Proteomic approaches to early diagnosis of Johne’s disease in dairy cows

by

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ABSTRACT

PROTEOMIC APPROACHES TO EARLY DIAGNOSIS OF JOHNE’S DISEASE IN DAIRY COWS

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*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis or Johne’s disease (JD), an infectious, chronic granulomatous enteritis of ruminants. Current diagnostic tests for JD have limited sensitivity and specificity for the identification of subclinically infected animals warranting an investigation of MAP species-specific and sensitive antigens that might improve diagnostic test sensitivity and specificity.

The aim of the first study was to identify antigenic proteins from the MAP cell envelope by comparing protein profiles from MAP, *Mycobacterium avium* subspecies *hominissuis* and *M. smegmatis* using a 2D-DIGE proteomic approach. Thirteen protein spots were selected and 15 proteins were subsequently identified by LC-MS/MS.

The aim of the second study was to generate antibodies to recombinantly expressed MAP cell envelope proteins as well as to an extract of MAP total cell envelope proteins that was subsequently used to identify MAP organisms by IHC and immunomagnetic separation. Six MAP cell envelope proteins (SdhA, FadE25_2, FadE3_2, MkI, DesA2 and hypothetical protein MAP1233) were recombinantly expressed, three of which (SdhA, FadE25_2 and DesA2) were suitable for polyclonal
antibody generation. Polyclonal antibodies generated to an extract of MAP cell envelope proteins detected MAP organisms in infected tissues using IHC and IF techniques thereby providing a more sensitive alternative to acid-fast staining of MAP in tissues for JD diagnosis. Furthermore, these antibodies were effective in immunomagnetic capture of MAP microorganisms in solution thereby providing proof-in-principle for a novel diagnostic approach.

The objective of third study was to use an extract of MAP total cell envelope proteins as well as the six recombinant MAP proteins in ELISA formats to detect MAP-specific antibodies in cattle serum. The diagnostic sensitivity and specificity of the MAP envelope protein ELISA after serum absorption was 75% and 96% respectively. While ELISAs using the six recombinant protein antigens had reasonably high sensitivities, specificities were comparatively less than the commercial IDEXX serum ELISA. The potential use of these recombinant proteins ELISAs for diagnosis of early MAP infection and control of JD requires further investigation and validation.
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Declaration of work performed

I declare that all the work performed in this thesis was performed by me and exceptions were given below.

Mr. Allison MacKay (Biomedical Sciences, University of Guelph) helped me with the 2D-DIGE experiments mentioned in the chapter 1. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis mentioned in the chapter 1 was performed at the SPARC BioCentre (SickKids Proteomics Analytics Robotics & Chemical Biology Centre) at the Hospital for Sick Children (Toronto, Canada). Immunization of rats and collection of blood samples mentioned in chapter 2 was done by technicians in the Central Animal Facility in University of Guelph, Canada.
# Table of Contents

Abstract.........................................................................................................................ii
Acknowledgements ........................................................................................................iv
Declaration of work performed .....................................................................................vii
Table of contents ...........................................................................................................viii
List of tables ..................................................................................................................xi
List of figures ................................................................................................................xii
List of abbreviations .......................................................................................................xiii

Literature Review...........................................................................................................1

1. Introduction .............................................................................................................1

2. History of paratuberculosis .......................................................................................1

3. Characteristic features of the MAP ......................................................................2

4. Characteristic features of Johne’s disease ..............................................................12

5. Economic impact .....................................................................................................15

6. Host ........................................................................................................................17

7. Zoonosis ..................................................................................................................18

8. Susceptibility ...........................................................................................................18

9. Transmission ...........................................................................................................19

10. Pathogenesis .........................................................................................................20

11. Stages in clinical manifestations ..........................................................................22

12. Diagnosis ...............................................................................................................24

13. Control ...................................................................................................................29

14. Research question ..................................................................................................31

14.1. Hypothesis and objectives .................................................................................31
14.2. Rationale.................................................................32
15. Expected outcome.......................................................36
16. References.................................................................36

Chapter 1........................................................................52
Identification of antigenic proteins from Mycobacterium avium subspecies paratuberculosis cell envelope by comparative proteomic analysis........52

1.1. Abstract..................................................................52
1.2. Introduction.............................................................53
1.3. Materials and methods.............................................56
1.4. Results.....................................................................63
1.5. Discussion..............................................................77
1.6. References..............................................................85

Chapter 2........................................................................90
Generation of polyclonal antibodies to recombinantly expressed and purified MAP cell envelope proteins for use in clinical detection of MAP bacteria.......90

2.1. Abstract.................................................................90
2.2. Introduction.............................................................91
2.3. Materials and methods.............................................94
2.4. Results..................................................................108
2.5. Discussion..............................................................123
2.6. References..............................................................129
Development and validation of novel ELISAs with *Mycobacterium avium* subspecies *paratuberculosis* cell envelope protein and MAP-specific recombinant protein antigens for the serodiagnosis of Johne’s disease

3.1. Abstract

3.2. Introduction

3.3. Materials and methods

3.4. Results

3.5. Discussion

3.6. References

General discussion

References

Summary and conclusion

Appendix I

Appendix II
List of Tables

Chapter 1
Table 1: Mass spectrometric identification of antigenic proteins from MAP cell envelope. .......................................................................................................................................................................................... 71

Table 2: Characteristics of MAP cell envelope proteins and orthologous mycobacterial proteins .......................................................................................................................................................................................... 74

Chapter 2

Table 1: List of primers used to amplify DNA fragments and cycling conditions.......................................................................................................................................................................................... 96

Table 2: Codon optimized MAP genes in reference with E. coli.................... 109

Chapter 3

Table 1: Details of the serum samples used in this study and classified based on the MAP test results. .......................................................................................................................................................................................... 140

Table 2: Calculated sensitivities, specificities and ROC curve for the total MAP cell envelope protein (with and without serum absorption) IDEXX serum ELISAs. ..... 146

Table 3: Mean OD_{450} value difference between MAP fecal culture positive and negative serum samples with recombinant protein antigens. ................................................. 150

Table 4: Calculated sensitivities and specificities at the selected cut-off points, ROC curve and Youden index J for the six recombinant protein antigens............................. 152
List of figures

Chapter 1
Figure 1: Comparative proteomic analysis of cell envelope proteins from Mycobacterium avium subsp. paratuberculosis and other mycobacterial species ........64
Figure 2: Proteomic analysis of cell envelope proteins from Mycobacterium avium subsp. paratuberculosis and other mycobacterial species .................................. 66
Figure 3: Assessment of reactivity of serum samples against cell envelope proteins from Mycobacterium avium subsp. paratuberculosis, M. avium subsp. hominisuis (MAH) and M. smegmatis (MS) by immunoblot analysis. ...................... 67
Figure 4: Identification of antigenic MAP-specific cell envelope proteins. Western blot (2D-PAGE) of cell envelope proteins of MAP probed with serum from JD test-positive cattle .......................................................... 69

Chapter 2
Figure 1: PCR amplification of codon optimized fragments of synthetic MAP DNA ..................................................................................................................... 110
Figure 2: Immunoblot analysis of recombinant proteins with anti-His antibodies ................................................................................................................. 112
Figure 3: Assessment of immunogenicity of recombinant proteins against polyclonal antibodies .................................................................................. 114
Figure 4: Assessment of specificity of polyclonal antibodies to Mycobacterium avium subsp. paratuberculosis and orthologous mycobacterial species .......................................................................................................................... 115
Figure 5: Assessment of specificity of polyclonal antibodies to Mycobacterium avium subsp. paratuberculosis .......................................................................................................................... 116
Figure 6: Assessment of specificity of rat polyclonal antibodies generated against cell envelope proteins from Mycobacterium avium subsp. paratuberculosis .................................................. 117
Figure 7: Immunohistochemical and Immunofluorescence staining of tissues sections using anti-MAP cell envelope antibodies .......................................................... 119
Figure 8: Immunohistochemical and immunofluorescence staining of control animal tissues sections with anti-MAP cell envelope antibodies ........................................ 120
Figure 9: PCR amplification of MAP microorganisms isolated by immunomagnetic capture using rat anti-MAP polyclonal antibodies ........................................... 122
Figure 10: PCR amplification of MAP microorganisms isolated by immunomagnetic capture using rat anti-MAP polyclonal antibodies ........................................ 123

Chapter 3
Figure 1: Receiver operating characteristic (ROC) curves for ELISA with unabsorbed serum and absorbed serum ................................................................. 145
Figure 2: Comparison of receiver operating characteristic (ROC) for ELISA with MAP envelope protein ELISA with and without serum absorption and IDEXX serum ELISA kit ........................................................................................................... 148
Figure 3: Receiver operating characteristic (ROC) for ELISA with recombinant protein antigens ........................................................................................................... 151
List of abbreviations

2-DE-Two-dimensional gel electrophoresis
BSA- Bovine serum albumin
CHAPS-3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CFU- Colony forming units
DNA-Deoxyribonucleic acid
DTT-Dithiothreitol
EDTA-Ethylenediaminetetraacetic acid
ELISA- Enzyme-linked immunosorbent assay
FITC -Fluorescein isothiocyanate
H and E-Hematoxylin and eosin
HRP- Horseradish peroxidase
IAA-Iodoacetamide
IEF -Isoelectric focusing
IPG -Immobilized pH gradient strips
MAP- *Mycobacterium avium* subsp. *paratuberculosis*
OD- Optical density
PBS-Phosphate-buffered saline
SDS-PAGE-sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE- Tris base-acetate-EDTA buffer
TEMED-Tetramethylethylenediamine
TMB -3, 3’, 5, 5’-Tetramethylbenzidine
Literature Review

1. Introduction

Johne’s disease or paratuberculosis is chronic granulomatous enteritis found in ruminants and non-ruminants worldwide (Arsenault et al., 2014; Banasure et al., 2001; Manning and Collins, 2001). The disease is caused by an intracellular pathogen Mycobacterium avium subsp. paratuberculosis (MAP) (Pal et al., 2015) and is responsible for major economic losses to the cattle industry (Garcia and Shalloo, 2015). Since the disease was first reported in 19th century, there have been considerable efforts to understand the complex nature of the disease, to diagnose and control its spread. In spite of these efforts, JD still represents a considerable anathema to the dairy industry due to its global prevalence, lack of effective diagnostics during the early phase of infection, considerable economic losses and lack of effective treatments and vaccines. In addition, possible involvement of MAP in irritable bowel syndrome, Crohn’s disease, type 1 diabetes, multiple sclerosis and autism are reasons for a growing concern from the public health perspective (Cossu et al., 2015; Dow, 2011; Scanu et al., 2007; Sechi and Dow, 2015a).

2. History of Johne’s disease

In 1895, German bacteriologist Heinrich Albert Johne and Langdon Frothingham examined the stomach, intestine and omentum of a cow that died due to wasting and found the intestinal mucosal thickening with enlargement of mesenteric lymph nodes (Twort and Ingram, 1912). Histologically, massive infiltration of leukocytes, epithelioid cells and a few giant cells in the intestinal walls were observed. Acid fast staining of the inflamed tissues revealed red-stained bacteria
(acid-fast) (Chiodini, 1993; Kennedy and Benedictus, 2001; Li et al., 2015). Subsequent injection of guinea pigs with inflamed tissues did not result in their death. They concluded that the cow died of a bacterial infection similar to tuberculosis in birds. Based on these findings, Drs Johne and Frothingham named the condition ‘pseudotuberculous enteritis’ in cattle (Chiodini, 1993; Kennedy and Benedictus, 2001). In 1906, John Mc Fadyean a leading British veterinary researcher on mycobacterium coined the term ‘Johne’s disease’ (Kennedy and Benedictus, 2001). In 1910, the etiological agent responsible for Johne’s disease (JD) was isolated (Twort and Ingram, 1912) and was named “Mycobacterium enteriditis chronicae pseudotuberculosa bovis Johne” (Chiodini, 1993). The bacterium was officially named Mycobacterium paratuberculosis in the 1923 edition of the Bergey’s Manual of Determinative Bacteriology (Chiodini, 1993). In 1990, based on DNA-DNA hybridization techniques Mycobacterium paratuberculosis was renamed as Mycobacterium avium subspecies paratuberculosis (Thorel et al., 1990).

