 | 0.88 | 0.88 | 0.82 | 0.82 | 0.83 | 0.82 | 0.84 | 0.02 | 2.29 | 0.01 | 0.73 | <0.00 | 0.43 |

B2. Adhesion to BBM cells by *M. tuberculosis* grown in the presence of $O_2$

| | Rate of adhesion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 Well1 | Run1 Well2 | Run1 Well3 | Run1 Mean | Run2 Well1 | Run2 Well2 | Run2 Well3 | Run2 Mean | Run3 Well1 | Run3 Well2 | Run3 Well3 | Run3 Mean | All3 Mean | Run1 SD | Run1 %CV | Run2 SD | Run2 %CV | Run3 SD | Run3 %CV |
| B1 | 6.99 | 7.10 | 7.03 | 7.04 | 7.09 | 7.07 | 7.09 | 7.08 | 7.19 | 7.20 | 7.21 | 7.20 | 7.11 | 0.06 | 0.78 | 0.01 | 0.16 | 0.01 | 0.17 |
| 2 | 21.90 | 22.00 | 22.05 | 21.98 | 21.88 | 21.69 | 21.73 | 21.77 | 21.90 | 22.01 | 22.04 | 21.98 | 21.91 | 0.08 | 0.36 | 0.10 | 0.46 | 0.08 | 0.35 |
| 3 | 1.90 | 1.98 | 1.91 | 1.93 | 1.90 | 1.95 | 1.95 | 1.93 | 1.95 | 1.94 | 1.91 | 1.94 | 1.93 | 0.04 | 2.21 | 0.03 | 1.55 | 0.02 | 1.06 |
| X1 | 2.06 | 2.05 | 2.05 | 2.06 | 2.02 | 2.02 | 2.03 | 2.02 | 2.10 | 2.11 | 2.10 | 2.10 | 2.06 | 0.01 | 0.40 | 0.01 | 0.32 | 0.01 | 0.29 |
| 2 | 3.80 | 3.90 | 3.98 | 3.89 | 3.60 | 3.66 | 3.76 | 3.67 | 3.69 | 3.70 | 3.84 | 3.74 | 3.77 | 0.09 | 2.34 | 0.08 | 2.31 | 0.08 | 2.24 |
| 3 | 13.14 | 13.13 | 13.22 | 13.17 | 13.11 | 13.11 | 13.13 | 13.12 | 13.16 | 13.18 | 13.20 | 13.18 | 13.15 | 0.05 | 0.38 | 0.01 | 0.10 | 0.02 | 0.14 |
| K1 | 1.21 | 1.25 | 1.26 | 1.24 | 1.22 | 1.25 | 1.23 | 1.23 | 1.24 | 1.30 | 1.20 | 1.24 | 1.24 | 0.02 | 2.00 | 0.02 | 1.50 | 0.05 | 4.00 |
| 2 | 2.32 | 2.30 | 2.43 | 2.35 | 2.35 | 2.36 | 2.38 | 2.36 | 2.32 | 2.38 | 2.36 | 2.36 | 2.35 | 0.07 | 3.13 | 0.02 | 0.72 | 0.03 | 1.38 |
| 3 | 3.27 | 3.28 | 3.31 | 3.29 | 3.19 | 3.20 | 3.20 | 3.20 | 2.99 | 3.15 | 3.13 | 3.09 | 3.19 | 0.02 | 0.65 | 0.01 | 0.20 | 0.09 | 2.95 |
| U1 | 0.71 | 0.72 | 0.74 | 0.72 | 0.73 | 0.74 | 0.75 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.01 | 1.44 | 0.01 | 1.74 | <0.01 | 0.28 |
| 2 | 0.68 | 0.69 | 0.68 | 0.68 | 0.61 | 0.61 | 0.61 | 0.61 | 0.59 | 0.60 | 0.59 | 0.59 | 0.63 | <0.01 | 0.65 | <0.01 | 0.20 | <0.01 | 0.82 |
| 3 | 1.72 | 1.56 | 1.59 | 1.63 | 1.62 | 1.60 | 1.64 | 1.62 | 1.63 | 1.71 | 1.58 | 1.64 | 1.63 | 0.08 | 5.21 | 0.02 | 1.45 | 0.06 | 3.93 |
| 4 | 0.71 | 0.72 | 0.74 | 0.72 | 0.72 | 0.73 | 0.74 | 0.73 | 0.71 | 0.71 | 0.71 | 0.71 | 0.72 | 0.01 | 1.69 | 0.01 | 1.09 | <0.01 | 0.37 |
| 5 | 0.69 | 0.67 | 0.67 | 0.68 | 0.71 | 0.70 | 0.70 | 0.70 | 0.76 | 0.75 | 0.76 | 0.76 | 0.71 | 0.01 | 1.30 | 0.01 | 1.01 | 0.01 | 0.99 |
| 6 | 1.17 | 1.18 | 1.17 | 1.17 | 1.20 | 1.23 | 1.24 | 1.22 | 1.15 | 1.15 | 1.15 | 1.15 | 1.18 | 0.01 | 0.74 | 0.02 | 1.88 | <0.01 | 0.20 |
| H37Rv | 0.65 | 0.68 | 0.69 | 0.67 | 0.64 | 0.64 | 0.67 | 0.65 | 0.66 | 0.66 | 0.69 | 0.67 | 0.66 | 0.02 | 2.49 | 0.02 | 2.35 | 0.02 | 2.58 |
| H37Ra | 0.24 | 0.24 | 0.24 | 0.24 | 0.25 | 0.25 | 0.29 | 0.26 | 0.28 | 0.28 | 0.29 | 0.29 | 0.26 | <0.01 | 1.11 | 0.02 | 8.97 | 0.01 | 2.56 |

