

CHAPTER 1

Preface

1. BACKGROUND AND MOTIVATION

Annually, approximately 9 million new tuberculosis (TB) cases are recorded and up to 2 million people die as a result of this disease (WHO, 2010 (a)). TB is most commonly localised in the lungs and is mainly caused by the bacterium, *Mycobacterium tuberculosis*. Although this is a worldwide epidemic, Africa (30%) and Asia (55%) accounts for 85% of all global TB cases. When evaluating recorded adult deaths in low- and middle-income countries, TB is ranked third, after HIV/AIDS and ischemic heart disease (WHO, 2010 (a)). These statistics are rather disturbing considering the fact that TB can be prevented and is, in most instances, a curable disease. From the 1950s to the 1980s, various first-line anti-TB drugs have been developed and when using combinations of these, patients with drug-susceptible TB can successfully be cured within 6 months. Approximately 0.4 - 0.5 million annual TB cases are, however, multi-drug resistant (MDR) (WHO, 2010 (b)), requiring the use of second-line drugs, which are costly, have severe side effects, and require almost 2 years of continual treatment, eventually still resulting in a dismal cure rate of only 50 – 70% (WHO, 2010 (a)). Since the 1980s, the rising HIV pandemic has become a significant obstacle in modern TB control. TB is considered to be the primary cause of death in most HIV infected patients, partly due to: the increased TB infection rate in HIV patients; a weakened immune system, and; the difficulties associated with diagnosing TB in HIV patients due to a lowered bacterial load in the lungs of these individuals (UNAIDS, 2006; U.S. Global Health Policy, 2010). Therefore, in order to eradicate this disease, the world is in urgent need of new, less toxic, faster acting TB treatment approaches, together with more sensitive, specific and rapid TB diagnostic methods, which are able to cope with the rising MDR-TB and HIV incidence.

The relatively new research field of metabolomics ('the non-biased identification and quantification of *all* metabolites in a biological system' using highly sensitive analytical techniques (Dunn *et al.*, 2005)) has successfully been used over the past few years to characterise a variety of diseases (Schoeman & Loots, 2011), including TB. Considering the metabolomics investigations done on TB, from a disease characterisation perspective, using a lipidomics approach, Jain *et al.* (2007) indicated that *M. tuberculosis* undergoes a metabolic shift to preferentially use host lipids as a carbon source during infection, consequently increasing their virulence associated lipid anabolism. In addition, using a similar approach, de Carvalho *et al.* (2010) successfully proved that *M. tuberculosis* can simultaneously catabolise multiple carbon sources. From a disease diagnostic perspective, metabolomics has been used to differentiate between various cultured *Mycobacterium species*, and to potentially diagnose TB from patient collected sputum samples, using electronic nose technology (Fend *et al.*, 2006). However, as the nature by which these sensors function is not yet fully understood, the identity and quantities of the differentiating volatiles cannot be determined, and may be seen as a

limitation to using this sensor array technology. Phillips *et al.* (2010) subsequently used a metabolomics approach and automated thermal desorption gas chromatography and mass spectrometry (ATD/GC-MS), in order to identify TB-specific volatile organic compounds (VOCs) from patient collected breath, for diagnostic purposes. Using these identified VOCs, they were able to differentiate TB-positive from TB-negative patient collected breath samples.

No investigation to date has explored the possibility of metabolite marker identification, using metabolomics, for differentiating and better characterising various different infectious *Mycobacterium* species, various drug-resistant strains of *M. tuberculosis*, or for general TB disease characterisation using patient collected sputum. Consequently, we developed and evaluated various compound isolation procedures for the purpose of extracting the most biologically relevant metabolite information from a sample. This investigation was done not only to better characterise various infectious *Mycobacterium* species and drug-resistant strains, but also for the purpose of identifying metabolite markers for better characterising TB from patient collected sputum. The developed methods were evaluated by considering their comparative extraction capacities (the number and intensity of compounds extracted per sample), repeatability, speed, simplicity, detection limits, and the nature of the metabolite markers extracted with regards to their biological relevance. The initial method development investigations in this study were done using cultured samples of various *Mycobacterium* species (*M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. avium*) and *P. aeruginosa*. The relevant methods were subsequently applied to: 1) better characterise rifampicin-resistance by comparing two different cultured *M. tuberculosis* *rpoB* mutants to a wild-type parent strain, and 2) to better characterise *M. tuberculosis* infection in humans, by comparing TB-positive to TB-negative patient collected sputa. Considering this, the current investigation not only contributes to the existing scientific knowledge base by being the first to develop and compare various extraction methods for a metabolomics investigation of TB, but also by identifying those metabolite markers differentiating and better characterising various *Mycobacterium* species, drug resistant strains, and a TB disease state in humans. Using these newly identified markers, novel disease mechanisms are proposed by interoperating the compound information in the light of previously generated knowledge collected from various other "omics" approaches (genomics, transcriptomics and proteomics).