3. Characteristic features of MAP

**Taxonomy and bacteriological properties**

*M. avium* subsp. *paratuberculosis* is a small rod-shaped, Gram-positive, 0.5 µM in wide and 1-2 µM long in size, acid-fast staining, non-motile, very slow-growing with a generation time of 22-26 h (5 to 16 weeks to visible colonies on agar medium), very fastidious, mycobactin-J-dependent, obligate intracellular pathogenic bacterium of ruminants and non-ruminants belonging to the genus Mycobacterium, the family Mycobacteriaceae and the order Actinomycetales (Meißner et al., 2015; Timms et al., 2011). MAP is grouped under the *Mycobacterium avium* complex (MAC). MAC includes *M. avium, M. intracellulare* and four new species including
M. chimaera, M. colombiense, M. arosiense and M. vulneris (Turenne and Alexander, 2010). Furthermore, M. avium contains four subspecies: M. avium subsp. paratuberculosis (MAP), M. avium subsp. avium, M. avium subsp. hominis suis (MAH) and M. avium subsp. silvicium (MAS) (Turenne et al., 2008). However, M. avium subspecies differ in host preferences, growth requirements, tissue tropism and environmental distribution (Rónai et al., 2015). M. avium subsp. avium causes tuberculosis-like lesions in birds (Dhama et al., 2011). Mijs et al., (2002) suggested that M. avium bird strains originated from human and pigs due to similarities in IS1245 RFLP, growth temperature, 16S rRNA sequence signatures and 16S-23S rDNA ITS analysis (Mijs et al., 2002). The M. avium species are commonly present in the environment, and are opportunistic pathogens of humans, and obligate pathogens of birds and livestock (Turenne and Alexander, 2010; Turenne et al., 2007). Based on phenotypic characteristics, host preference, growth requirements, molecular strain typing, comparative genomic studies, whole genome sequencing studies and phylogenetic analysis, MAP is classified into three lineages including the extremely slow growing and fastidious type I strain (Type S/sheep/ovine), the type II strain (Type C/cattle/bovine) and type III strain (subtypes of Type S strain) (Bannantine et al., 2012; Bryant et al., 2016; Collins, 2010; Stevenson, 2010). Recently another strain type has been isolated from bison in Montana, US. Based on pathogenesis, growth requirements and molecular analysis this strain has been named as Type B or Bison strain (Stevenson, 2015b). Type B strains isolated in Indian bison were named as “Indian bison type” and molecular analysis revealed that they were different from US bison strains (Sohal et al., 2013). To understand the biology of MAP, epidemiology and disease transmission, genomic characterization of different MAP strains from
different geographical and animals species are essential (Bryant et al., 2016; Li et al., 2016).

**MAP Envelope**

MAP cell envelope structure is similar to that of other mycobacterial species (Rowe and Grant, 2006). The cell envelope consists of the cytoplasmic membrane and the cell wall core (Kaur et al., 2009). The cytoplasmic membrane is made up of a lipid bilayer and contains membrane proteins. The cell wall core is made up of a peptidoglycan backbone (Tessema et al., 2001) covalently attached via phosphoryl-N-acetylglucosaminosylrhamnosyl linkage units with arabinogalactan (AG) (Kaur et al., 2009; Tessema et al., 2001). The highly branched AG is esterified with fatty acids known as the mycolic acids (Kaur et al., 2009; Tessema et al., 2001). These mycolic acids (C₆₀₋₉₀) form the outermost layer, which is thick, waxy and hydrophobic in nature (Bansal-Mutalik and Nikaido, 2014; Salem et al., 2013). Mycolic acids constitute 30 to 40% of the cell envelope mass (Rastogi et al., 2001). In addition to these components, the cell wall consists of a variety of non-covalently attached lipoglycans (phosphatidyl-myoinositol mannosides, lipomannan and lipoarabinomannan), glycolipids (trehalose-containing lipids and the phenolic glycolipids), polysaccharides (α-D-glucan, arabinomannan and mannan), galactose, glycoproteins, mycothiol, alanine and proteins (Kaur et al., 2009; Rastogi et al., 2001). These complex substances are responsible for bacterial survival in adverse environmental conditions like desiccation, disinfectants, heat and mechanical stress (Houben et al., 2014; Rowe and Grant, 2006) and play an important role in host cell entry, interference with phagosome maturation and modulation of the immune system (Tessema et al., 2001).
**Genome**

The MAP strain K-10 (type C, clinical isolate from bovine) was sequenced and significant findings have been documented by Li et al., (2005). MAP strain K-10 genome contains 4,829,781 base pair sequences with 69.3% of G+C content. The MAP genome contains 4,350 predicted open reading frames (MAP001-MAP4341). MAP has a total of 39 predicted unique proteins with no identifiable homologous protein sequences in the database. The MAP K-10 genome contains repetitive DNA sequences (1.5%) such as insertion sequences (IS) (Total 58 IS), multigene families, and duplicated housekeeping genes. Some of the IS (IS900, IS1311 and ISMav2) are specific to MAP, and they are useful as diagnostic targets (Li et al., 2005). The MAP K-10 genome was re-sequenced and a total of 90 single nucleotide errors and 51bp indels of the MAP K-10 genome were corrected resulting in 27 frame-shift mutations in the ORF (Wynne et al., 2010). PCR screening and a BLAST search of MAP K-10 against other mycobacterial species shows that 13 ORF are unique to MAP (Turenne et al., 2007). Genomic analysis of MAP S397 (S-type strain) showed that this genome has 4,814,922 bp in size and contains 4,700 predicted ORF. Comparative genomic analysis revealed that MAP S397 strain had acquired 10 large sequence regions and were specific to this strain and some of them were shared with MAH 104 but absent in MAP K-10 (Bannantine et al., 2012). For instances, genomic insertions due to large sequence polymorphism (LSP) lead to addition of 70 ORFs into sheep strain (MAPS397) but were absent in MAP K-10 strain, of these 13 were absent MAH104 and 57 were presented in MAH104 (Bannantine et al., 2012). Comparative genomic analysis also showed that 30 ORF were present in MAP K-10 but absent in MAP S397 genome (Bannantine et al., 2012). Significant genomic differences between C-type and S-type were originated from large sequence polymorphisms,
single nucleotide polymorphisms, repeat and insertion sequences and genetic stability and mutations (Stevenson, 2015a). In the evolutionary biology, it is believed that MAP sheep strains were diverged from MAH104 by one step inversion event and subsequently MAP bovine strains were diverged from MAP sheep strains by a second inversion event (Alexander et al., 2009). Genomic comparison of the MAP strain K-10 and the *M. avium* strain104 (5.48 Mb, 4480-4987 ORF) shows that ~ 161 sequences are unique to MAP strain K-10 (Turenne et al., 2007). However, genetic studies such as genetic diversity, gene plasticity, comparative genomics and genomic homogeneity between MAP and *M. avium* species reveal that MAP shares more than 97% of DNA homology (nucleotide level) with *M. avium* strain 104 (Bannantine et al., 2003a; Wu et al., 2006). This close genetic relatedness reduces sensitivity and specificity, and thus hampers the diagnosis of JD by gene-based and serological assays (Britton et al., 2016; Gilardoni et al., 2012).

**MAP Antigens**

Identification and characterization of MAP antigens and their use in the diagnosis of JD by measuring cell-mediated and humoral immune responses are a major focus in MAP research (Olsen et al., 2002). Several antigens have been discovered in MAP for JD diagnostic purposes but only a few have been characterized and tested for their clinical utility (Olsen et al., 2000).

**CMI antigens**

In general, CMI-based assays suffer from low sensitivity and specificity due the antigens used in the assay and most of the studies were conducted with purified protein derivatives (PPDs) (Mikkelsen et al., 2011b). Stimulation of whole blood
samples with a PPD preparation from *M. avium* and a subsequent IFN-γ release assay had a sensitivity of 36.8% and 58.3% for focal and multifocal lesions of MAP-infected animals respectively (Vazquez et al., 2013). However, the IFN-γ release assay had very low specificity (68.9% to 76.4%) in the identification of MAP infection and therefore measuring CMI responses is not adequate for the identification of MAP-infected animals (Vazquez et al., 2013). However, many studies have been conducted with more specific antigens to measure the CMI responses. Fourteen novel recombinant proteins were tested to measure CMI responses in blood samples and their specificity in the diagnosis of early stages of MAP infection in 26 heifers from MAP-infected herd. Of the 14 recombinant proteins, three latent proteins such as putative carbonate dehydrogenase (LATP-1), putative PhiRv2 prophage integrase (LATP-2), and hypothetical conserved protein (LATP-3) showed specific and consistent IFN-γ responses in MAP-infected animals (Mikkelsen et al., 2011a). Immunogenicity of 30 MAP-specific proteins for the induction of CMI responses in subclinically infected sheep was evaluated and three proteins (MAP1297, MAP1365 and MAP3651c) induced high IFN-γ levels in subclinically infected sheep in comparison to the healthy animals (Hughes et al., 2013). Olsen et al. (2000) used MAP alkyl hydroperoxide reductase C and D (Ahp C and D) to induce specific CMI responses. Results revealed that these antigens were strong inducers of IFN-γ production and MAP-specific CMI suggesting their potential use for JD diagnosis (Olsen et al., 2000).

Heat shock proteins such as HspX, Hsp65, GroES, Hsp70, MAP3840 and MAP3841 are strongly immunogenic, but the fact that they are conserved in other bacteria makes them unsuitable for MAP diagnosis (Olsen et al., 2002). Another in-house ELISA-based study tested five recombinant antigens including antigen
complex 85A, B and C, 35-kDa (p35) and superoxide dismutase (SOD) for their immunogenicity (Shin et al., 2004). However, these protein antigens share epitopes with other mycobacterial species. In silico screening of B cell and T cell epitopes of MAP proteins up-regulated under in vitro stress conditions revealed that MAP2698c, MAP2312c, MAP3651c, MAP2872c, MAP3523c, MAP0187c, MAP3567 and MAP1168c, and a hypothetical protein carry large numbers of B cell and T cell epitopes (Gurung et al., 2012a). Some of the epitopes were specific to MAP and these proteins could be useful for study of T and B cell-mediated immune responses within infected hosts and serodiagnostic assays (Gurung et al., 2014b; Gurung et al., 2012a).

Secreted antigens

Secreted proteins are located in the extracellular environment and are available to the sensitized immune cells. Therefore, secreted proteins are considered to be more immunogenic. Immunoblot analysis using field serum samples from JD test-positive animals by fecal culture showed higher immunoreactivity to secreted proteins than to proteins of intracellular origin (Cho and Collins, 2006). Proteomic analysis of MAP culture filtrate fraction identified a total of 121 proteins, out of which 25 were specific to MAP (Leroy et al., 2007). Five proteins including MAP1693c, MAP2677c, MAP3199, MAP0586c and MAP4308c were further tested in ELISA with a panel of serum samples from 19 MAP-positive and 48 negative cases. Three (MAP1693c, MAP4308c and MAP2677) out of five proteins showed a sensitivity and specificity of 73.68% and 91.67% respectively (Leroy et al., 2007). Another recent study identified 162 proteins from MAP culture filtrate using a 2-step fractionation method and reverse-phase liquid chromatography. Out of these, 66 proteins were not reported earlier in the MAP culture filtrates (Facciuolo et al., 2013). Subsequent immunoblot analysis of serum (n=14) samples from JD test-positive cattle by fecal culture and
ELISA showed that proteins of various molecular weights including 28 kDa [i.e. two co-migratory proteins (MAP1981c and MAP0471)], 47 kDa [i.e. two co-migratory proteins (MAP0196c and MAP1569)] and 52 kDa (MAP0196c) were strongly immunogenic and incorporation of these antigens in an ELISA may aid in JD diagnosis and control (Facciuolo et al., 2013). Five recombinantly expressed stress-associated secretory proteins including MAP2411, ClpP (MAP2281c), Ppa (MAP0435c), MAP0593c and GreA (MAP1027c) have been tested for their immunogenicity by ELISA with sheep serum samples from positive and healthy controls, but test results were inconclusive (Gumber et al., 2009).