**Invasion assay**

B3. Invasion of A594 cells by *M. tuberculosis* grown in the presence of $O_2$

| | Rate of Invasion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 Well1 | Run1 Well2 | Run1 Well3 | Run1 Mean | Run2 Well1 | Run2 Well2 | Run2 Well3 | Run2 Mean | Run3 Well1 | Run3 Well2 | Run3 Well3 | Run3 Mean | All3 Mean | Run1 SD | Run1 %CV | Run2 SD | Run2 %CV | Run3 SD | Run3 %CV |
| B1 | 7.50 | 7.86 | 7.64 | 7.67 | 9.30 | 9.43 | 9.61 | 9.44 | 8.40 | 8.49 | 8.44 | 8.44 | 8.52 | 0.18 | 2.41 | 0.16 | 1.64 | 0.05 | 0.56 |
| 2 | 12.30 | 12.49 | 12.34 | 12.38 | 13.30 | 13.61 | 13.21 | 13.38 | 13.70 | 13.83 | 13.73 | 13.75 | 13.17 | 0.10 | 0.82 | 0.21 | 1.57 | 0.07 | 0.49 |
| 3 | 5.95 | 6.12 | 6.12 | 6.06 | 6.21 | 5.97 | 6.00 | 6.06 | 7.14 | 7.30 | 7.38 | 7.27 | 6.46 | 0.10 | 1.64 | 0.13 | 2.22 | 0.12 | 1.71 |
| X1 | 9.85 | 10.11 | 10.03 | 10.00 | 8.43 | 8.69 | 9.13 | 8.75 | 9.90 | 10.21 | 9.89 | 10.00 | 9.58 | 0.13 | 1.34 | 0.36 | 4.06 | 0.19 | 1.85 |
| 2 | 11.90 | 11.94 | 12.16 | 12.00 | 11.29 | 11.37 | 11.35 | 11.33 | 9.78 | 9.97 | 10.25 | 10.00 | 11.11 | 0.14 | 1.17 | 0.04 | 0.36 | 0.23 | 2.34 |
| 3 | 13.62 | 13.50 | 13.38 | 13.50 | 14.75 | 14.60 | 14.65 | 14.67 | 13.47 | 13.45 | 13.58 | 13.50 | 13.89 | 0.12 | 0.90 | 0.08 | 0.55 | 0.07 | 0.51 |
| K1 | 7.90 | 7.86 | 8.05 | 7.94 | 6.76 | 6.75 | 6.96 | 6.83 | 6.38 | 6.32 | 6.34 | 6.35 | 7.04 | 0.10 | 1.31 | 0.12 | 1.77 | 0.03 | 0.47 |
| 2 | 8.98 | 9.13 | 9.16 | 9.09 | 8.33 | 8.25 | 8.65 | 8.41 | 9.25 | 9.30 | 9.41 | 9.32 | 8.94 | 0.10 | 1.10 | 0.22 | 2.57 | 0.08 | 0.90 |
| 3 | 11.70 | 11.97 | 12.05 | 11.90 | 11.14 | 11.20 | 11.23 | 11.19 | 10.17 | 10.25 | 10.30 | 10.24 | 11.11 | 0.18 | 1.54 | 0.04 | 0.40 | 0.07 | 0.65 |
| U1 | 2.32 | 2.35 | 2.39 | 2.35 | 2.36 | 2.39 | 2.62 | 2.46 | 2.70 | 2.97 | 2.84 | 2.84 | 2.55 | 0.04 | 1.55 | 0.14 | 5.80 | 0.14 | 4.77 |
| 2 | 1.38 | 1.42 | 1.48 | 1.43 | 1.05 | 1.13 | 1.03 | 1.07 | 1.15 | 1.23 | 1.37 | 1.25 | 1.25 | 0.05 | 3.48 | 0.05 | 5.10 | 0.11 | 9.14 |
| 3 | 3,20 | 3.28 | 3.39 | 3.29 | 2.66 | 2.83 | 2.89 | 2.79 | 2.72 | 2.69 | 2.81 | 2.74 | 2.94 | 0.10 | 2.93 | 0.12 | 4.36 | 0.06 | 2.16 |
| 4 | 2.49 | 2.48 | 2.53 | 2.50 | 2.99 | 2.96 | 3.15 | 3.03 | 2.79 | 2.83 | 2.78 | 2.80 | 2.78 | 0.03 | 1.03 | 0.10 | 3.34 | 0.03 | 1.01 |
| 5 | 1.57 | 1.68 | 1.75 | 1.67 | 1.99 | 1.98 | 2.10 | 2.02 | 1.78 | 1.86 | 1.96 | 1.87 | 1.85 | 0.09 | 5.36 | 0.06 | 3.19 | 0.09 | 4.60 |
| 6 | 2.19 | 2.31 | 2.44 | 2.31 | 1.99 | 1.90 | 2.28 | 2.06 | 1.70 | 1.86 | 1.84 | 1.80 | 2.06 | 0.13 | 5.47 | 0.20 | 9.90 | 0.09 | 4.85 |
| Rv | 2.38 | 2.49 | 2.42 | 2.43 | 2.71 | 2.68 | 2.61 | 2.67 | 2.69 | 2.73 | 2.78 | 2.73 | 2.61 | 0.06 | 2.29 | 0.05 | 1.87 | 0.05 | 1.69 |
| Ra | 0.95 | 0.99 | 1.07 | 1.00 | 0.96 | 0.97 | 0.99 | 0.98 | 0.97 | 1.03 | 1.00 | 1.00 | 0.99 | 0.06 | 6.14 | 0.02 | 1.81 | 0.03 | 2.76 |

B4. Invasion of BBM cells by *M. tuberculosis* grown in the presence of $O_2$

| | Rate of Invasion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 | Run1 | Run1 | Run1 | Run2 | Run2 | Run2 | Run2 | Run3 | Run3 | Run3 | Run3 | All3 | Run1 | Run1 | Run2 | Run2 | Run3 | Run3 |
| | Well1 | Well2 | Well3 | Mean | Well1 | Well2 | Well3 | Mean | Well1 | Well2 | Well3 | Mean | Mean | SD | %CV | SD | %CV | SD | %CV |
| B1 | 2.35 | 2.43 | 2.34 | 2,38 | 2.33 | 2.36 | 2.43 | 2.38 | 2.35 | 2.36 | 2.41 | 2.38 | 2.38 | 0.05 | 2.10 | 0.05 | 2.27 | 0.03 | 1.15 |
| 2 | 2.65 | 2.77 | 2.91 | 2.78 | 3.29 | 3.32 | 3.39 | 3.33 | 2.77 | 2.69 | 2.87 | 2.78 | 2.96 | 0.13 | 4.58 | 0.05 | 1.55 | 0.09 | 3.19 |
| 3 | 2.14 | 2.20 | 2.15 | 2.16 | 2.47 | 2.60 | 2.64 | 2.57 | 2.18 | 2.13 | 2.17 | 2.16 | 2.30 | 0.03 | 1.50 | 0.09 | 3.47 | 0.03 | 1.23 |
| X1 | 3.65 | 3.69 | 3.86 | 3.73 | 3.98 | 3.96 | 4.05 | 4.00 | 4.24 | 4.22 | 4.34 | 4.27 | 4.00 | 0.11 | 2.98 | 0.05 | 1.17 | 0.07 | 1.53 |
| 2 | 3.99 | 4.18 | 4.19 | 4.12 | 3.87 | 4.23 | 4.19 | 4.10 | 4.13 | 3.99 | 4.12 | 4.08 | 4.10 | 0.11 | 2.77 | 0.20 | 4.88 | 0.08 | 1.96 |
| 3 | 4.13 | 4.28 | 4.34 | 4.25 | 3.96 | 4.03 | 4.01 | 4.00 | 4.17 | 4.26 | 4.32 | 4.25 | 4.17 | 0.11 | 2.54 | 0.04 | 0.91 | 0.08 | 1.82 |
| K1 | 1.99 | 1.99 | 2.04 | 2.00 | 2.13 | 2.13 | 2.10 | 2.12 | 2.10 | 1.99 | 2.10 | 2.06 | 2.06 | 0.03 | 1.63 | 0.02 | 0.81 | 0.07 | 3.23 |
| 2 | 3.30 | 3.53 | 3.52 | 3.45 | 3.50 | 3.57 | 3.46 | 3.51 | 3.42 | 3.25 | 3.31 | 3.33 | 3.43 | 0.13 | 3.77 | 0.05 | 1.56 | 0.09 | 2.71 |
| 3 | 3.57 | 3.65 | 3.77 | 3.66 | 3.26 | 3.34 | 3.54 | 3.38 | 3.37 | 3.35 | 3.51 | 3.41 | 3.48 | 0.10 | 2.84 | 0.14 | 4.17 | 0.09 | 2.69 |
| U1 | 0.73 | 0.74 | 0.77 | 0.75 | 0.73 | 0.75 | 0.79 | 0.76 | 0.71 | 0.72 | 0.73 | 0.72 | 0.74 | 0.02 | 3.26 | 0.03 | 3.79 | 0.01 | 1.29 |
| 2 | 0.53 | 0.54 | 0.54 | 0.54 | 0.64 | 0.62 | 0.64 | 0.64 | 0.61 | 0.63 | 0.60 | 0.61 | 0.60 | 0.01 | 1.29 | 0.01 | 1.92 | 0.01 | 1.86 |
| 3 | 0.99 | 1.21 | 0.81 | 1.00 | 1.03 | 1.04 | 1.24 | 1.10 | 1.18 | 1.19 | 1.22 | 1.19 | 1.10 | 0.20 | 20.25 | 0.12 | 11.01 | 0.02 | 1.63 |
| 4 | 0.72 | 0.73 | 0.74 | 0.73 | 0.63 | 0.64 | 0.65 | 0.64 | 0.74 | 0.76 | 0.80 | 0.77 | 0.71 | 0.01 | 1.40 | 0.01 | 1.80 | 0.03 | 3.78 |
| 5 | 0.61 | 0.62 | 0.60 | 0.61 | 0.65 | 0.68 | 0.68 | 0.67 | 0.51 | 0.51 | 0.52 | 0.51 | 0.60 | 0.01 | 1.67 | 0.01 | 2.16 | <0.01 | 0.38 |
| 6 | 0.74 | 0.82 | 0.78 | 0.78 | 0.62 | 0.70 | 0.74 | 0.69 | 0.87 | 0.89 | 0.89 | 0.88 | 0.78 | 0.04 | 4.94 | 0.06 | 8.56 | 0.01 | 1.54 |
| Rv | 1.13 | 1.28 | 1.42 | 1.28 | 1.24 | 1.24 | 1.28 | 1.25 | 1.13 | 1.15 | 1.17 | 1.15 | 1.23 | 0.14 | 11.22 | 0.02 | 1.75 | 0.02 | 1.55 |
| Ra | 0.62 | 0.64 | 0.69 | 0.65 | 0.65 | 0.62 | 0.67 | 0.65 | 0.65 | 0.64 | 0.65 | 0.65 | 0.65 | 0.03 | 5.19 | 0.02 | 3.69 | 0.01 | 0.97 |