2. AIM AND OBJECTIVES OF THE STUDY

2.1 Aim

The aim of this study is to investigate the potential of metabolomics as a tool for better characterising TB.

2.2 Objectives

The above-mentioned aim will be accomplished by completing the following objectives:

1. The development of the most optimal methodological approach for the metabolomics investigations of cultured samples.
2. The application of the relevant developed methodology in objective 1 to metabolite marker identification for the purpose of better characterising rifampicin-resistance conferring *rpoB*-mutations in *M. tuberculosis*.
3. The application of the relevant developed methodology in objective 1 to metabolite marker identification for the purpose of better characterising a pulmonary TB disease state in humans using patient collected sputa.

3. STRUCTURE OF THESIS

This thesis is a compilation of chapters written specifically to comply with the requirements of the North-West University, Potchefstroom Campus, South Africa, for the completion of the degree Philosophiae Doctor (Biochemistry) in thesis format. In order ensure easy reading and a logical flow, all chapters comprise of their own introduction, materials and methods, results, discussion, conclusions, and reference sections.

The current chapter (Chapter 1) gives a brief background of the conducted study, focussing on the problem statement, aim and objectives. This chapter also discusses the structure of the thesis and the outcomes of the study and clarifies the contributions and roles of each co-author and co-worker towards the completion of this study and the resultant patent and publications which emanated.

Chapter 2 provides an overview of the relevant literature required as a basis for understanding the results, discussion and conclusions in the chapters that follow. A part of this chapter has been published in the *Journal of Cell and Tissue Research* (Appendix A).

Chapter 3 describes the development of a GC-MS, fatty acid, metabolomics methodology for characterising various cultured *Mycobacterium* species. In this chapter, three fatty acid extraction procedures, prior to GC-MS analyses, were compared based on their respective repeatability and extraction efficiencies. The GC-MS generated data of the best performing extraction approach was consequently analysed using multivariate statistical data analysis (PCA and PLS-DA), in order to identify those metabolite markers contributing most to the variation between the compared bacterial species groups. These metabolite markers were then validated and the capacity of the method to extract markers of biological relevance, using previously described literature, are described. The detection limit or minimum amount of sample required for this approach was subsequently determined. A part of this chapter has been published in the *Journal of Microbiological Methods* (Appendix A).

In Chapter 4, the above mentioned GC-MS fatty acid, metabolomics approach was used to differentiate between two rifampicin-resistance conferring *rpoB* mutants and a wild-type *M. tuberculosis* parent strain. Furthermore, metabolite markers were identified in order to investigate the effect these *rpoB* mutations on the fatty acid metabolism and the related metabolic pathways of these organisms. A part of this chapter has been submitted for publication to *OMICS* (Appendix A).

Chapter 5 describes our efforts to adapt, modify and improve the metabolomics methodology developed in Chapter 3, and applied in Chapter 4. This improved approach was, furthermore, compared to another, similar approach, which has the ability to extract a much wider variety of compounds considering that, as per definition, metabolomics takes "all metabolites in a biological sample" into account. These methods were compared with regards to their repeatability, extraction capacity, speed, simplicity, detection limits, and the markers identified by each (validated from a biological relevance perspective), using the same organism culture sample repeats as described in Chapter 3. Parts of this chapter have been published in the *African Journal of Microbiology Research* (Appendix A) and were used for the compilation of the registered preliminary patent (Appendix B).