**Cell envelope antigens**

Cell envelope proteins (cell wall and membrane) play critical roles in the bacterial pathophysiology during attachment / colonization and infection of target tissues / cells and are accessible to the host immune system and are considered to be very immunogenic (Leite et al., 2015). A study involving cell surface “shaving” of live MAP cells with trypsin and subsequent LC-MS/MS analysis of cleaved protein products to identify proline–proline–glutamic acid protein families (PPE) on the MAP cell wall revealed two PPE proteins (MAP1506 and MAP3420c) that were surface-exposed and MAP3420c induced humoral immunresponses in MAP infected animals (Newton et al., 2009). However, orthologous genes are also present in MAH and MAA genomes, and could affect the sensitivity and specificity of diagnostic assays (Newton et al., 2009). Blue native PAGE and subsequent 2-DE PAGE analyses of MAP envelope proteins identified ten antigenic proteins (Leite et al., 2015). From these, five proteins, MAP2121c (MMP), MAP2120c (cysteine desulfurase), Cfp29 (MAP0630c), MAP2855 (phage shock protein A), and MAP3290c (Mpt64), reacted strongly by immunoblotting and IFN-γ release assay using serum and whole blood
samples from Holstein dairy cows naturally infected with MAP (Leite et al., 2015). Animals experimentally and naturally infected with MAP showed strong humoral immune responses to two cell wall proteins namely MAP 1204 and MAP1087 (Bannantine et al., 2008b). However, these antigens were not MAP species-specific resulting in cross-reactivity with serum samples from *M. tuberculosis* positive cases (Bannantine et al., 2008b). An ELISA developed with a recombinant immunodominant 34 kDa extracellular protein that is MAP species-specific correctly identified 18 MAP positive and 50 negative cattle (Malamo et al., 2006). Comparative proteomic and genomic analysis of MAP purified protein derivatives (PPD) and subsequent recombinant expression of three selected proteins (MAP1138c, MAP1718c and MAP3515c) and analysis of their immunogenicity revealed that MAP1138c (LprG) had greater serum antibody responses to JD test-positive by fecal culture (n=20) than negative (n=20) animals, despite the protein being conserved among the mycobacterial species (Santema et al., 2009). Proteomic characterization of a purified protein derivate from MAP (Johnin PPD or PPDj), a purified protein derivate from *Mycobacterium avium* subspecies *avium* (PPDa) and a purified protein derivate from *Mycobacterium bovis* (PPDb) showed that PckA, Mdh, Tig and Eno proteins were specific to PPDj and their immunogenicity needs to be evaluated (Wynne et al., 2012). Another study screened MAP-specific coding sequences and found that 87 sequences were specific to MAP. Among these, three sequences were recombinantly expressed and evaluated in ELISA format with 18 JD-positive and 48 negative serum samples to measure MAP-specific antibodies and two out of three proteins namely antigen 6 and MAP1637c showed a 72 and 82% sensitivity respectively with specificities of 98% (Leroy et al., 2009).
**Lipoprotein antigens**

Lipids are major constituents of the mycobacterial cell wall and are accessible to the host immune system thereby generating antibodies to the lipid components. For example, the lipoarabinomannan and lipid-free arabinomannan are strong immunogenic macromolecules on the MAP cell wall, but they are also widely conserved in mycobacteria (Olsen et al., 2002). Lipopentapeptide (L5P) is MAP species-specific and immunogenic. To prove this, L5P was tested with a panel of serum samples from MAP or, *M. bovis* or *Mycobacterium avium* subsp. *avium* (MAV) infected animals and MAV and *M. avium* subsp. *intracellulare* positive human cases. The test results revealed that L5P is more sensitive and specific in the diagnosis of MAP infection in comparison to the MAP-PPDj-based diagnostic test (Biet et al., 2008). Lipid Para-LP-01 is a major cell wall lipopeptide component of MAP that shows seroreactivity with MAP-positive serum samples (Eckstein et al., 2006). PstA, another MAP-specific lipoprotein is involved in MAP invasion, biofilm formation and immunogenicity. Immunoblot analysis of serum samples from a cow subclinically infected with MAP strongly reacted with synthetic PstA peptides in comparison to cows in clinical stages of JD. Negative serum samples were non-reactive to PstA peptides (Wu et al., 2009). Diagnostic utility of L5P, Para-LP-01 and PstA needs to be evaluated with suitable sample sizes (Mikkelsen et al., 2011b).

To date, no obvious fully characterized and evaluated MAP species-specific antigens are included in the commercially available JD diagnostics for either detection of cell-mediated immune responses (IFN-γ) or antibody mediated immune responses for antigen or antibody detection. This is due to the fact that the majority of MAP antigens contain epitopes shared with/amongst other closely related mycobacteria and there is no single MAP-specific antigen that could detect all infected animals at
different stages of infection. This poses a considerable challenge in the selection of suitable antigens (Facciuolo et al., 2013).

4. Characteristic features of Johne’s disease

Prevalence of JD

JD is worldwide in distribution (Over et al., 2011) and is endemic in many countries (Gilardoni et al., 2012). Different methods, such as measuring of i) MAP antibodies in serum and milk, ii) culturing of fecal, environmental and tissues samples, and iii) PCR-based techniques applied to feces, milk, blood and tissues have been routinely used to estimate prevalence rate of JD (Barkema et al., 2010).

In 1908, Pearson described the first case of JD in Pennsylvania (US) and thereafter, the disease has been reported in many other parts of the USA and the world (Twort and Ingram, 1912). The disease has subsequently crossed the boundaries of many countries due to animal trade and inadequate biosecurity measures. In Asia and Africa, JD was reported in 1920s. From the 1970s onwards, the disease has been reported globally (Barkema et al., 2010).

Herd level

In Canada, 30% of dairy herds have a minimum of two cows seropositive for JD (Sorge et al., 2010). In Alberta, the herd-level prevalence is 26.8% ± 9.6% and 27.6% ± 6.5% to 57.1% ± 8.3%, determined by ELISA and fecal culture respectively (Sorensen et al., 2003). Another study reported that in Alberta, 58.8% of herds were seropositive for MAP (Scott et al., 2006). The seroprevalence at the herd level for MAP in Maritime cattle in Canada is 16.7% (VanLeeuwen et al., 2001). In Ontario, herd level prevalence of MAP is 27.2% as determined by milk ELISA based on testing of all milking cows in the herd at one point of time (Pieper et al., 2015).
In the USA, serosurveillance studies revealed that 21.6% of dairy herds (Wells and Wagner, 2000) and 7.9% of beef herds were test-positive for JD (Dargatz et al., 2001b). In Europe, the herd level prevalence of JD is more than 50% (Nielsen and Toft, 2009). MAP has been reported in free-living and captive wild animals and an average of 2.4% of wildlife are JD test-positive (Carta et al., 2013). Analysis of 486 rabbit’s revealed a MAP prevalence rate of between 23 to 39.7% (Judge et al., 2006).

Environmental samples collected from Alberta (n=360) and Saskatchewan (n=166) dairy farms were screened for presence of live MAP to estimate herd-level prevalence of MAP. A subsequent Bayesian computation model was used to estimate a true herd prevalence of 68% and 76% for Alberta and Saskatchewan dairy farms respectively (Wolf et al., 2014). Environmental samples from 80 dairy herds in Minnesota showed that 78% of the herds were culture positive for MAP (Raizman et al., 2004). Lombard et al (2006) collected 483 environmental samples from 98 dairy herds from 21 American states for MAP culture analysis and found that 45.5% of the environmental samples were culture positive (Lombard et al., 2006a). They concluded that in US 70% of dairy operations were affected with MAP (Lombard et al., 2006a). Another study examining the seroprevalence of MAP in 21 Californian dairy herds reported that 43% of dairy herds were test positive for MAP ELISA (Tavornpanich et al., 2008).

**Cow level**

A seroprevalence study conducted by Wells and Wagner (2000) showed that 3.4% of dairy cows in the USA were infected with MAP (Wells and Wagner, 2000). Bacteriological and histological examination of lymph nodes and ileum samples from 994 dairy cows from Eastern Canada and Maine slaughter houses showed a
prevalence of 16.1% and 3.6% respectively at the cow level (McKenna et al., 2005a). Results of a serological survey of 4579 beef cattle showed a prevalence of 3.0% and 43.8% at the cattle and herd levels respectively (Roussel et al., 2005). Cow level seroprevalence of MAP in seven Canadian provinces (n=315 herds) was 3.6% (Tiwari et al., 2009). The seroprevalence rate of MAP in Maritime cattle in Canada is 2.6% at the cow level (VanLeeuwen et al., 2001). In Europe, cattle level prevalence rate varies from 3-20% and this prevalence variation was due to the inherent limitation of the diagnostic tests and experimental designs (Nielsen and Toft, 2009).

**Milk, milk products, meat, soil and drinking water**

A number of studies have reported the presence of live MAP organisms or genomic material in raw and/or pasteurized milk and milk products (Gill et al., 2011; Hruska et al., 2011; Paolicchi et al., 2012). It is a growing concern due the involvement of MAP in some patients with Crohn’s disease. A total of 702 pasteurized milk samples purchased from California, Minnesota and Wisconsin were tested for live MAP organisms (Ellingson et al., 2005). Viable MAP has been isolated from 20 (2.8%) milk samples that were further confirmed by PCR (Ellingson et al., 2005). A total of 100 raw milk samples from Swiss cattle were analysed to estimate the prevalence rate by real time PCR targeting the f57 gene and test results revealed that 3% of milk samples were test-positive for MAP (Bosshard et al., 2006). Stephan et al (2007) used a f57 gene-based real-time PCR technique to analyse raw milk and Swiss cheese samples (n=143) and found a prevalence rate of 4.2% (Stephan et al., 2007). Sixty-three (28.6%) out of 220 bulk milk tank samples from dairy herds from Cyprus were test-positive for MAP IS900 and f57 gene based on real-time quantitative PCR assay (Slana et al., 2009).
MAP has been isolated and confirmed from natural water sources and sediments by MAP culture and PCR-based techniques (Gill et al., 2011). In Ohio, drinking water and bio-film samples were collected from 33 households and/or commercial buildings and were tested for MAP. Test results revealed that 88 and 76 % of water and bio-film samples were positive for MAP IS900 PCR, respectively (Beumer et al., 2010). Rhodes et al (2013) analysed 1902 soil samples from various regions of Great Britain for MAP prevalence rate by real-time qPCR and results revealed that 115 (10.5 %) soil core samples were test-positive (Rhodes et al., 2013).

However, these reported prevalence estimates are most likely underestimates, due to the variability in sensitivity and specificity of the current diagnostic techniques, and improperly designed experiments (Barkema et al., 2010). Because present diagnostic tests are best suited for later stages of the disease than earlier stages, the JD prevalence rate is likely underestimated globally at the herd and/or cow level (Magombedze et al., 2013; Whitlock and Buergelt, 1996). It is believed that currently available diagnostic tests are able to detect only the tip of the iceberg regarding JD-infected animals (Whitlock and Buergelt et al., 1996).

5. Economic impact

MAP affects dairy and beef herds but it incurs significant economic losses to the cattle industry. Economic losses associated with MAP are due to reduced milk production (McAlloon et al., 2016) and quality (Gonda et al., 2007), reduced body weight gain (Kudahl and Nielsen, 2009), premature culling (Lombard et al., 2005), reduced carcass value (More et al., 2015), increased calving interval (Smith et al., 2010), poor feed conversion ratio, diagnosis and treatment costs, death of infected animals, heifer replacement costs, loss of good germplasm and negative impact on
animal trade (Vidić et al., 2013). Estimated annual losses to the Canadian dairy industry due to JD is 15 million dollars (Cho et al., 2013). As per a 1996 national dairy study, the estimated annual losses to the US agriculture and dairy industry due to JD was more than 200 million dollars (Ott et al., 1999). Economic losses increased from $35 to $72/cow/year over a twenty year period (Groenendaal and Galligan, 2003). The estimated annual loss due to a JD-infected 50 cow herd is $2,472 in Canada (Chi et al., 2002). Another study reported that, in Canada, an average herd with 61 cows with seropositivity of 12.7% to MAP incurs an annual loss of $2,992 (Tiwari et al., 2008).

**Milk production and culling risk**

There is a strong negative correlation between milk production and JD. Furthermore, this is associated with the various stages of JD infection; early phase of infection has less impact on milk production than later stages which has more impact on milk production. This is due to reduced absorption of nutrients from inflamed intestinal tissues (Smith et al., 2009). Animals that test positive have a decline in milk production of 173 to 458 kg (6 to 8%) in a 305–day lactation period than animals that were test-negative (Hendrick et al., 2005). Another study which tested 569 herds (n=15,490 cows) showed that ELISA-positive, PCR- or fecal culture-positive, and Ziehl-positive cases produce less by 1.58 to 3.30, 2.03 to 2.51 and 5.36 to 7.20 kg/day respectively than animals that were test-negative (Beaudeau et al., 2007). Cows that test positive for JD by milk ELISA produce 2.9 to 6.8% less milk and cows with high test positive results in 1st, 2nd and 4th lactation produced 466, 514, and 598 kg less milk respectively than cows with low test-positive (Sorge et al., 2011). Raizman et al. (2009) analysed data from 1,048 cows to calculate economic losses associated with
MAP and found that MAP fecal culture-positive cows recorded 1,355 kg less milk production than culture-negative cows (Raizman et al., 2009). Cows that were test positive for JD by ELISA or fecal culture or test positive by both diagnostic tests produce 2.5 kg less of 4% fat corrected milk (FCM), 2.2 kg less of 4% FCM, and 4.7 kg less of 4% FCM per day respectively than animals that were test negative (Aly et al., 2010).