B5. Invasion of A594 cells by *M. tuberculosis* grown under $O_2$ deprivation

| | Rate of Invasion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 Well1 | Run1 Well2 | Run1 Well3 | Run1 Mean | Run2 Well1 | Run2 Well2 | Run2 Well3 | Run2 Mean | Run3 Well1 | Run3 Well2 | Run3 Well3 | Run3 Mean | All3 Mean | Run1 SD | Run1 %CV | Run2 SD | Run2 %CV | Run3 SD | Run3 %CV |
| B1 | 13.87 | 13.91 | 13.92 | 13.90 | 13.8 | 13.78 | 13.82 | 13.8 | 14.28 | 14.3 | 14.32 | 14.3 | 14.00 | 0.03 | 0.19 | 0.02 | 0.14 | 0.02 | 0.14 |
| 2 | 15.29 | 15.38 | 15.41 | 15.36 | 14.89 | 14.97 | 14.96 | 14.94 | 14.81 | 14.89 | 14.85 | 14.85 | 15.05 | 0.06 | 0.41 | 0.04 | 0.29 | 0.04 | 0.27 |
| 3 | 11.78 | 11.77 | 11.73 | 11.76 | 11.82 | 11.77 | 11.84 | 11.81 | 11.53 | 11.58 | 11.57 | 11.56 | 11.71 | 0.03 | 0.22 | 0.04 | 0.31 | 0.03 | 0.23 |
| X1 | 19.1 | 18.92 | 18.98 | 19.00 | 19.26 | 19.35 | 19.29 | 19.3 | 19.71 | 19.66 | 19.73 | 19.7 | 19.33 | 0.09 | 0.48 | 0.05 | 0.24 | 0.04 | 0.18 |
| 2 | 33.37 | 33.43 | 33.46 | 33.42 | 33.87 | 33.92 | 33.91 | 33.9 | 34.32 | 34.38 | 34.41 | 34.37 | 33.90 | 0.05 | 0.14 | 0.03 | 0.08 | 0.05 | 0.13 |
| 3 | 35.15 | 35.04 | 35.08 | 35.09 | 34.88 | 34.84 | 34.86 | 34.86 | 35.14 | 35.05 | 35.08 | 35.09 | 35.01 | 0.06 | 0.16 | 0.02 | 0.06 | 0.05 | 0.13 |
| K1 | 19.03 | 18.99 | 18.92 | 18.98 | 17.81 | 17.91 | 17.92 | 17.88 | 17.71 | 17.61 | 17.72 | 17.68 | 18.18 | 0.06 | 0.29 | 0.06 | 0.34 | 0.06 | 0.34 |
| 2 | 22.35 | 22.33 | 22.25 | 22.31 | 23.03 | 22.96 | 23.04 | 23.01 | 21.95 | 21.84 | 21.91 | 21.9 | 22.41 | 0.05 | 0.24 | 0.04 | 0.19 | 0.06 | 0.25 |
| 3 | 35.21 | 35.29 | 35.28 | 35.26 | 34.89 | 34.95 | 34.98 | 34.94 | 34.08 | 34.12 | 34.13 | 34.11 | 34.77 | 0.04 | 0.12 | 0.05 | 0.13 | 0.03 | 0.08 |
| U1 | 7.59 | 7.54 | 7.58 | 7.57 | 7.59 | 7.64 | 7.66 | 7.63 | 7.56 | 7.49 | 7.54 | 7.53 | 7.58 | 0.03 | 0.35 | 0.04 | 0.47 | 0.04 | 0.48 |
| 2 | 6.17 | 6.23 | 6.26 | 6.22 | 6.53 | 6.59 | 6.62 | 6.58 | 6.89 | 6.94 | 6.96 | 6.93 | 6.58 | 0.05 | 0.74 | 0.05 | 0.70 | 0.04 | 0.52 |
| 3 | 9.46 | 9.52 | 9.55 | 9.51 | 9.64 | 9.61 | 9.67 | 9.64 | 8.96 | 9.07 | 9.09 | 9.04 | 9.40 | 0.05 | 0.48 | 0.03 | 0.31 | 0.07 | 0.77 |
| 4 | 7.99 | 7.91 | 7.96 | 7.95 | 8.02 | 8.08 | 8.11 | 8.07 | 7.74 | 7.69 | 7.76 | 7.73 | 7.92 | 0.04 | 0.51 | 0.05 | 0.57 | 0.04 | 0.47 |
| 5 | 6.82 | 6.93 | 6.95 | 6.90 | 6.55 | 6.64 | 6.67 | 6.62 | 7.18 | 7.12 | 7.21 | 7.17 | 6.90 | 0.07 | 1.01 | 0.06 | 0.94 | 0.05 | 0.64 |
| 6 | 6.81 | 6.92 | 6.94 | 6.89 | 7.35 | 7.26 | 7.32 | 7.31 | 7.26 | 7.21 | 7.25 | 7.24 | 7.15 | 0.07 | 1.02 | 0.05 | 0.63 | 0.03 | 0.37 |
| Rv | 6.91 | 6.84 | 6.89 | 6.88 | 7.11 | 7.15 | 7.16 | 7.14 | 7.19 | 7.12 | 7.17 | 7.16 | 7.06 | 0.04 | 0.52 | 0.03 | 0.37 | 0.04 | 0.50 |
| Ra | 3.03 | 3.01 | 3.05 | 3.03 | 2.71 | 2.67 | 2.72 | 2.7 | 2.55 | 2.62 | 2.63 | 2.6 | 2.78 | 0.02 | 0.66 | 0.03 | 0.98 | 0.04 | 1.68 |