In Chapter 6, the best performing approach, developed in Chapter 5, was modified for application to the metabolomics analyses of patient collected sputa. This entailed a comparison of four sputum preparation methods, prior to extraction and GCxGC-TOFMS analyses, considering their comparative repeatability, extraction efficiency, and capacity to differentiation between *M. tuberculosis* spiked and control sputum sample repeats. We additionally identified those compounds best explaining the variation between the two sample groups, validated these from a biological relevance perspective considering published literature, and determined the

minimum number of cells required (detection limit) for detecting the variation between the sample groups.

Chapter 7 describes the application of the metabolomics experimental approach developed in Chapters 5 and 6 to metabolite marker identification using 95 patient collected TB-positive and TB-negative sputum samples. Furthermore, the markers identified in this chapter, and those identified previously from the cultured samples in the preceding chapters, were discussed by focussing on their various origins including those detected due to: 1) the physical presence of *M. tuberculosis* in these sputum samples; 2) *in vivo* growth conditions of *M. tuberculosis*; and 3) markers related to the host's response to infection.

Chapter 8 is a comprehensive discussion and conclusion of the results obtained in Chapters 3, 4, 5, 6, and 7. Additional recommendations and future research prospects, potentially emanating from this research, are discussed.

4. OUTCOMES OF THE STUDY

The publications and preliminary patent which originated from this study are attached in Appendix A and B respectively, should the reader be interested.

Please note that the regulations of the patent act of South Africa prohibited the distribution of the work done in this study to the public domain before the successful registration of the preliminary patent. Therefore, the submission of the work done in this study for publication was temporarily delayed and done only at a belated stage of the study.

4.1 Manuscripts - Appendix A

OLIVIER I., LOOTS, D.T. 2011. An overview of tuberculosis treatments and diagnostics. What role could metabolomics play? *J. Cell Tissue Res.* 11(1):2655-2671.

OLIVIER, I., LOOTS, D.T. 2012. A metabolomics approach to characterise and identify various *Mycobacterium* species. *J. Microbiol. Meth.* 88:419-426.

OLIVIER, I., LOOTS, D.T. Altered fatty acid metabolism due to rifampicin-resistance conferring mutations in the *rpoB* gene of *M. tuberculosis*. *OMICS*. Submitted. Manuscript nr. OMI-2012-0028.

OLIVIER, I., LOOTS, D.T. 2012. A comparison of two extraction methods for differentiating and characterizing various *Mycobacterium* species and *Pseudomonas aeruginosa* using GC-MS metabolomics. *Afr. J. Microbiol. Res.* 6(13): 3159-3172.

4.2 Registered preliminary patent - Appendix B

LOOTS, D.T., OLIVIER, I. 2011. Method of distinguishing between different pathogens.
Preliminary patent: ZA, 2011/03029. 54 p.

5. AUTHOR CONTRIBUTIONS

The principle author of this thesis is Ilse Olivier. The contribution of the co-authors, co-workers, and collaborators made towards this work is given in Table 1.1.

The following is a statement from the co-authors confirming their individual roles in the study and giving their permission that the data generated and conclusions made may form part of this thesis.

I declare that my role in this study, as indicated in Table 1.1, is representative of my actual contribution and I hereby give my consent that this work may be published as part of the Ph.D. thesis of Ilse Olivier.



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Prof. D.T. Loots



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I. Olivier

Table 1.1: Research team

Co-author	Co-worker	Collaborator	Contribution
I. Olivier (B.Sc. Hons. Biochemistry)			Responsible, together with the promoter, for the planning, execution, data analyses, and writing of the thesis, patent, publications, and all other documentation associated with this study.
Prof. D.T. Loots (Ph.D. Biochemistry)			Promoter: Co-ordinated and supervised all aspects of the study including: study design, planning, execution, and the writing of the thesis, patent, publications, and all other documentation associated with this study.
	Dr. G. Koekemoer (Ph.D. Statistics)		Assisted with the study design and data analyses from a statistical perspective.
	J.C. Schoeman (B.Sc. Hons. Biochemistry)		Assisted with sample analyses in Chapter 6.
		The Royal Tropical Institute (KIT)	Performed the bacterial culturing and provided access to all bacterial samples.
		AMPATH laboratories	Collected and provided all patient sputum samples.

6. REFERENCES

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