The chances of culling of MAP culture-positive cows was 3 times higher than culture negative cows (Raizman et al., 2009; Smith et al., 2010). A total of 1,014 slaughter house carcasses of adult Friesian cattle that were tested to determine the impact of JD on carcass weight found that mean body weight loss varied from 3.7 and 12.4 % cases for histological JD and MAP antibody ELISA test respectively (Vázquez et al., 2012). Reduction of 22 and 26% body weight was observed in animals with heavy bacterial loads and visible gross pathological lesions respectively (Vázquez et al., 2012). Carcass weight and quality was compared with MAP milk ELISA, and test-positive animals showed a reduction of 10 and 17% carcass weight and quality respectively than test negative animals (Kudahl and Nielsen, 2009). Similarly, a reduction of 15% in carcass weight and 31% in carcass quality were noted in fecal culture-positive animals than negative animals (Kudahl and Nielsen, 2009). Animals with enteritis or edema revealed a reduction of 20 and 31% for carcass weight and quality respectively (Kudahl and Nielsen, 2009).

6. Host

MAP primarily affects domesticated and non-domesticated ruminants such as cattle, buffalo, sheep, goat, camels, llamas, giraffes, white-tailed deer, red deer, fallow deer, elk, bighorn sheep, mountain goats, bison, antelope and wildebeests (Carta et al.,
The spill-over of MAP into non-ruminants such as pigs, horses, donkeys, dogs, red fox, feral cats, rabbits, brown rat, field mouse, birds and non-human primates has also been reported (Florou et al., 2008; Matos et al., 2014; Mönki et al., 2015). MAP has also been isolated from testes, uterus, placenta, fetuses and milk of rabbits (Judge et al., 2006). It has been concluded that, rabbits play a vital role in environmental persistence of MAP and inter-species transmission to domesticated ruminants (Judge et al., 2006).

7. Zoonosis

MAP has been implicated in the development of Crohn's disease (CD) in humans and MAP has been isolated from tissues of some CD patients (McNees et al., 2015). Humans are also as equally susceptible to MAP infection as cattle (Davis, 2015). MAP has been linked with other diseases such as multiple sclerosis, Hashimoto thyroiditis, type 1 diabetes and Blau syndrome. Genetic susceptibility of humans to mycobacterial infections and exposure of such individuals to MAP might play an important role in zooses (Sechi and Dow, 2015a). Certain MAP proteins (e.g. HSP65, HSP70, MAP3865c, MAP_2694, MAP4027, and MAP_2619c) mimic human proteins (Glutamic acid decarboxylase (GAD), ZnT8-pancreatic, ZnT8-thyroid and myelin basic proteins) and antibodies against these proteins may cross-react with human tissues. This may lead to autoimmune diseases in humans (Sechi and Dow, 2015a).

8. Susceptibility

Young calves less than 6 months (Lombard, 2011) to 12 months of age are more susceptible to MAP infection than adult animals (Marcé et al., 2011), but adults
are also susceptible to MAP infection (Pradhan et al., 2011). Calves in their first 24 hrs of life are at risk to MAP infection due to increased intestinal permeability (Lombard, 2011). The susceptibility of young ruminants is also linked to the developing immune system, and the presence of large numbers of transient ileal Peyers Patches (PP) compared to adult ruminants that provide more M-cells to favour the uptake of MAP organisms (Koets et al., 2015b). Calves less than 6 months of age have a higher likelihood (75%) to contract JD infection upon contact with MAP organisms and this susceptibility becomes 50% for 6 to 12-month-old calves, whereas only 20% of cattle older than 12 months get infection after coming in contact with MAP (Windsor and Whittington, 2010). Adult animals also get infected with MAP depending on the frequency of exposure and dose (Stabel, 2006).

9. Transmission

Subclinically and clinically infected animal and MAP super shedders in a herd are source of infection to the uninfected and susceptible herd-mates. Infected animals with subclinical disease, a cohort that is not detected by current JD-tests, intermittently shed MAP organisms in the feces and thereby contaminate teats, milk, calving environments, feed, water and pasture. Neonates, young animals and adult animals are infected by the fecal-oral route. This is the major route of disease transmission in which susceptible animals get infected by consumption of milk, feed, water and pasture contaminated with fecal materials containing viable MAP (Marcé et al., 2011; Whittington and Sergeant, 2001). Indeed, viable MAP has been recovered from mammary tissues, colostrum and milk (Stabel et al., 2014). Therefore, feeding of young calves with colostrum and milk from infected animals is also a feasible route in the transmission cycle of MAP (Donat et al., 2016; Nielsen et al., 2008).
Intra-uterine and placental barrier transmission of MAP from infected dam to fetus have been reported by many researchers (Adaska and Whitlock, 2012; Whittington and Windsor, 2009). Meta-analysis of data from reported studies revealed that 9 and 39% of fetuses from subclinically and clinically infected cows were respectively infected with MAP (Whittington and Windsor, 2009). MAP has been isolated from testes, semen, epididymis and vesicular gland. Therefore, semen from MAP test-positive breeding bulls can transmit the disease (Abbas et al., 2011; Khol et al., 2010; Münster et al., 2013).

10. Pathogenesis

After ingestion MAP reaches the small intestine and specialized intestinal epithelial cells (M cells) and/or enterocytes, thereafter within few minutes to hours which then translocate MAP into the lamina propria (Arsenault et al., 2014; Lamont et al., 2012; Momotani et al., 1988). MAP is then phagocytosed by subepithelial macrophages and dendritic cells where they survive or are killed by host defence mechanisms (Koets et al., 2015a). Macrophages use different pathways to engulf MAP bacteria including complement receptors (CR1, CR3 and CR4), immunoglobulin receptors (FcR), mannose receptor and scavenging receptors. Depending on the route of entry, MAP can be killed or can survive in macrophages and induce different immune responses (Arsenault et al., 2014; Bannantine and Bermudez, 2013). At this stage, infected macrophages are activated by cytokines such as interferon gamma (IFN-γ), interleukin-1 alpha (IL-1α), IL-6, IL-2 and tumor necrosis factor-alpha (TNF-α) from Th1- type T-helper lymphocytes (Stabel, 2010). These cytokines play a vital role in MAP clearance and in containment of the infection (Coussens, 2001; Stabel, 2010). During the earlier stages of infection γ δ T
cells plays a vital role in the granuloma formation, maintenance and protective immune responses against mycobacterial infections (Plattner et al., 2009). Thus may affect the adaptive immune responses to mycobacterial infections (Plattner et al., 2009). Subcutaneous infection of bovine calves with live MAP cells using matrigel recruited more $\gamma \delta$ T cells within 7 days post infection than 30 days post infection and released more IFN-\(\gamma\). Experimental results suggest the role of $\gamma \delta$ T cells in the protective innate early immune responses against MAP infections (Plattner et al., 2012). However, in the majority of cases this mechanism is not sufficient enough to clear MAP infection. Survival of MAP bacteria within macrophages is dependant on its ability to prevent phagosome acidification, phagosome maturation and phagolysosome fusion to evade oxidative and hydrolytic damage to MAP (Stevenson, 2015b). Phagolysosome fusion is affected by the mycobacterial cell envelope components that disrupt lysosomal membranes by preventing phagosomal maturation, phagosome acidification and apoptosis (Coussens, 2001; Goren et al., 1987). MAP proliferates slowly over a period of time (2 years or more) and inexorably and progressively spreads the infection (Tiwari et al., 2006). During this subclinical period animals appear healthy but can shed live MAP organisms intermittently through the feces. The disease progression was thought to be associated with a shift from Th1 to Th2-type T-helper lymphocyte-mediated immune responses (Coussens, 2001; Stabel, 1998; Tiwari et al., 2006), although recent studies showed the existence of both cell mediated and antibody mediated immune responses during early stages of MAP infection suggesting a complex interplay between MAP and host immune system. This may significantly affects the disease manifestation or resistance (Begg et al., 2011; Waters et al., 2003). Th2 T-helper lymphocytes become dominant and secrete cytokines such as IL-4, IL-10 and transforming growth factor (TGF)-\(\beta\) (Koets et al.,
These cytokines down-regulate the IFN-γ response and produce a Th2 response, which leads to antibody production though B cells (Coussens, 2001). The increased cytokine (IL-4, IL-5 and IL-10) production deregulates the immune response and attracts higher numbers of lymphocytes and macrophages results in the thickening of the inflamed intestinal mucosa (Salem et al., 2013). The loss of Th1 dominant immune responses and killing of infected cells leads to growth of the pathogen, shedding of MAP in the feces, milk and colostrum without any clinical signs. Eventually, fecal shedding of MAP organisms and serum antibodies can be detectable once clinical signs of JD become apparent (Coussens, 2001).

11. Stages in clinical manifestations

There are four stages in clinical manifestation of JD: silent infection, subclinical infection, clinical infection and advanced clinical infection.

Silent infection

Calves, heifers, young and adult cattle are included in this stage (Fecteau and Whitlock, 2010). Sometimes the host defense mechanism successfully contains the MAP infection, and MAP proliferates slowly and spreads into ileal submucosa and mesenteric lymph nodes. This stage may last for a minimum period of 2 years or more (Lavers, 2013). During this stage, infected animals appear healthy (Fecteau and Whitlock, 2010), but can shed MAP in the feces intermittently (Tiwari et al., 2006). High levels of anti-inflammatory markers have been reported in the circulation and localized tissues upon early infection with MAP. At postmortem examination, there may be little or no inflammatory changes in the infected intestinal tissues during this
stage of infection. Current serological tests are insensitive for the diagnosis of silent infection (Everman, 2014).

**Subclinical infection**

During this stage, contained MAP slowly overcomes the host immune responses by down-regulating the cell-mediated immune responses and resulting in an increased humoral immune response (Lavers, 2013). At this stage, infected animals appear healthy (Tiwari et al., 2006), with intermittent, low level fecal shedding of MAP, which contaminates the environment and spreads the infection throughout the herd (Stabel, 2006). This phase may continue for many years. Diagnostic tests such as fecal and milk culture, fecal PCR and measuring of humoral immune responses are inconsistent in the identification of the subclinically infected animals (Everman, 2014).

**Clinical and advanced stages of infection**

After a long period of 2 to 10 years of subclinical disease progression, animals enter into the clinical phase. In this stage, animals have gradual weight loss, normal or increased appetite, intermittent or persistent diarrhea, increased thirst, decreased milk production, emaciation and cachexia (Barkema et al., 2010; Tiwari et al., 2006). During the clinical phase, fecal shedding of MAP may exceed $10^{10}$ cfu/g of feces (Stabel, 2006). The advanced clinical infection stage is characterized by chronic severe watery diarrhea, dehydration, weakness, lethargy, hypoproteinemia, intermandibular edema, and emaciation, eventually leading to the death of the infected animals (Barkema et al., 2010; Lavers, 2013; Tiwari et al., 2006).
12. Diagnosis

Currently the disease is diagnosed by detection of MAP bacteria in the feces or milk and tissues via culture, detection of MAP bacterial DNA in feces and milk by PCR and immunological responses by serological assays.

Culture methods

In general, culturing and isolation of MAP from feces, tissues and milk is considered to be a gold standard test for the JD diagnosis (Salem et al., 2013). Modified Lowenstein-Jensen medium (LJ), Herrold’s egg yolk medium (HEY), and Middlebrook 7H10 and 7H11 supplemented with mycobactin J are commonly used solid media to isolate MAP from clinical samples (Whittington, 2010). Middlebrook 7H9, BACTEC 12B, MGIT 960, MB/BacT and ESP culturing system II are commonly used liquid media to isolate MAP (Bradner, 2013). However, fecal cultures have certain disadvantages such as lack of sensitivity in low shedders or at the sub-clinical stage of infection. Low sensitivity may be attributed to low numbers of MAP in the early stages of fecal shedding and/or loss of viable cells due to chemical decontamination and anti-microbial compounds to kill other unwanted fast growing microbial organisms (Laurin et al., 2015). Culture techniques and media vary between diagnostic laboratories due to lack of analytical sensitivity (Timms et al., 2011; Whittington and Sergeant, 2001; Whittington, 2009). A time period of 5 to 16 weeks is required to detect viable MAP from clinical samples; colonies are then identified by PCR. Other disadvantages with fecal culture are laboriousness, cost and lack of a minimum detection level of MAP in the feces (Khol et al., 2012). Overall sensitivity for bovine fecal culture is 50%-70% (Stabel, 1998). Another fecal culture study revealed a sensitivity of 70% for clinical cases and a sensitivity of 23-49% for
subclinically infected animals (Bosward et al., 2010). Culturing of MAP from infected tissue sample is more accurate than fecal culture. This is because passive shedding of MAP in the feces rather than from infected animals has been reported and may lead to false positive reporting of JD (Britton et al., 2016). Comparison of culture results of ileum and associated lymph nodes collected from 994 cows with fecal culture revealed that 16.5% of animals were MAP-positive with the former and only 3.6% with the latter (McKenna et al., 2005a).