B6. Invasion of BBM cells by *M. tuberculosis* grown under $O_2$ deprivation

| | Rate of Invasion (%) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iso | Run1 Well1 | Run1 Well2 | Run1 Well3 | Run1 Mean | Run2 Well1 | Run2 Well2 | Run2 Well3 | Run2 Mean | Run3 Well1 | Run3 Well2 | Run3 Well3 | Run3 Mean | All3 Mean | Run1 SD | Run1 %CV | Run2 SD | Run2 %CV | Run3 SD | Run3 %CV |
| B1 | 3.59 | 3.51 | 3.56 | 3.55 | 3.54 | 3.51 | 3.54 | 3.53 | 3.26 | 3.24 | 3.19 | 3.23 | 3.44 | 0.04 | 1.14 | 0.02 | 0.49 | 0.04 | 1.12 |
| 2 | 4.4 | 4.35 | 4.39 | 4.38 | 4.45 | 4.53 | 4.52 | 4.50 | 4.46 | 4.48 | 4.41 | 4.45 | 4.44 | 0.03 | 0.60 | 0.04 | 0.97 | 0.04 | 0.81 |
| 3 | 3.41 | 3.48 | 3.46 | 3.45 | 3.27 | 3.22 | 3.26 | 3.25 | 3.4 | 3.15 | 3.35 | 3.30 | 3.33 | 0.04 | 1.05 | 0.03 | 0.81 | 0.13 | 4.01 |
| X1 | 5.18 | 5.28 | 5.29 | 5.25 | 5.57 | 5.25 | 5.53 | 5.45 | 5.36 | 5.23 | 5.31 | 5.30 | 5.33 | 0.06 | 1.16 | 0.17 | 3.20 | 0.07 | 1.24 |
| 2 | 14.31 | 14.18 | 14.26 | 14.25 | 14.22 | 14.05 | 14.18 | 14.15 | 13.75 | 14.03 | 14.07 | 13.95 | 14.12 | 0.07 | 0.46 | 0.09 | 0.63 | 0.17 | 1.25 |
| 3 | 14.45 | 14.61 | 14.55 | 14.54 | 14.79 | 14.61 | 14.73 | 14.71 | 14.61 | 14.52 | 14.67 | 14.60 | 14.62 | 0.08 | 0.56 | 0.09 | 0.62 | 0.08 | 0.52 |
| K1 | 9.05 | 9.03 | 8.89 | 8.99 | 9.11 | 9.21 | 9.25 | 9.19 | 9.07 | 9.11 | 9.09 | 9.09 | 9.09 | 0.09 | 0.97 | 0.07 | 0.78 | 0.02 | 0.22 |
| 2 | 9.57 | 9.64 | 9.59 | 9.60 | 9.64 | 9.71 | 9.69 | 9.68 | 9.71 | 9.76 | 9.78 | 9.75 | 9.68 | 0.04 | 0.38 | 0.04 | 0.37 | 0.04 | 0.37 |
| 3 | 13.49 | 13.57 | 13.59 | 13.55 | 13.18 | 13.26 | 13.31 | 13.25 | 13.23 | 13.16 | 13.21 | 13.20 | 13.33 | 0.05 | 0.39 | 0.07 | 0.49 | 0.04 | 0.27 |
| U1 | 2.77 | 2.81 | 2.82 | 2.80 | 2.81 | 2.78 | 2.81 | 2.80 | 2.73 | 2.65 | 2.72 | 2.70 | 2.77 | 0.03 | 0.94 | 0.02 | 0.62 | 0.04 | 1.61 |
| 2 | 2.68 | 2.64 | 2.69 | 2.67 | 2.79 | 2.77 | 2.75 | 2.77 | 2.54 | 2.51 | 2.51 | 2.52 | 2.65 | 0.03 | 0.99 | 0.02 | 0.72 | 0.02 | 0.69 |
| 3 | 2.84 | 2.79 | 2.83 | 2.82 | 2.91 | 2.96 | 2.98 | 2.95 | 2.88 | 2.86 | 2.82 | 2.85 | 2.87 | 0.03 | 0.94 | 0.04 | 1.22 | 0.03 | 1.07 |
| 4 | 2.82 | 2.77 | 2.81 | 2.80 | 2.84 | 2.81 | 2.84 | 2.83 | 2.72 | 2.67 | 2.71 | 2.70 | 2.78 | 0.03 | 0.94 | 0.02 | 0.61 | 0.03 | 0.98 |
| 5 | 2.66 | 2.71 | 2.73 | 2.70 | 2.69 | 2.69 | 2.66 | 2.68 | 2.74 | 2.73 | 2.69 | 2.72 | 2.70 | 0.04 | 1.34 | 0.02 | 0.65 | 0.03 | 0.97 |
| 6 | 2.81 | 2.79 | 2.77 | 2.79 | 2.68 | 2.72 | 2.67 | 2.69 | 2.76 | 2.71 | 2.75 | 2.74 | 2.74 | 0.02 | 0.72 | 0.03 | 0.98 | 0.03 | 0.97 |
| Rv | 2.88 | 2.83 | 2.87 | 2.86 | 2.76 | 2.71 | 2.75 | 2.74 | 2.78 | 2.71 | 2.76 | 2.75 | 2.78 | 0.03 | 0.93 | 0.03 | 0.97 | 0.04 | 1.31 |
| Ra | 1.14 | 1.19 | 1.18 | 1.17 | 1.05 | 1.03 | 1.01 | 1.03 | 1.14 | 1.11 | 1.14 | 1.13 | 1.11 | 0.03 | 2.26 | 0.02 | 1.94 | 0.02 | 1.53 |

## Lactate dehydrogenase assay - % cytotoxicity

B7. Cytotoxicity of *M. tuberculosis* grown in the presence of $O_2$ to A549 cells

| Strain | % cytotoxicity | | | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|
| B1 | 20.57 | 20.45 | 19.58 | 20.69 | 20.93 | 20.56 | 18.81 | 19.38 | 19.50 | 20.05 |
| B2 | 22.92 | 22.68 | 24.29 | 23.38 | 23.13 | 23.13 | 23.62 | 23.28 | 23.51 | 23.33 |
| B3 | 13.69 | 15.41 | 14.55 | 10.50 | 11.84 | 11.57 | 13.53 | 13.20 | 12.76 | 13.01 |
| XKZN1 | 16.12 | 16.24 | 16.61 | 14.66 | 15.79 | 15.41 | 15.56 | 15.33 | 15.22 | 15.66 |
| XKZN2 | 20.86 | 20.50 | 20.62 | 20.49 | 20.37 | 19.88 | 20.52 | 20.52 | 20.75 | 20.50 |
| XKZN3 | 22.22 | 22.47 | 22.47 | 22.56 | 22.81 | 22.56 | 22.10 | 21.87 | 22.21 | 22.36 |
| KZN1 | 14.74 | 15.96 | 13.86 | 13.78 | 14.27 | 13.54 | 13.75 | 14.43 | 13.98 | 14.26 |
| KZN2 | 16.09 | 16.21 | 16.45 | 16.34 | 16.22 | 16.71 | 16.27 | 16.63 | 16.39 | 16.37 |
| KZN3 | 17.69 | 19.83 | 18.97 | 19.25 | 19.52 | 18.71 | 19.36 | 18.92 | 19.25 | 19.06 |
| U1 | 4.99 | 4.71 | 4.56 | 5.25 | 5.65 | 5.38 | 5.28 | 5.06 | 5.06 | 5.11 |
| U2 | 3.33 | 3.16 | 2.98 | 3.66 | 3.54 | 3.78 | 3.61 | 3.72 | 3.49 | 3.47 |
| U3 | 8.60 | 7.72 | 7.02 | 8.78 | 9.63 | 9.15 | 8.00 | 8.68 | 8.46 | 8.45 |
| U4 | 4.28 | 3.91 | 4.03 | 4.51 | 4.39 | 4.64 | 4.85 | 4.96 | 4.85 | 4.49 |
| U5 | 3.51 | 3.51 | 3.68 | 3.90 | 3.90 | 3.68 | 3.95 | 4.06 | 4.28 | 3.83 |
| U6 | 7.03 | 7.27 | 7.75 | 8.05 | 8.17 | 7.68 | 6.96 | 7.43 | 7.31 | 7.52 |
| Rv | 3.57 | 3.42 | 3.42 | 4.04 | 3.77 | 4.17 | 4.29 | 4.07 | 3.96 | 3.86 |
| Ra | 2.50 | 2.74 | 2.15 | 3.66 | 3.29 | 3.17 | 2.95 | 2.71 | 3.07 | 2.92 |

B8. Cytotoxicity of *M. tuberculosis* grown under $O_2$ deprivation to A549 cell

| Strain | % cytotoxicity | | | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|
| B1 | 24.09 | 24.74 | 25.00 | 24.16 | 23.90 | 24.29 | 22.56 | 23.05 | 23.17 | 23.88 |
| B2 | 27.29 | 27.80 | 27.12 | 27.04 | 27.30 | 27.17 | 27.57 | 27.45 | 28.31 | 27.45 |
| B3 | 17.06 | 16.15 | 16.41 | 18.69 | 18.19 | 18.56 | 18.32 | 17.95 | 17.70 | 17.67 |
| XKZN1 | 31.72 | 31.34 | 31.72 | 31.79 | 31.43 | 31.55 | 30.27 | 30.15 | 30.51 | 31.16 |
| XKZN2 | 34.75 | 34.58 | 34.92 | 32.96 | 32.70 | 32.33 | 31.00 | 30.88 | 31.00 | 32.79 |
| XKZN3 | 35.16 | 35.03 | 35.16 | 34.78 | 37.50 | 38.00 | 34.53 | 35.15 | 35.64 | 35.66 |
| KZN1 | 17.58 | 18.10 | 18.23 | 18.44 | 18.83 | 17.92 | 19.02 | 18.78 | 18.66 | 18.40 |
| KZN2 | 20.34 | 20.68 | 21.19 | 19.50 | 19.87 | 20.00 | 19.24 | 19.49 | 19.73 | 20.00 |
| KZN3 | 25.75 | 25.37 | 24.81 | 24.36 | 23.63 | 24.73 | 23.77 | 24.39 | 24.02 | 24.54 |
| U1 | 7.94 | 7.42 | 7.55 | 6.31 | 6.31 | 6.68 | 7.05 | 7.43 | 7.67 | 7.15 |
| U2 | 5.04 | 5.22 | 5.22 | 5.12 | 5.36 | 5.48 | 5.64 | 5.39 | 5.27 | 5.30 |
| U3 | 11.57 | 11.19 | 10.63 | 10.72 | 10.11 | 10.35 | 10.91 | 10.54 | 10.66 | 10.74 |
| U4 | 5.93 | 6.10 | 5.76 | 5.66 | 5.66 | 6.04 | 6.25 | 6.99 | 6.74 | 6.13 |
| U5 | 5.73 | 5.60 | 5.60 | 5.57 | 5.57 | 5.32 | 6.06 | 5.94 | 5.94 | 5.70 |
| U6 | 9.14 | 9.70 | 10.07 | 9.50 | 8.89 | 9.26 | 8.95 | 9.31 | 9.56 | 9.38 |
| Rv | 5.73 | 5.47 | 5.21 | 5.84 | 5.45 | 5.58 | 5.85 | 5.98 | 6.10 | 5.69 |
| Ra | 3.13 | 3.52 | 3.39 | 3.38 | 3.38 | 3.12 | 3.54 | 3.41 | 3.05 | 3.32 |

**Induced projections to *M. tuberculosis* bacilli on A549 cells**

B9 *M. tuberculosis* grown in the presence of $O_2$

| Strain | Image_1_means | | Image_2_means | | Image_3_means | | Image_4_means | | All 4 images mean | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection |
| B1 | 11 | 109 | 5 | 89 | 6 | 85 | 8 | 104 | 8 | 97 |
| XKZN | 9 | 94 | 9 | 110 | 12 | 158 | 7 | 137 | 9 | 125 |
| KZN | 6 | 20 | 8 | 32 | 4 | 28 | 14 | 53 | 8 | 33 |
| U1 | 1 | 11 | 2 | 12 | 4 | 17 | 1 | 9 | 2 | 12 |
| U2 | 5 | 18 | 5 | 25 | 5 | 24 | 4 | 16 | 5 | 21 |
| Rv | 4 | 12 | 4 | 18 | 2 | 10 | 5 | 19 | 4 | 15 |

B10 *M. tuberculosis* grown under $O_2$ deprivation

| Strain | Image_1_means | | Image_2_means | | Image_3_means | | Image_4_means | | All 4 images mean | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection | Bacilli | Projection |
| B1 | 15 | 169 | 5 | 164 | 10 | 153 | 12 | 181 | 11 | 167 |
| XKZN | 8 | 181 | 11 | 191 | 13 | 197 | 16 | 189 | 12 | 190 |
| KZN | 12 | 96 | 7 | 80 | 13 | 91 | 5 | 76 | 9 | 86 |
| U1 | 3 | 45 | 9 | 49 | 13 | 99 | 1 | 14 | 7 | 52 |
| U2 | 5 | 23 | 8 | 31 | 8 | 37 | 4 | 36 | 6 | 32 |
| Rv | 6 | 24 | 5 | 31 | 5 | 33 | 3 | 28 | 5 | 29 |

## Gene expression

B11 Mean gene expression level in *M. tuberculosis* grown in the presence of $O_2$ before exposure to A549 cells