**PCR based assays**

PCR approaches target MAP-specific sequences such as IS900, ISMAP02, ISMav2, F57 and hspX and ISMap02 for detection of MAP in feces, milk and tissue materials (Coelho et al., 2013; Wadhwa et al., 2012). Among these sequences, IS900 is most frequently used to identify MAP (Bradner, 2013). However, similar kinds of sequences have been reported in other mycobacterial species as well (Turenne et al., 2007) thereby affecting test sensitivity and specificity and necessitating use of two different genome sequences to confirm the MAP. IS sequences in MAP can be found in unrelated bacteria due to the mobile nature of these sequences (Turenne et al., 2007). Therefore, it is necessary to use an array of techniques for appropriate characterization of MAC isolates (Li et al., 2005; Solovera, 2012). This may be time-consuming and expensive. PCR assays have a few other limitations such as not being useful to differentiate live and dead bacteria. For example, goat milk samples were (n=304) collected and tested with culture and PCR targeting IS900. Test results revealed that 7.5% of goats were positive by PCR, but none of them were culture positive (Djønne et al., 2003) and this may be due to sample treatment or failure of MAP to recover from dormant state. Extraction methods for the lysis of the MAP cell
to purify MAP DNA from the feces and effective removal of PCR inhibitors such as polysaccharides, phytic acid and bile salts are also affect the fecal PCR sensitivity (Donat et al., 2015). For example, fecal samples from MAP positive cases were processed with 6 different DNA extraction kits revealed that DNA yield and purity varied significantly between the kits evaluated. Therefore selection of a correct method is important to identify JD though fecal PCR (Leite et al., 2013). Overall sensitivity ranges from 57.1% to 85.3% depending on the PCR target sequence. However, comparison of PCR results with fecal cultures yields a relative sensitivity of 4.1% for low to moderate fecal shedders, and a sensitivity of 76% for heavy shedders (Hasonova and Pavlik, 2006). Even though PCR can be used in the diagnosis of JD, it has limited use in prevalence studies and eradication programs (Britton et al., 2016).

**Cell-mediated immune responses**

Intradermal injection of purified protein derivatives (PPDs) and measuring of delayed hypersensitivity 72 h after PPD injection is commonly used in the diagnosis of mycobacterial infections (Antognoli et al., 2007). PPDs are prepared from *M. bovis* (PPDb), MAP (PPDj) or *M. avium* subsp. *avium* (MAA) (PPDa) (Wynne et al., 2012). Increase in the skin thickness after injecting PPD gives an indication about the exposure of animals to MAP or infected with MAP (Capsel, 2015). However, current skin tests have certain disadvantages due to crude antigens in the PPDs preparation. Thus, exposure of animals to environmental mycobacteria generates antibodies and induces delayed hypersensitivity reactions after injection with PPDs (Mikkelsen et al., 2011b). This results in false positive reactions in the diagnosis of MAP. Other issues associated with measuring of CMI using PPD is the lack of standardized strains, MAP-specific antigens with MAP-specific T-cell epitopes because of reported high
genomic variability among the organisms used in the PPD preparations (Capsel, 2015). In addition, lack of standard protocols in the preparation of PPD also affects the measurement of CMI (Singh et al., 2014).

Inconsistency in skin tests leads to in vitro evaluation of CMI responses, in which whole blood samples are collected and cultured with MAP antigens to stimulate and to release IFN-γ from the immune cells. Released IFN-γ is measured by ELISA. Whole blood samples from MAP-infected herds (n=252 cattle) and paratuberculosis free herds (n=117 cattle) were stimulated with MAP-PPD, avian and bovine purified protein derivatives (PPD) and IFN-γ levels was measured. Stimulation with MAP-PPD revealed 95 to 99% specificities for the diagnosis JD (Jungersen et al., 2002). However, false positive results in animals less than 15 months of age were also reported and therefore interpretation of IFN-γ test results must be interpreted within the context of individual test needs (Jungersen et al., 2002). A total of 147 whole blood samples from high and low prevalence herds infected with MAP were stimulated with different PPD preparations and 50 to 75% of the infected animals were correctly diagnosed by IFN-γ release assay upon stimulation with johnin PPD (PPDj) (Stabel and Whitlock, 2001). For in vitro stimulation, MAP-PPDj is routinely used and this method also suffers from low specificity due to the crude antigens in the stimulation of blood cells. This test is not useful to predict the number of animals that would progress to JD, but it is useful to identify calves that have been exposed to MAP earlier in their life (Huda et al., 2003). In addition to this, test performances may be affected by anticoagulant type, sample collection time, storage conditions of the blood, cell culture population and concentration, incubation time and plastic wares used in the assay (Hartmann et al., 2016; Whittington et al., 2012).
**Humoral immune responses**

ELISA is the most commonly used technique to measure humoral immune responses to JD. The currently available commercial ELISA technique uses complex mixtures of antigens such as whole-cell sonicated protein preparations, PPA (protoplasmic antigens), LAM (lipoarabinomannan) and a mixture of proteins (Casey et al., 2011; Mon et al., 2012) to measure the MAP antibodies in milk or serum. But these preparations contain cross-reacting antigens to other genetically related environmental mycobacteria (Osterstock et al., 2007) and this affects the sensitivity and specificity of the assay. In addition to JD sero-conversion occurs at later stages of infection only, so ELISA is not useful to detect subclinically infected animals (Mikkelsen et al., 2011b). Whitlock et al (2000) reported a sensitivity of 15% for low shedders and 75% for high shedders for the serum ELISA (Whitlock et al., 2000). Evaluation of milk and serum ELISA results relative to fecal culture from 1,921 revealed a sensitivity of 21.2 and 23.5% respectively at cow level (Lombard et al., 2006b). Overall sensitivity and specificity of ELISA varies between 7 to 94% and 41 to 100% respectively (Khol et al., 2012). McKenna et al (2005) tested PPA (absorbed ELISA) and LAM (non-absorbed ELISA) to measure immune responses from 383 cows and results were compared with fecal culture to calculate the sensitivity and specificity. The diagnostic sensitivity and specificity was 72.4% and 98.4%, respectively, for absorbed ELISA. Non-absorbed ELISA revealed a sensitivity and specificity of 65.5% and 87.9% respectively. They concluded that non-absorbed ELISA had low sensitivity and specificity in comparison to absorbed ELISA (McKenna et al., 2005b). Use of in-house ELISA showed improved sensitivity and specificity in the diagnosis of JD in comparison of commercial ELISA kits.
Extraction of MAP subspecies-specific surface antigens by treating MAP with ethanol and ELISA with these antigens resulted in 97.4% and 100% sensitivity and specificity respectively, an ELISA result that was better than that of the Biocor ELISA result (Eda et al., 2006). Serum samples from fecal culture-positive (n=444 cows) and culture-negative (n=412) cows were tested with JTC-ELISA (coating of ELISA plates with culture filtrate antigens from MAP strain JTC303) and results were compared with commercial ELISAs. JTC-ELISA was more sensitive (40%) than five commercial ELISA kits (20%) for low fecal shedders (Shin et al., 2008). A total of 300 JD negative and 22 positive serum samples were tested with an in house ELISA using protoplasmic antigen (PPA) from a MAP filed strain and results revealed 68.18% sensitivity and 97.0% specificity for PPA ELISA (Adji et al., 2015). Sensitivities and specificities of the commercial ELISAs for milk samples vary from 29 –61% and 83–100% respectively (Nielsen and Toft, 2008). Serum samples (n=994) collected from the slaughter houses were tested with three commercial ELISAs and the agreement between ELISA results was poor (McKenna et al., 2006a). None of the commercial ELISAs can be used as a single test to diagnose early MAP infection in dairy cattle (Khol et al., 2012).

13. Control

The MAP infection can be controlled in three ways: i) preventing entry of infected animals into JD-free herds, ii) in JD-positive herds, minimizing the exposure of susceptible animals (particularly young stock) to infectious agents, and iii) in a JD-positive herds, on-farm hygiene and biosecurity, and periodic testing and removal of infected animals from the herd (Garry, 2011; McKenna et al., 2006b; Olsen et al., 2002). An ideal diagnostic test with high sensitivity and specificity to detect all
animals in a herd infected with MAP at different stages of the disease would be preferable to control JD. Unfortunately this expectation is not fulfilled with currently available diagnostic tests (Jungersen et al., 2012). Current tests are specific to MAP, but are low in sensitivity. However, this low sensitivity is partly associated with the complex nature of MAP infection. This bacterium elicits a very low level of immune response and intermittent shedding of MAP below the detection limit during early stages of infection. Currently available tests using undefined complex antigen mixtures are ineffective in the identification low level of antibodies and low numbers of MAP organisms shed in the feces. This warrants an investigation of MAP species-specific, sensitive antigens and their incorporation into diagnostic tests to improve their sensitivity and specificity in JD diagnosis.

An ideal antigen candidate would be specific to MAP, immunogenic, expressed and detectable throughout disease progression and detectable in all infected animals at different stages of infection with high sensitivity and specificity. Unfortunately this expectation is not fulfilled completely with the antigens tested so far because MAP shares genetic similarity with other M. avium species (Bannantine et al., 2003a) and shares antigens with other pathogenic and non-pathogenic mycobacterial species (Stabel et al., 2013). Moreover, the complex nature of the MAP infection and host responses leads to differential expression of antigens depending upon on the interaction between the host and MAP. MAP differs from other mycobacterial species in many key aspects such as growth requirements, tissue tropism, host preference and disease progression. Therefore, we speculate that MAP may differ from other closely related members of the mycobacterial family in terms of differential protein expression and structure, orientation, isoforms and post-
translational modifications, which may provide uniqueness to MAP. This uniqueness cannot be predicted by comparison of gene and/or amino acid sequences of related mycobacterial species alone. Therefore, we propose to investigate other suitable options and techniques to identify MAP-species specific antigens.

14. Research question: Can MAP-infected cattle be accurately diagnosed at pre-clinical stages of the disease through the detection of antigens and antibodies unique to MAP cell wall epitopes?

14.1. Hypothesis

Antibodies to unique MAP cell envelope proteins can be used to identify infected cattle by analyzing serum antibody titers or by antibody targeting of MAP organisms in the feces or tissues. To test the hypothesis, our study has the following objectives:

i) Identification of antigenic cell envelope proteins from *Mycobacterium avium* subspecies *paratuberculosis* by comparative proteomic analysis.

ii) Cloning, expression, purification of MAP specific cell envelope proteins, generation of associated polyclonal antibodies and detection of MAP organisms using antibodies to MAP-specific cell envelope epitopes.

iii) Detection of serum antibodies to MAP-specific cell envelope wall proteins.
Objective 1: Identification of MAP-specific cell envelope antigenic proteins by comparative proteomic analysis.

14.2. Rationale

MAH and MAP share genetic similarity of more than 97% at their nucleotide level, but they have divergent phenotypes and different disease causing ability (Bannantine et al., 2003a). Considering these facts, we speculate that there may be unique antigens among these species particularly in the cell envelope (cell wall and cytoplasmic membrane) components. The envelope plays a vital role in host cell entry, modulation of phagosome membrane, defence against host immune system, and environmental survival (He and De Buck, 2010). Different strategies and techniques such as immunoproteomics, screening of expression libraries, identification of unique MAP coding sequences, genomic comparisons and 2-DE are used to discover the MAP-specific antigens candidates (Bannantine et al., 2004; White et al., 1994). The diagnostic ability of antigen candidates such as secreted antigens, cell wall and membrane antigens, lipoproteins, heat shock proteins and hypothetical proteins from MAP has been reviewed (Mikkelsen et al., 2011b). However, most of these diagnostic antigens are not MAP-specific due to the presence of orthologues in other mycobacteria (Mikkelsen et al., 2011b). As a result, we plan to investigate other suitable options and techniques to identify MAP species-specific antigens. The recent development in the 2D-DIGE technique for comprehensive proteomic approaches is an available option. In 2D-DIGE technique, protein samples are prelabelled with fluorescent dyes before 2-DE and protein samples (up to 3) can be run in a single gel. As such, this technique is more advantageous than traditional 2-DE (Cacciotto et al., 2010; Lilley and Friedman, 2004) and antigenicity of individual MAP proteins can be assessed using 2-DEimmunoblot assays. Therefore, the first objective was to identify
unique antigenic peptides from the cell wall of MAP organism by comparing MAP with MAH, and *M. smegmatis* (ubiquitous in the environment) cell envelope protein profiles through 2D-DIGE proteomic approaches. Immunogenicity of the differentially expressed MAP cell envelope proteins were tested by 2-DE western blot analysis and immunogenic proteins were identified by LC-MS/MS (Liquid chromatography-tandem mass spectrometry). We predict that the MAP species-specific cell wall proteins will be more useful in the diagnosis of JD with more consistency, sensitivity and specificity and for differentiating MAP from other cross-reacting bacteria.

**Objective 2: Cloning, expression, purification of MAP-specific cell envelope proteins, generation of associated polyclonal antibodies and detection of MAP organisms using antibodies to MAP-specific cell envelope epitopes.**

**Rationale**

After identification of antigens exposed on the cell surface and cell wall, it is necessary to screen them for their utility as diagnostic makers for the identification of MAP organisms in the target material (feces or tissues). For this purpose, the first step is to acquire enough antigens for further studies. This can be done by recombinant expression of the selected antigens in the heterologous expression systems like *Escherichia coli* with subsequent generation of polyclonal antibodies against the selected MAP cell envelope proteins. Polyclonal antibodies can then be assessed for their ability to bind to epitopes on MAP organisms in the infected tissues by immunohistochemistry and immunomagnetic capturing of MAP organisms. Therefore, aim of the second study was to generate antibodies to recombinantly expressed MAP cell envelope proteins as well as to an extract of MAP total cell
envelope proteins that would be used to identify MAP organisms by IHC and immunomagnetic separation.

Identification or isolation of MAP from infected tissue is a more accurate approach in JD diagnosis. Although tissues are collected at post-mortem procedures, it is possible to collect the samples by surgical biopsies (Britton et al., 2016). However, due to the low numbers of MAP and disparate tissue distribution, multiple tissue samples are necessary to isolate MAP from tissue and other disadvantages of MAP culture have been mentioned earlier. Alternatively, direct detection on MAP in infected tissue by immunohistochemistry (IHC) using MAP-specific polyclonal antibodies is more sensitive and specific. In addition, both intact and lysed MAP cells can be detected in IHC techniques. Even though IHC is more sensitive and reliable, this can be done from slaughter house samples or necropsy samples only and may not be suitable for routine diagnosis of MAP under field conditions.

Isolation of MAP from the fecal culture is considered to be a gold standard diagnostic test. However, in JD, subclinically infected animals intermittently shed very low levels of MAP in the feces and milk and current fecal culture methods are insensitive in the detection of low level MAP from these materials. This may be due to use of chemical decontaminants and antibiotics to kill the fast growing contaminant bacterial species thereby affecting the viability and recovery of MAP from the feces and milk. Currently, PCR is also used to detect MAP-DNA. Isolation of high quality MAP DNA from the fecal sample for PCR is also a challenging task due to low copy number of MAP DNA mixed in the feces and the difficulty in lysing MAP cells to extract DNA. In addition, the presence of PCR inhibitors in the fecal matter affects the sensitivity of PCR-based identification of MAP. Therefore, rapid, sensitive and
selective detection of low concentrations MAP organisms in the clinical material without further processing offers versatility in JD diagnosis and control.

Immunomagnetic capture of pathogenic bacteria is routinely used in veterinary and food microbiology (Cao et al., 2012; Linda et al., 2001; Xiong et al., 2014). The target bacterium is selectively concentrated from other non-specific bacteria and from other inhibitory substances (Foddai et al., 2010). Subsequently, captured bacteria can be identified by other methods like culture, bacteriophage and PCR (Foddai et al., 2010; O’Brien et al., 2016). Therefore, coating of magnetic beads with MAP species-specific polyclonal antibodies and immunomagnetic capturing of MAP from feces and milk may improve the sensitivity and specificity of the subsequent detection methods such as culture and PCR.

**Objective 3: Detection of serum antibodies to MAP-specific cell envelope wall proteins.**

**Rationale**

After identification of antigens, they must be screened for their utility as diagnostic markers for the identification of MAP-specific antibodies in serum samples in an ELISA format or by immunoblot analysis. ELISA is the most commonly used quantitative assay to measure the specific antibody responses to MAP due to its simplicity, cost-effectiveness and suitability to automation. However, current ELISA has low sensitivity and specificity for animals subclinically infected with MAP. Identification and incorporation of MAP specific antigens in JD diagnostics may improve their sensitivity and specificity. It is believed that in the early stages of MAP infection, the host CMI is more predominant than the humoral immune responses, and seroconversion occurs at later stages of infection. Thus, the use of humoral
immunoassays is not suitable in the early stages of infection for the antigens currently in use. However, recent reports indicate that both CMI and humoral immune responses coexist in the early phase of MAP infection and have a major role in disease progression or resistance (Begg et al., 2011). Humoral immune responses to MAP have been reported at as early as 70 days post infection in cattle experimentally infected with MAP (Bannantine et al., 2008a). Therefore, we believe that MAP-specific proteins or a cocktail of antigens in ELISA or immunoblot assays could be useful in the diagnosis of MAP infection. Therefore, the objective of the third study was to use recombinant MAP proteins (SdhA, FadE25_2, FadE3_2, Mkl, DesA2 and hypothetical protein MAP1233) as well as MAP total cell envelope proteins in an ELISA format to detect MAP-specific antibodies in cow serum.

15. Expected outcome

In these experiments, we anticipate identifying MAP-species specific antigens from the cell envelope of MAP bacteria. The availability of MAP species-specific antigens and their incorporation into the JD diagnostic tests will help in the diagnosis of MAP infections with improved sensitivity and specificity.

16. References


Bansal-Mutalik, R., Nikaüdo, H., 2014, Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. Proceedings of the National Academy of Sciences 111, 4958-4963.


Bradner, L.K., 2013, Optimization of methods for culturing Mycobacterium avium subsp. paratuberculosis from bovine milk and colostrum and application to samples collected from naturally infected dairy cows.


Capsel, R.T., 2015, Development of an improved production method, determination of protein composition, and potency characterization of Mycobacterium avium subsp. paratuberculosis purified protein derivative.


Coelho, A.C., Matos, A., Matos, M., de Lurdes Pinto, M., dos Anjos Pires, M., 2013, Mycobacterium avium complex in domestic and wild animals. INTECH Open Access Publisher.


Davis, W.C., 2015, On deaf ears, Mycobacterium avium paratuberculosis in pathogenesis Crohn’s and other diseases. World journal of gastroenterology 21, 13411.


Donat, K., Kube, J., Dressel, J., Einax, E., Pfeffer, M., Failing, K., 2015, Detection of Mycobacterium avium subspecies paratuberculosis in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiology and Infection 143, 975-985.


Everman, J.L., 2014, Disease models and infectious phenotypes of Mycobacterium avium subspecies paratuberculosis.


Gurung, R.B., Begg, D.J., Purdie, A.C., de Silva, K., Bannantine, J.P., Whittington, R.J., 2014, Lymphoproliferative and gamma interferon responses to stress-


Laurin, E., McKenna, S., Chaffer, M., Keefe, G., 2015, Sensitivity of solid culture, broth culture, and real-time PCR assays for milk and colostrum samples from Mycobacterium avium ssp. paratuberculosis-infectious dairy cows. Journal of dairy science 98, 8597-8609.

Lavers, C.J., 2013. Evaluation of diagnostic tests for detection of Mycobacterium avium subsp. paratuberculosis (MAP) at the herd-level and cow-level. University of Prince Edward Island,


National Academy of Sciences of the United States of America 102, 12344-12349.

Li, L., Kataki, R., Schilling, M., Kapur, V., 2015, Molecular Epidemiology of Mycobacterium avium subsp. paratuberculosis on Dairy Farms. Annual review of animal biosciences.


Mönki, J., Hewetson, M., Hahn, S., Vainio, K., Skrzypczak, T., 2015, Disseminated alimentary mycobacteriosis in the horse: a retrospective study of nine cases. Equine Veterinary Education.


Plattner, B., Huffman, E., Hostetter, J., 2012, Gamma-delta T-cell responses during subcutaneous *Mycobacterium avium* subspecies *paratuberculosis* challenge in
sensitized or naive calves using matrix biopolymers. Veterinary Pathology Online, 0300985812463404.


Rowe, M., Grant, I., 2006, Mycobacterium avium ssp. paratuberculosis and its potential survival tactics. Letters in applied microbiology 42, 305-311.


Scott, H.M., Sorensen, O., Wu, J.T., Chow, E.Y., Manninen, K., VanLeeuwen, J.A., 2006, Seroprevalence of Mycobacterium avium subspecies paratuberculosis,
Neospora caninum, Bovine leukemia virus, and Bovine viral diarrhea virus infection among dairy cattle and herds in Alberta and agroecological risk factors associated with seropositivity. Canadian veterinary journal 47, 981.


47
Stevenson, K., 2015a, Genetic diversity of Mycobacterium avium subspecies paratuberculosis and the influence of strain type on infection and pathogenesis: a review. Veterinary research 46, 1.


Wells, S.J., Wagner, B.A., 2000, Herd-level risk factors for infection with Mycobacterium paratuberculosis in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. Journal of the American Veterinary Medical Association 216, 1450-1457.

White, W., Whipple, D., Stabel, J., Bolin, C., 1994, Comparison of cellular and extracellular proteins expressed by various isolates of Mycobacterium


Whittington, R., 2010, 22 Cultivation of Mycobacterium avium subsp. paratuberculosis. Paratuberculosis: organism, disease, control, 244.


Whittington, R.J., Begg, D.J., de Silva, K., Plain, K.M., Purdie, A.C., 2012, Comparative immunological and microbiological aspects of paratuberculosis as a model mycobacterial infection. Veterinary immunology and immunopathology 148, 29-47.


Chapter 1

Identification of antigenic proteins from *Mycobacterium avium* subspecies *paratuberculosis* cell envelope by comparative proteomic analysis

1.1. Abstract

Johne’s disease (JD) is infectious, chronic granulomatous enteritis of ruminants and non-ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Currently MAP infection is incurable and no preventative vaccine is available to control MAP infection. The aim of the present study was to identify antigenic proteins from the MAP cell envelope (i.e. cell wall and cytoplasmic membranes) by comparing MAP, *Mycobacterium avium* subspecies *hominissuis* and *M. smegmatis* cell envelope protein profiles using a 2D-DIGE proteomic approach. Composite 2D-DIGE images revealed thirteen protein spots that were identified only in the MAP cell envelope. Using serum from JD test-positive (serum and milk ELISA) cattle, western blot analysis of 2D gels revealed that proteins in the thirteen spots were immunoreactive. Gel plugs of these spots were removed and proteins were subsequently identified by LC-MS/MS as the products of the following genes: *sdhA*, *fadE25_2*, *mkl*, *citA*, *gapdh*, *fadE3_2*, *moxR1*, *mmp*, *purC*, *mdh*, *atpG*, *fbpB* and *desA2* as well as two proteins without annotated gene names, which were identified as a MAP0035 transcriptional regulator protein and as a MAP1233 hypothetical protein. Regarding functional aspects, seven proteins (*SdhA*, *FadE25_2*, *FadE3_2*, *Mdh*, *CitA*, *AtpG* and *Gapdh*) are involved in energy generation through enzymatic activity in the TCA cycle, glycolysis, fatty acid and cholesterol metabolism. Other proteins are associated with cell wall biosynthesis (*DesA2* and *FbpB*), protein maturation
(MoxR1), bacterial replication (PurC) and invasion of epithelial cells (Mmp). The majority of these proteins are considered to be essential to MAP virulence and intracellular survival. Identification and characterization of MAP species-specific proteins (either pathogen-specific or having specific epitopes) in this manner may be useful in developing new diagnostic tests and vaccines for JD diagnosis and prevention.

Key words: 2D-DIGE, MAP cell envelope, proteome, antigenicity

1.2. Introduction

Johne’s disease (JD)/paratuberculosis is an uncurable chronic granulomatous enteritis of ruminants and non-ruminants caused by the bacteria Mycobacterium avium subsp. paratuberculosis (MAP) grouped under the Mycobacterium avium complex (MAC) (Harris and Barletta, 2001). JD is global in distribution and incurs huge economic losses to the cattle industry(Salem et al., 2013). Neonates and young animals are more susceptible to MAP infection than adult cattle. The major route of transmission is by the feco-oral route, however, transmission through colostrum, milk and the intra-uterine route has also been reported (Salem et al., 2013). JD is characterized by a long subclinical phase followed by a clinical phase. Subclinically infected animals appear healthy but can intermittently shed MAP in feces, milk and colostrum thereby acting as a source of infection for susceptible animals. The clinical phase is characterized by chronic watery diarrhoea, progressive emaciation, edema, reduced milk production and eventual mortality (Salem et al., 2013). Moreover, there are no effective treatments and/or vaccines available to control JD. In addition to the economic burden, the possible involvement of MAP in irritable bowel syndrome, Crohn’s disease, type 1 diabetes and autism are growing concerns to public health (Dow, 2011).
Currently, JD is diagnosed by detection of MAP bacteria in feces or milk via culture, detection of MAP bacterial DNA by PCR and immunological responses to MAP by serological assays. Fecal culture is time-consuming, costly and lacks sensitivity (i.e. 23 to 49%) particularly in low shedders or in the pre-clinical stage of infection (Timms et al., 2011). PCR analyses that amplify MAP-specific sequences such as IS900 and ISMAP02 are used for detection of MAP DNA in feces, milk and tissues (Timms et al., 2011). However, test sensitivity and specificity is diminished due to difficulties in recovering high quality MAP DNA as well as PCR interference due to inhibitors in the feces. ELISA is commonly used for JD diagnosis by measuring milk or serum antibodies to complex mixtures of MAP proteins such as whole-cell sonicated protein preparations, cell wall preparations and/or purified protein derivatives. However, these preparations contain cross-reacting proteins to other genetically related environmental mycobacteria. In addition, seroconversion typically occurs at later stages of JD infection such that current commercially available ELISA diagnostic tests are not useful in detecting sub-clinically infected animals (Mikkelsen et al., 2011b). Therefore, rapid, sensitive and specific diagnostic tests are needed to identify animals infected with MAP to prevent and control MAP transmission and JD. In view of this, there is a need to identify MAP species-specific antigens that can be incorporated into JD diagnostic tests with improved sensitivity and specificity (Willemsen et al., 2006).