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $hbhA/_{16S}$ | | | $hbhA/_{ftsZ}$ | | | $mdp1/_{16S}$ | | | $mdp1/_{ftsZ}$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 1.0817 | 1.0813 | 1.0812 | 1.0406 | 1.0408 | 1.0404 | 0.9171 | 0.9182 | 0.9184 | 0.6707 | 0.6701 | 0.6713 |
| B2 | 1.7659 | 1.7654 | 1.7658 | 1.4898 | 1.4903 | 1.4905 | 1.3152 | 1.3154 | 1.3141 | 1.1145 | 1.1146 | 1.115 |
| B3 | 1.0422 | 1.0429 | 1.0427 | 1.0209 | 1.0211 | 1.0213 | 0.7548 | 0.7556 | 0.7555 | 0.5415 | 0.5409 | 0.5415 |
| XKZN1 | 1.2016 | 1.2018 | 1.2011 | 1.1545 | 1.1552 | 1.1553 | 0.9459 | 0.9458 | 0.9454 | 0.7668 | 0.7661 | 0.7675 |
| XKZN2 | 1.531 | 1.5315 | 1.5308 | 1.3255 | 1.3251 | 1.325 | 0.8568 | 0.8559 | 0.8568 | 0.6576 | 0.6565 | 0.6578 |
| XKZN3 | 1.8128 | 1.8136 | 1.8135 | 1.6988 | 1.699 | 1.6995 | 1.2968 | 1.2961 | 1.2969 | 1.0852 | 1.0844 | 1.0851 |
| KZN1 | 1.1432 | 1.1427 | 1.1425 | 1.1214 | 1.1212 | 1.1213 | 2.7485 | 2.7481 | 2.7483 | 2.4704 | 2.4711 | 2.4712 |
| KZN2 | 1.3043 | 1.3036 | 1.3044 | 1.1901 | 1.1909 | 1.1908 | 1.3801 | 1.3809 | 1.3808 | 1.1471 | 1.1479 | 1.1481 |
| KZN3 | 2.2943 | 2.2945 | 2.2938 | 1.8587 | 1.8579 | 1.8586 | 1.3828 | 1.3821 | 1.3826 | 1.3336 | 1.3341 | 1.3343 |
| U1 | 1.0969 | 1.0976 | 1.0971 | 1.0755 | 1.0753 | 1.0751 | 1.3522 | 1.3517 | 1.3521 | 1.1241 | 1.1234 | 1.1242 |
| U2 | 1.3484 | 1.3486 | 1.3485 | 1.2568 | 1.2569 | 1.2576 | 1.382 | 1.381 | 1.383 | 1.1611 | 1.1614 | 1.1614 |
| U3 | 1.1459 | 1.1451 | 1.1458 | 1.1247 | 1.1242 | 1.1249 | 1.2501 | 1.2514 | 1.2512 | 1.0241 | 1.0241 | 1.0235 |
| U4 | 1.1001 | 1.1008 | 1.1009 | 1.0948 | 1.0948 | 1.0951 | 1.2001 | 1.2995 | 1.2992 | 1.0241 | 1.0249 | 1.0248 |
| U5 | 0.0351 | 0.0362 | 0.0364 | 1.0189 | 1.0191 | 1.0196 | 1.1986 | 1.1981 | 1.1988 | 1.0191 | 1.0187 | 1.0192 |
| U6 | 1.0307 | 1.0312 | 1.0314 | 0.0758 | 0.0751 | 0.0756 | 1.0379 | 1.0381 | 1.0371 | 1.0145 | 1.0147 | 1.0146 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0053 | 0.0048 | 0.0049 | 0.0034 | 0.0035 | 0.0034 | 1.0025 | 1.0021 | 1.0026 | 1.0007 | 1.0004 | 1.0006 |

B12 Mean gene expression level in *M. tuberculosis* grown in the presence of $O_2$ thereafter exposed to A549 cells

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $hbhA/_{16S}$ | | | $hbhA/_{ftsZ}$ | | | $mdp1/_{16S}$ | | | $mdp1/_{ftsZ}$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 1.7069 | 1.7071 | 1.7073 | 1.3385 | 1.3389 | 1.339 | 1.1449 | 1.1441 | 1.1448 | 1.0903 | 1.0901 | 1.0902 |
| B2 | 2.0259 | 2.0254 | 2.0252 | 1.7647 | 1.7641 | 1.7647 | 0.6128 | 0.6141 | 0.6139 | 0.3259 | 0.3266 | 0.3264 |
| B3 | 1.6022 | 1.6021 | 1.6026 | 1.3189 | 1.3191 | 1.3193 | 1.7454 | 1.7449 | 1.7456 | 1.6732 | 1.6734 | 1.6739 |
| XKZN1 | 1.5251 | 1.5261 | 1.5262 | 1.0721 | 1.0725 | 1.0726 | 0.8049 | 0.8047 | 0.8051 | 0.5971 | 0.5973 | 0.5978 |
| XKZN2 | 1.9852 | 1.9849 | 1.9855 | 1.6009 | 1.6017 | 1.6016 | 0.6229 | 0.6224 | 0.6225 | 0.3979 | 0.3986 | 0.3981 |
| XKZN3 | 2.0369 | 2.0376 | 2.0374 | 2.0098 | 2.0104 | 2.0101 | 0.9025 | 0.9029 | 0.903 | 0.7621 | 0.7622 | 0.7614 |
| KZN1 | 1.7813 | 1.7812 | 1.7814 | 1.5108 | 1.5112 | 1.511 | 1.0139 | 1.0138 | 1.0146 | 1.0096 | 1.0091 | 1.0095 |
| KZN2 | 1.5309 | 1.531 | 1.5314 | 1.2845 | 1.2838 | 1.284 | 1.0761 | 1.0771 | 1.0772 | 1.0527 | 1.0523 | 1.0524 |
| KZN3 | 2.6735 | 2.6729 | 2.6738 | 2.2097 | 2.2099 | 2.2107 | 1.0067 | 1.0068 | 1.0066 | 1.0017 | 1.0018 | 1.0018 |
| U1 | 0.2723 | 0.2722 | 0.2724 | 0.1496 | 0.1491 | 0.1495 | 2.2593 | 2.2599 | 2.2602 | 1.9984 | 1.9981 | 1.9978 |
| U2 | 0.9191 | 0.9201 | 0.9202 | 0.8822 | 0.8819 | 0.8825 | 2.1868 | 2.1861 | 2.1866 | 1.9815 | 1.9818 | 1.9809 |
| U3 | 0.9699 | 0.9701 | 0.9706 | 0.8987 | 0.8981 | 0.8987 | 0.7284 | 0.7292 | 0.7294 | 0.5188 | 0.5193 | 0.5195 |
| U4 | 0.8949 | 0.8953 | 0.8957 | 0.7628 | 0.7639 | 0.7635 | 1.7328 | 1.7332 | 1.7333 | 1.5135 | 1.5128 | 1.5136 |
| U5 | 0.0161 | 0.0155 | 0.0158 | 0.1629 | 0.1639 | 0.1637 | 1.8601 | 1.8608 | 1.8609 | 1.7231 | 1.7238 | 1.7236 |
| U6 | 0.6181 | 0.6189 | 0.6188 | 0.5551 | 0.5556 | 0.5558 | 1.6119 | 1.6114 | 1.6121 | 1.4041 | 1.4051 | 1.4052 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0009 | 0.0012 | 0.0012 | 0.0004 | 0.0006 | 0.0003 | 2.0124 | 2.0131 | 2.0132 | 1.9484 | 1.9486 | 1.9479 |

B13 Mean gene expression level in *M. tuberculosis* grown under oxygen deprivation before exposure to A549 cells

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $hbhA/_{16S}$ | | | $hbhA/_{ftsZ}$ | | | $mdp1/_{16S}$ | | | $mdp1/_{ftsZ}$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 0.3208 | 0.3209 | 0.3207 | 0.2098 | 0.2095 | 0.2098 | 1.3289 | 1.3291 | 1.3293 | 1.1195 | 1.1188 | 1.119 |
| B2 | 1.4521 | 1.4523 | 1.4522 | 1.238 | 1.248 | 1.246 | 1.6599 | 1.6602 | 1.6602 | 1.4275 | 1.4284 | 1.4284 |
| B3 | 0.3234 | 0.3239 | 0.3238 | 0.2188 | 0.2199 | 0.2195 | 1.0424 | 1.0429 | 1.0428 | 1.0009 | 1.0006 | 1.0003 |
| XKZN1 | 1.6727 | 1.6724 | 1.6724 | 1.4546 | 1.4552 | 1.4555 | 1.3958 | 1.3961 | 1.3961 | 1.1938 | 1.1952 | 1.1951 |
| XKZN2 | 1.3391 | 1.3396 | 1.3395 | 1.1542 | 1.1529 | 1.154 | 1.2289 | 1.2294 | 1.2293 | 1.0096 | 1.0094 | 1.0092 |
| XKZN3 | 1.8379 | 1.8378 | 1.8383 | 1.6592 | 1.6581 | 1.6594 | 1.8276 | 1.8274 | 1.8272 | 1.6571 | 1.6579 | 1.6578 |
| KZN1 | 1.5216 | 1.5219 | 1.5219 | 1.3661 | 1.3671 | 1.3672 | 1.1757 | 1.1761 | 1.1762 | 1.0028 | 1.0023 | 1.0027 |
| KZN2 | 1.3152 | 1.3157 | 1.3156 | 1.1331 | 1.1329 | 1.1321 | 0.8306 | 0.8311 | 0.831 | 0.6837 | 0.6843 | 0.6846 |
| KZN3 | 1.3119 | 1.3123 | 1.3121 | 1.1128 | 1.1124 | 1.1126 | 1.6282 | 1.6277 | 1.6281 | 1.4438 | 1.4431 | 1.4439 |
| U1 | 0.6369 | 0.6365 | 0.6364 | 0.5479 | 0.5468 | 0.5478 | 0.6534 | 0.6539 | 0.6538 | 0.4648 | 0.4654 | 0.4657 |
| U2 | 0.4747 | 0.4746 | 0.4745 | 0.3121 | 0.3126 | 0.3128 | 0.5819 | 0.5822 | 0.5819 | 0.3415 | 0.3413 | 0.3411 |
| U3 | 0.3161 | 0.3165 | 0.3163 | 0.2078 | 0.2087 | 0.2084 | 0.2152 | 0.2157 | 0.2156 | 0.0831 | 0.0842 | 0.0844 |
| U4 | 0.6343 | 0.6346 | 0.6346 | 0.5129 | 0.5131 | 0.5132 | 0.5299 | 0.5304 | 0.5303 | 0.3998 | 0.3993 | 0.3994 |
| U5 | 0.0816 | 0.082 | 0.0821 | 0.0667 | 0.0672 | 0.0674 | 0.4856 | 0.4859 | 0.4859 | 0.2387 | 0.2392 | 0.2394 |
| U6 | 0.1694 | 0.1699 | 0.1698 | 0.0928 | 0.0935 | 0.0939 | 0.2167 | 0.2171 | 0.2172 | 0.0928 | 0.0934 | 0.0937 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0285 | 0.0291 | 0.0291 | 0.0185 | 0.0187 | 0.0183 | 0.0297 | 0.0293 | 0.0295 | 0.0091 | 0.0094 | 0.0091 |

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $fdxA/16S$ | | | $fdxA/ftsZ$ | | | $hspX/16S$ | | | $hspX/ftsZ$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 1.0079 | 1.0085 | 1.0088 | 1.0046 | 1.0042 | 1.0047 | 1.3645 | 1.3662 | 1.3658 | 1.1968 | 1.1973 | 1.1975 |
| B2 | 1.2241 | 1.2244 | 1.2253 | 1.0997 | 1.0997 | 1.0994 | 1.5351 | 1.5361 | 1.5362 | 1.3392 | 1.3392 | 1.3395 |
| B3 | 0.7626 | 0.7623 | 0.7629 | 0.5195 | 0.5193 | 0.5185 | 0.9412 | 0.9421 | 0.9418 | 0.7279 | 0.7283 | 0.7284 |
| XKZN1 | 1.0916 | 1.0932 | 1.0936 | 1.0631 | 1.0625 | 1.0631 | 1.1486 | 1.1497 | 1.1499 | 1.1155 | 1.1151 | 1.1156 |
| XKZN2 | 1.0221 | 1.0219 | 1.0226 | 1.0091 | 1.0099 | 1.0098 | 1.2654 | 1.2663 | 1.2666 | 1.1238 | 1.1231 | 1.1239 |
| XKZN3 | 1.8317 | 1.8334 | 1.8336 | 1.6398 | 1.6401 | 1.6407 | 1.9977 | 1.9996 | 1.9994 | 1.7321 | 1.7322 | 1.7314 |
| KZN1 | 1.5587 | 1.5598 | 1.5594 | 1.3789 | 1.3807 | 1.3804 | 1.4321 | 1.4336 | 1.4339 | 1.2593 | 1.2592 | 1.2594 |
| KZN2 | 1.0558 | 1.0565 | 1.0566 | 1.0324 | 1.0317 | 1.0325 | 1.0938 | 1.0953 | 1.0956 | 1.0697 | 1.0692 | 1.0693 |
| KZN3 | 1.0023 | 1.0037 | 1.0039 | 1.0006 | 1.0002 | 1.0007 | 1.0321 | 1.0329 | 1.0328 | 1.0142 | 1.0136 | 1.0142 |
| U1 | 0.6643 | 0.6656 | 0.6654 | 0.4897 | 0.4894 | 0.4897 | 0.9701 | 0.9711 | 0.9712 | 0.7685 | 0.7693 | 0.7692 |
| U2 | 0.3861 | 0.3876 | 0.3879 | 0.1541 | 0.1551 | 0.1549 | 0.4309 | 0.4317 | 0.4319 | 0.2209 | 0.2201 | 0.2211 |
| U3 | 0.3356 | 0.3366 | 0.3364 | 0.1219 | 0.1231 | 0.1228 | 0.4605 | 0.4601 | 0.4603 | 0.2701 | 0.2709 | 0.2708 |
| U4 | 0.6173 | 0.6184 | 0.6186 | 0.4517 | 0.4511 | 0.4517 | 0.8418 | 0.8432 | 0.8437 | 0.6771 | 0.6778 | 0.6776 |
| U5 | 0.1617 | 0.1627 | 0.1625 | 0.0976 | 0.0994 | 0.0991 | 0.2076 | 0.2095 | 0.2093 | 0.1171 | 0.1169 | 0.1164 |
| U6 | 0.6038 | 0.6049 | 0.6048 | 0.3923 | 0.3912 | 0.3922 | 0.7943 | 0.7956 | 0.79254 | 0.5825 | 0.5827 | 0.582 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0089 | 0.0096 | 0.0097 | 0.0021 | 0.0024 | 0.0021 | 0.0172 | 0.0174 | 0.0172 | 0.0091 | 0.0099 | 0.0098 |

B14 Mean gene expression level in *M. tuberculosis* grown under oxygen deprivation thereafter exposed to A549 cells