In spite of genotypic similarities, there are major phenotypic differences that exist between bacterial strains within the MAC. For example, MAP and Mycobacterium avium sub-species hominissuis (MAH) share genetic similarity of more than 95% at their nucleotide level, but MAP is very slow growing, mycobactin J-dependent and an obligatory intracellular pathogen of intestinal tissues. In contrast,
MAH is a fast growing and opportunistic pathogen of the respiratory tract (Bannantine et al., 2003b). In view of this, we speculate that there may be unique antigenic-proteins or epitopes among these species, particularly in the cell envelope (i.e. cell wall and cytoplasmic membrane), that could be used in diagnostic test development. The cell envelope plays a vital role in host cell entry, modulation of the phagosome membrane, defense against the host immune system, and environmental survival (He and De Buck, 2010).

Proteomic comparisons using gel-based 2-Dimensional Electrophoresis (2-DE) and gel-free approaches have revolutionized protein identification and provide a useful complement to gene expression studies (Målen et al., 2011). In particular, 2-DE is a valuable tool for differential comparisons of cell membrane proteins from closely related bacterial strains (Cacciotto et al., 2010). In addition to characterization of isoelectric points, molecular weights, isoforms and post-translational modifications, the identity and antigenicity of proteins can be achieved by combining 2-DE with Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and immunoblotting (Oliveira et al., 2014; Rabilloud, 2012). In addition, Differential In Gel Electrophoresis (DIGE) is a useful technological refinement involving labeling of proteins with fluorescent Cy dyes and subsequent 2-DE that allows for the assessment of quantitative differences in proteins between samples. Indeed, a comparison of membrane proteins of three strains of *Mycoplasma agalactiae* by 2D-DIGE showed considerable protein differences between strains (Cacciotto et al., 2010).

The aim of this study was to identify antigenic MAP species-specific proteins from the cell envelope of MAP organisms by comparing MAP, MAH and *M. smegmatis* cell envelope protein profiles using 2D-DIGE, 2-DE immunoblot and LC-MS/MS proteomic approaches.
1.3. Materials and methods

Source bacterial strains, media and growth conditions

Three bacterial species, *Mycobacterium smegmatis* strain MC^2^155, *Mycobacterium avium* sub species *hominissuis* (MAH) strain 104 and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Madonna, gc86 and gd30 strains), graciously provided by Dr. L. Mutharia, University of Guelph, were used in the experiments. The environmental mycobacteria *M. smegmatis* was examined as a possible source of antibodies that are cross-reactive to MAP as most cattle are exposed to this bacterium in their environment. *M. smegmatis* was grown in Luria Bertani broth medium (BD Difco™, NJ, USA) at 37°C for three days in an orbital shaker incubator (120 RPM). MAH is another common opportunistic pathogen in the environment and has genetic similarity with other members of MAC such as *M. avium* subsp. *avium* and MAP. Exposure of cattle to this bacterium also generates cross-reacting antibodies and affects MAP diagnosis. MAH was cultured as described by (McNamara et al., 2012). In brief, MAH was grown in Middlebrook 7H9 broth medium (BD Difco™, NJ, USA) supplemented with glycerol (0.5% v/v), 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) at 37°C for 7 to 14 days. Three MAP strains isolated from clinical cases from southern Ontario (Canada) were used instead of MAP-K10 reference strain. The advantages of clinical strains for proteomic comparison have been well documented (Radosevich et al., 2007). Three MAP strains (Madonna, Gc86 and GD30 strains) were grown in Middlebrook 7H9 broth medium supplemented with glycerol (0.5% v/v), 10% (v/v) oleic acid-albumin-dextrose-catalase and mycobactin-J (2 μg/mL) (Allied Monitor Inc, Fayette, MO, USA) at 37°C for 6 to 8 weeks. All bacterial cultures were harvested by centrifugation at 3500 rpm for 30 min at 4°C and
washed three times with ice-cold phosphate buffered saline (PBS) (pH 7.4). Bacterial pellets were then washed with 0.16 M sodium chloride solution and the weight of wet cells was determined (He and De Buck, 2010). The bacterial pellets were preserved at -80°C until further analysis.

**Subcellular fractionation and protein extraction**

Subfractionation of *M. smegmatis*, MAH and MAP was done to obtain cell wall core and cytoplasmic membranes as described (He and De Buck, 2010; Zheng et al., 2012) with a few modifications. Briefly, 2 mL of lysis buffer (0.05 M potassium phosphate, 0.022% (v/v) β- mercaptoethanol, pH 6.5) was added to each gram of frozen bacterial cells (wet cell weight). Lysozyme (final concentration 4.8 mg/mL w/v) (Sigma-Aldrich, Oakville, CA) and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were also added to the cells and incubated for 4 h at 37°C with continuous stirring. Bacterial lysates were transferred into 2 mL screw-capped centrifuge tubes containing sterile zirconium-silica beads (0.1mm) (Biospec Products, Inc) and disrupted by 12 cycles of high velocity bead beating with intermittent cooling on ice after each bead beating. Lysates were centrifuged at 1000 g for 15 min at 4°C to remove unbroken cells and debris. Supernatants were centrifuged at 20,000 g for 30 min at 4°C to obtain the pelleted cell walls and the supernatants containing cytoplasmic membrane and cytosolic fractions. Supernatants were centrifuged at 150,000 g for 90 min at 4°C to obtain cytoplasmic membranes and cytosolic fractions. Pellets containing cells wall and membrane fractions [cell envelope] were then washed twice with PBS (pH 7.4) containing protease inhibitor cocktail to remove cytoplasmic contamination.

Cell envelope pellets were re-suspended in standard cell lysis buffer containing 7 M urea (Sigma-Aldrich, St.Louis, MO, USA), 2 M thiourea (GE
Healthcare, Montreal, QC, Canada), 30 mM Tris/HCl, 4% CHAPS (pH 8.5) (Affymetrix Inc, Maumee, Ohio, USA), incubated on ice for 30 min with intermittent mixing and centrifuged at 10,000 g for 20 min at 4°C to remove insoluble materials. Supernatants containing soluble proteins were stored at -20°C until further 2D-DIGE analysis.

2D-Difference Gel Electrophoresis (2D-DIGE)

Protein labeling

Prior to 2D-DIGE analysis, proteins samples were processed with a 2-DE clean-up kit (GE Healthcare, Montreal QC, Canada) as per manufacturer protocols to remove impurities and total protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc, USA). For analytical 2D-DIGE gel analysis, 50 µg of protein samples from MAP (pooled proteins from three strains), MAH and M. smegmatis cell envelopes were labeled with 100 picomoles of Cy2, Cy3 and Cy5 CyDye DIGE fluors (GE Healthcare, Montreal QC, Canada) respectively according to the minimal dye labeling procedure described by the manufacturer. Protein samples were incubated with CyDyes on ice for 1 h in the dark followed by 10 min on ice with 1 µL of 10 mM lysine in the dark to stop the labeling reaction.

First and second dimensional isoelectric focusing

Labelled protein samples were pooled and mixed with 450 µl of rehydration buffer ([7 M urea, 2 M thiourea and 2% w/v CHAPS supplemented with 1% w/v DTT] (Fisher Scientific, Pittsburgh, USA) and 0.5% v/v Pharmalytes (GE Healthcare Bio-Sciences AB, SE-75184 Uppsala, Sweden)]. Samples were loaded onto 24 cm, nonlinear, pH 3–7 Immobiline DryStrips (GE Healthcare), placed on a IPGphor isoelectric focusing unit (GE Healthcare) and rehydrated at 30 V for 14–16 h. First dimensional focusing occurred over 21 h under the following conditions: 150 V for 3
h, 300 V for 3 h, 1000 V for a 6 h gradient, 8000 V for a 3 h gradient, 8000 V for a 6 h and 500 V for 5 h (Weigoldt et al., 2011).

After first dimensional isoelectric focussing, strips were equilibrated in 10 mL of equilibration buffer containing 70 mM Tris-HCl (pH 8.8), 2% SDS, 6 M urea, 30% glycerol, supplemented with 1% w/v DTT for 15 min under shaking conditions, and again in 10 mL of equilibration buffer supplemented with 2.5% w/v iodoacetamide for another 15 min under shaking conditions. For second dimensional separation, pH gradient strips were rinsed with SDS running buffer and placed onto 12.5% SDS-PAGE gels with a lane allocated for pre-stained molecular markers (ECL Plex Rainbow; GE Healthcare). Electrophoresis was carried out at 6 W/gel for 17 h at 20º C in a DALT six gel electrophoresis unit (GE Healthcare). Gel images were obtained by a Typhoon 9410 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned with laser excitation at 488 nm, 532 nm and 633 nm respectively; and with emission filters at 520 nm, 580 nm and 670 nm respectively. Gel images were transferred to DeCyder software 6.5 (GE Healthcare, Montreal, QC), and data analysis was performed for the proteomes of all three Mycobacterial species as previously described (Cacciotto et al., 2010).

**Animal Sera**

A total of 15 serum samples were used in this experiment. Twelve serum samples were obtained from cows on dairy farms participating in the Ontario Johne’s Education and Management Assistance Program (OJEMAP) that tested positive using Paracheck© milk, IDEXX milk and IDEXX serum and IDEXX hyper ELISAs as per manufacturer’s instructions. Dr. Niel Karrow (University of Guelph) generously provided three serum samples from calves experimentally infected with MAP and
control serum from 2-month old Jersey and Holstein calves procured within 24 h after calving from herds with no reported cases of JD.

**Western blot analysis of 2-DE gels of MAP cell wall and membrane proteins**

For western blot analysis, 150 µg of MAP cell envelope proteins in standard cell lysis buffer underwent isoelectric focusing and equilibration as per the 2D-DIGE protocol mentioned earlier. Equilibrated 24 cm strips were cut into two pieces (12.5 cm x 2) in order to perform 2-DE in a Hoefer SE600 Ruby electrophoresis unit (GE Healthcare), and the second dimension separation was performed on a 12.5% SDS PAGE gel at 10 W/gel for 3 h. Proteins were then electrophoretically transferred onto nitrocellulose membranes and incubated in blocking buffer (2% BSA in Tris-buffered saline pH 7.6 contains 0.1% Tween 20 (TBST) at room temperature for 1 h and then incubated with JD ELISA-positive, pooled cattle serum (1:100 in 2% BSA in TBST buffer) for 13 h at 4°C on a shaker. Membranes were then incubated with horseradish peroxidase-conjugated affinity-purified rabbit anti-bovine IgG (Jackson Immunoresearch Laboratories Inc., West Gove, PA) antibodies (1:10000 in 0.5% BSA-TBST buffer) for 1 h at room temperature. After washing with TBST and TBS buffers, membranes were visualized by enhanced chemiluminescence.

**Protein identification**

2-DE electrophoresis: Preparatory Gel

In order to identify the antigenic proteins that were observed on 2-DE western blots, a total of 200 µg of unlabelled MAP proteins was loaded into a sample cup on Immobiline DryStrips (pH 3 to 7, NL, 24 cm) that underwent first dimensional isoelectric focusing as mentioned in the 2D-DIGE protocol above. The gel was then fixed in 7% acetic acid and 10% methanol for 2 h, stained overnight with SYPRO® Ruby Protein Gel Stain (Fisher Scientific, Ottawa, ON), destained and scanned on a
Typhoon 9410 scanner (GE Healthcare, Montreal, QC) at a wavelength of 532 nm. The gel image was analysed by DeCyder software 6.5 (GE Healthcare, Montreal, QC) and gel spots to be picked were cross-matched with the 2D-DIGE MAP proteome gel image. MAP-specific antigenic proteins were selected based on complementarity with the 2-D-DIGE MAP proteome gel and associated western blot images. Selected protein spots were picked by an Ettan automated spot picker (GE Healthcare) and gel plugs containing proteins were submitted to the Mass Spectrometry Research facility at the Hospital for Sick Children (Toronto, Canada) for identification by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

**LC-MS/MS analysis**

SYPRO Ruby-stained gel plugs were processed to extract peptides for LC-MS/MS analysis as described by (Gundry et al., 2009). Samples containing the peptides were desalted and concentrated using C18 Zip Tips (EMD, Millipore). The samples were run on a linear ion trap-Orbitrap hybrid analyzer (LTQ-Orbitrap) outfitted with a nano-spray source and an EASY-nLC split-free nano-LC system (ThermoFisher). Peptide mixtures were dissolved in 0.1% formic acid and loaded at constant 800 Bar onto a column for injection for mass spectrometry analysis. Thermo Easy Spray 75 µM X 15 cm PepMax RSLC Easy-Spray columns filled with 3 µM C18 beads were used. Peptides were eluted over 60 min at a rate of 250 nL/min using 0%-35% ACN gradient in 0.1% formic acid. One MS full scan (400-1500 m/z) in the Orbitrap mass analyzer was performed. Automatic gain control target of 500000, max injection time of 100 ms, one micro scan, resolution of 60000 were performed. Six data dependent scans, automatic gain control target of 30000, max injection time of 50 ms were applied. Minimum ion intensity of 1000 was required to trigger a MS/MS event. Dynamic exclusion was applied using a maximum exclusion list of 500 with
one repeat count; a repeat duration of 30 seconds and exclusion duration of 10 seconds were applied.