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $hbhA/_{16S}$ | | | $hbhA/_{ftsZ}$ | | | $mdp1/_{16S}$ | | | $mdp1/_{ftsZ}$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 1.0702 | 1.0721 | 1.0728 | 1.0561 | 1.0568 | 1.0569 | 1.8601 | 1.8604 | 1.8607 | 1.6569 | 1.6561 | 1.6571 |
| B2 | 2.7451 | 2.7461 | 2.7462 | 2.5569 | 2.5561 | 2.5571 | 2.5198 | 2.5201 | 2.5207 | 2.2475 | 2.2468 | 2.2473 |
| B3 | 1.0342 | 1.0338 | 1.0343 | 1.0117 | 1.0125 | 1.0127 | 1.0353 | 1.0355 | 1.0354 | 1.0161 | 1.0156 | 1.0154 |
| XKZN1 | 2.5371 | 2.5381 | 2.5379 | 2.3258 | 2.3251 | 2.3253 | 1.4868 | 1.4879 | 1.4878 | 1.2643 | 1.2637 | 1.2643 |
| XKZN2 | 2.0298 | 2.0303 | 2.0302 | 1.7835 | 1.7839 | 1.784 | 1.0137 | 1.0142 | 1.0144 | 1.0097 | 1.0099 | 1.0092 |
| XKZN3 | 2.0987 | 2.0992 | 2.0994 | 1.9742 | 1.9753 | 1.9755 | 2.1716 | 2.1722 | 2.1725 | 1.9473 | 1.9482 | 1.9485 |
| KZN1 | 2.0558 | 2.0566 | 2.0565 | 1.8244 | 1.8262 | 1.8259 | 2.0498 | 2.0504 | 2.0501 | 1.8891 | 1.8884 | 1.8889 |
| KZN2 | 2.0731 | 2.0724 | 2.0729 | 1.8678 | 1.8666 | 1.8672 | 1.0627 | 1.0633 | 1.0636 | 1.0485 | 1.0483 | 1.0478 |
| KZN3 | 2.0206 | 2.0215 | 2.0212 | 1.7459 | 1.7469 | 1.7467 | 2.0328 | 2.0331 | 2.0331 | 1.7841 | 1.7835 | 1.7838 |
| U1 | 0.7769 | 0.7778 | 0.7778 | 0.5401 | 0.5411 | 0.5412 | 0.8003 | 0.8007 | 0.8011 | 0.6017 | 0.6022 | 0.6018 |
| U2 | 0.7171 | 0.7181 | 0.7185 | 0.5193 | 0.5196 | 0.5193 | 0.7173 | 0.7181 | 0.7183 | 0.5849 | 0.5851 | 0.5856 |
| U3 | 0.6459 | 0.6451 | 0.6452 | 0.5193 | 0.4978 | 0.4972 | 0.5507 | 0.5513 | 0.5516 | 0.3144 | 0.3152 | 0.3151 |
| U4 | 0.1901 | 0.1905 | 0.1906 | 0.0918 | 0.0916 | 0.0911 | 0.8713 | 0.8719 | 0.8722 | 0.6512 | 0.6508 | 0.6513 |
| U5 | 0.1142 | 0.1145 | 0.1148 | 0.0645 | 0.0638 | 0.0646 | 0.8149 | 0.8161 | 0.8158 | 0.6143 | 0.6145 | 0.6135 |
| U6 | 0.2832 | 0.2835 | 0.2835 | 0.1163 | 0.1156 | 0.1155 | 0.5817 | 0.5825 | 0.5827 | 0.3719 | 0.3715 | 0.3714 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0211 | 0.0222 | 0.0221 | 0.0102 | 0.0109 | 0.0107 | 0.1232 | 0.1238 | 0.1235 | 0.1025 | 0.103 | 0.1032 |

| Strain | Mean gene expression levels per run | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $^{fdxA}/_{16S}$ | | | $^{fdxA}/_{ftsZ}$ | | | $^{hspX}/_{16S}$ | | | $^{hspX}/_{ftsZ}$ | | |
| | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| B1 | 2.004 | 2.011 | 2.012 | 1.8743 | 1.8739 | 1.8744 | 2.2012 | 2.2018 | 2.2021 | 2.0028 | 2.0021 | 2.0029 |
| B2 | 2.3781 | 2.3792 | 2.3794 | 2.123 | 2.1225 | 2.1232 | 2.5261 | 2.5266 | 2.5268 | 2.3506 | 2.3501 | 2.3502 |
| B3 | 1.0288 | 1.0293 | 1.0295 | 1.0096 | 1.0092 | 1.0097 | 1.0443 | 1.0452 | 1.0449 | 1.0135 | 1.0127 | 1.0134 |
| XKZN1 | 2.0201 | 2.0203 | 2.0208 | 2.0094 | 2.0098 | 2.0096 | 2.3516 | 2.3524 | 2.3523 | 2.1971 | 2.1969 | 2.1964 |
| XKZN2 | 2.0199 | 2.0201 | 2.0206 | 2.0093 | 2.0092 | 2.0091 | 2.1191 | 2.1196 | 2.1198 | 1.9301 | 1.9303 | 1.9308 |
| XKZN3 | 2.5581 | 2.5589 | 2.5591 | 2.3811 | 2.3806 | 2.3807 | 3.0851 | 3.0859 | 3.0861 | 2.9723 | 2.9719 | 2.9724 |
| KZN1 | 2.2798 | 2.2802 | 2.2803 | 2.0641 | 2.0637 | 2.0645 | 2.3328 | 2.3329 | 2.3333 | 2.1683 | 2.1692 | 2.1689 |
| KZN2 | 2.0983 | 2.0985 | 2.0987 | 2.0716 | 2.0711 | 2.0712 | 2.2262 | 2.2268 | 2.2271 | 2.0049 | 2.0041 | 2.0051 |
| KZN3 | 2.1352 | 2.1359 | 2.1357 | 2.0915 | 2.0919 | 2.0926 | 2.4257 | 2.4262 | 2.4264 | 2.2841 | 2.2854 | 2.2852 |
| U1 | 1.2123 | 1.2125 | 1.2124 | 1.0967 | 1.0975 | 1.0968 | 1.4218 | 1.4221 | 1.4224 | 1.2581 | 1.2578 | 1.2578 |
| U2 | 1.0969 | 1.0975 | 1.0972 | 1.0709 | 1.0713 | 1.0708 | 1.122 | 1.1199 | 1.1211 | 1.0901 | 1.0906 | 1.0905 |
| U3 | 0.8815 | 0.8822 | 0.8826 | 0.6963 | 0.6956 | 0.6964 | 1.0148 | 1.0153 | 1.0155 | 1.0077 | 1.0083 | 1.0086 |
| U4 | 0.9619 | 0.9626 | 0.9624 | 0.7728 | 0.7737 | 0.7728 | 1.1019 | 1.1021 | 1.1023 | 1.0755 | 1.0753 | 1.0748 |
| U5 | 0.5244 | 0.5251 | 0.5252 | 0.3593 | 0.3603 | 0.3601 | 0.6031 | 0.6035 | 0.6033 | 0.4619 | 0.4612 | 0.4617 |
| U6 | 0.9277 | 0.9281 | 0.9279 | 0.7184 | 0.7182 | 0.7186 | 1.3122 | 1.3131 | 1.3131 | 1.1525 | 1.1532 | 1.1524 |
| Rv | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Ra | 0.0203 | 0.0211 | 0.0213 | 0.0089 | 0.0081 | 0.0082 | 0.0349 | 0.0359 | 0.0357 | 0.0125 | 0.0129 | 0.0127 |