Database searching

Tandem mass spectra were extracted using Scaffold Software version 4.3.0. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The GPM, thegpm.org; version CYCLONE 2010.12.01.1). Sequest was set up to search Uniprot-Mycobacterium paratuberculosis.fasta (unknown version, 9222 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the Uniprot-Mycobacterium avium subsp. paratuberculosis database (unknown version, 17721 entries) also assuming trypsin. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 2.0 Da. Deamidation of asparagine and glutamine, oxidation of methionine and carbamidomethylation of cysteine were specified in Sequest as variable modifications. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, deamidated asparagine and glutamine, oxidation of methionine and carbamidomethyl of cysteine were specified in X! Tandem as variable modifications.

Criteria for protein identification

Scaffold (version Scaffold_4.3.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Protein probabilities were
assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

1.4. Results

**Extraction of bacterial cell wall and membrane proteins**

The objective of this study was to identify antigenic MAP-specific cell envelope proteins by comparative gel-based proteomics with minimal protein labeling with CyDye fluorophors. Proteins were extracted from *M. smegmatis* strain mc²155, MAH strain 104 and MAP (Madonna, gc86 and gd30 strains). Initially, we tested different methods to extract the cell envelope proteins such as phase extraction with Triton-X-114 and protein precipitation with acetone and methanol; delipidation of the cell envelope with chloroform, methanol, water, and treatment with Na₂CO₃ to remove loosely attached surface proteins. However, these protocols were time consuming and did not generate sufficient quantities of cell envelope proteins for further analysis. We eventually solved this issue by modifying the methods described by He and De Buck, 2010; Zheng et al., 2012 i.e. lysozyme concentration in the lysis buffer was increased to a final concentration of 4.8 mg/ mL and incubated at 37° C for 4 h with continuous stirring. The concentrations of proteins extracted per gram wet weight of cells ranged from 1.0 to 3.0 mg for *M. smegmatis*; 0.4 to 3.0 mg for MAH; and 0.2 to 0.9 mg for MAP.

**2-DE proteome map of MAP, MAH and M. smegmatis**

We assessed Immobilon strips with differing pH and lengths that ranged from pH 3 to 11 and lengths from 7 to 24 cm. Preliminary experiments revealed that most proteins were optimally focused between pH 3 and 7 with a strip length of 24 cm length so cell envelope proteins from MAP, MAH and *M. smegmatis* were
subsequently compared by 2D-DIGE, using pH 3 to 7 NL Immobilon strips that were 24 cm long to cover a wide range of proteins. A 2-DE proteomic analysis map clearly resolved numerous cell envelope proteins for all three mycobacterial species (fig. 1). The coloured spots shown in Figure 1 indicate differences in the proteome profiles for each mycobacterium species with only a few protein spots (white colour) that are shared by all three bacterial species. More importantly, we observed that many protein spots (blue) had pI and MW characteristics that were specific to the MAP cell envelope.

**Figure 1:** Comparative proteomic analysis of cell envelope proteins from *Mycobacterium avium* subsp. *paratuberculosis*, MAH and *M. smegmatis*. 2D-DIGE composite image of cell wall and membrane proteins (Panel 1A) from *Mycobacterium avium* subsp. *paratuberculosis* (blue spots), *M. avium* subsp. *hominisuis* (green spots) and *Mycobacterium smegmatis* (red spots).
By acquiring the images under a single channel for each Cy dye the labelled protein profiles were visualized separately also indicating differences in protein profile patterns (fig. 2A, B and C). For all three bacterial species, molecular masses of the proteins ranged from <12 to 150 kDa. For MAP and M. smegmatis, the majority were distributed within a range between ~26 and 76 kDa. For MAH, proteins were widely distributed between 12 and 150 kDa (fig. 2B). Proteins from all three mycobacterial species were located between ~ pH 3.5 and pH 7 ranges. However, most of the proteins were located in the acidic region (~pH 4-6). Several strings of protein spots with the same mass but with different pI values were observed in the 2-DE map of all three bacterial species, and some of these strings were located close to each other. These patterns imply that there were post-translational modifications of some of the proteins.
Figure 2: Proteomic analysis of cell envelope proteins from *Mycobacterium avium* subsp. *paratuberculosis* and other mycobacterial species. Extracted images of Cyanine Dye-labelled cell wall and membrane proteins from *Mycobacterium avium* subsp. *paratuberculosis* (Panel 2A), *M. avium* subsp. *hominisuis* (Panel 2B) and *Mycobacterium smegmatis* (Panel 2C).
Assessing antigenicity of mycobacterial cell envelope proteins

Routine exposure of animals to *M. smegmatis* and MAH in the environment may recognize MAP epitopes and interfere with the sensitivity and specificity of serological assays used for JD diagnosis. We validated this concept via immunoblot analysis by probing MAP, *M. smegmatis* and MAH cell envelope proteins, separated by 1-D PAGE, with serum from JD test-positive cattle (hereafter called JD-sera). Our results revealed that serum from JD-positive cattle reacted strongly with MAP proteins, but there was also considerable reactivity to *M. smegmatis* and MAH cell wall and membrane proteins (fig3). These results demonstrate that MAP contains proteins with epitopes common to/with other mycobacterium species.

Figure 3: Assessment of reactivity of serum samples against cell envelope proteins from *Mycobacterium avium* subsp. *paratuberculosis*, *M.avium* subsp. *hominisuis* (MAH) and *M. smegmatis* (MS) by immunoblot analysis. Panel A: Immunoblot of JD test-negative serum samples. Panel B: Immunoblot of JD test-positive serum samples.
Panel C: Immunoblot of serum from control calves not exposed to MAP. Panel D: Immunoblot of serum from calves experimentally infected with MAP (60 days post infection). MW: molecular weight protein ladder.

The immunoreactivity of MAP-specific proteins identified by 2D-DIGE was determined in order to identify antigen candidates for JD diagnostic purposes. Western blot analysis of 2D-PAGE gels of MAP cell envelope proteins was performed by probing nitrocellulose membranes with pooled serum samples from 12 dairy cows naturally infected with MAP and 3 experimentally infected calves (60 days post infection). Western blot analysis revealed serum reactivity to many MAP proteins (fig. 4A). A pattern of 13 of these spots aligned with the MAP-specific protein spots identified on the composite 2D-DIGE image of the three mycobacterial cell envelope proteins (fig.4B). Thus, the 13 protein spots that were selected based on complementarity with the western blot image were antigenic, specific to MAP and were sufficiently isolated from other proteins to allow for picking from preparatory gels for protein analysis.
Figure 4: Identification of antigenic MAP-specific cell envelope proteins. Western blot (2D-PAGE) of cell envelope proteins of MAP probed with serum from JD test-positive cattle. Panel A: Western blot showing antigenic proteins (red circles) that correspond to MAP-specific protein spots on the 2-DE proteome map. Panel B: 2D-DIGE image indicating antigenic proteins that are specific to MAP (white circles).
Protein identification by LC-MS/MS

Based on matching of protein spots on the composite 2D-DIGE gel and the 2-DE western blot, 13 antigenic MAP-specific protein spots were selected and picked for further analysis by LC-MS/MS. From these protein spots, fifteen proteins were identified (Table 1). Spot numbers 1, 2 and 3 were identified as succinate dehydrogenase iron-sulfur subunit (SdhA), spot 4 and 5 as acyl-CoA dehydrogenase (FadE25_2), and spots 11, 12 and 13 as fibronectin-binding antigen 85 complex B (FbpB). Spot number 9 was identified as ATP synthase gamma chain. Another five protein spots including 6, 7, 8, 10 and 13 were identified as having more than one protein per spot (Table 1). Whenever possible, proteins were identified by names, gene bank accession numbers, gene names, locus tags, molecular masses, theoretical pIs, their cellular localization and their predicted functions (Table 2).
Table 1. Mass spectrometric identification of antigenic proteins from MAP cell envelope.

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<th>Exclusive unique peptide count</th>
<th>Exclusive unique spectrum count</th>
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<th>% of sequence coverage</th>
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Table 2: Characteristics of MAP cell envelope proteins and orthologous mycobacterial proteins.(ND- not described and tested as candidate antigen Source: (*He and De Buck, 2010, McNamara et al., 2012)).

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<th>MS Ortholog</th>
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1.5. Discussion

To the best of our knowledge, this is the first study to compare the cell envelope proteomes of three mycobacterial species, i.e. MAP, MAH and *M. smegmatis*, by 2D-DIGE. By comparing differential expression of cell envelope protein profiles between three bacterial species, the objective of this study was to identify and characterize MAP-specific proteins that might eventually be used as target proteins for novel diagnostic and vaccine studies. We used three clinical isolates of MAP for proteomic comparisons as strains isolated from natural infections are more relevant for this purpose than are laboratory-adapted reference strains due to variable protein expression. For example, comparison of membrane and cytoplasmic proteins from the MAP-K10 reference strain with the clinical isolate MAP-187 revealed up-regulation of AtpC, RpoA and proteins involved in fatty acid metabolism in MAP-187 (Radosevich et al., 2007). Similarly proteomic comparison of two *M. tuberculosis* strains, i.e. H37Rv (virulent) and *M. tuberculosis* H37Ra (avirulent), revealed upregulation of many proteins in the virulent strain that are involved in disease pathogenesis (Målen et al., 2011).

Our proteomic analysis showed that several strings of protein spot distribution patterns were similar in the three mycobacterial species. Indeed, many of the proteins identified in this study have homologous gene sequences with other sub-species of the MAC and are conserved in the mycobacterial species. However, many of the cell envelope proteins were found to be distinct in the three mycobacterial species with many spots specific to MAP. This finding may be due to subtle differences in amino acid sequences or post-translational modifications of homologous proteins across species that cause shifts in DIGE coordinates. Structural and functional relationships of proteins, protein-protein interactions, catalytic functions of enzymes, formation of
protein complexes and antigenic epitopes are often modified by post-translational modifications (PTM) (Cain et al., 2014). This suggests that differential expression patterns or isoforms may represent specific epitopes or protein regions that may elicit pathogen-specific immune responses in their hosts. Indeed, as others have indicated, sequence homology alone may not be a good predictor of protein function as single amino acid changes can lead to differences in the protein function (Henderson and Martin, 2011; Jeffery, 2015). For example, bio-informatic analysis of two forms of Cpn60 protein (cpn60.1 and 2) in M. tuberculosis, with >60% nucleotide sequence similarity have markedly different functions in MTB pathogenesis (Henderson and Martin, 2011).

A previous study which compared the proteomes of MAP and MAA whole cell lysates by 2-DE analysis revealed that expression levels of 32 proteins were increased in MAP in comparison to MAA (Hughes et al., 2008). Out of these 32 proteins, the gene sequences of 19 proteins had little or no differences with MAA gene sequences. The MAP proteins identified as being differentially expressed in our study have homologous gene sequences with MAH and M. smegmatis and are conserved in other mycobacterial species. Authors of a recent study suggest that diversity in PTM amongst closely related mycobacterial species affords uniqueness to the proteins and/or species-specific epitopes (Facciuolo and Mutharia, 2014). As such, we speculate that these unique epitopes can elicit subspecies-specific immune responses in their hosts. Indeed, preliminary results from our ongoing studies indicate that antibodies to several of the MAP cell envelope proteins are specific to MAP and do not cross-react with cell envelope proteins from MAH and M. smegmatis.
LC-MS/MS results were also suggestive of different isoforms or PTM for three proteins that were represented by multiple spots on 2D-DIGE. These include iron-sulfur subunit of succinate dehydrogenase (identified in spots 1, 2 and 3), acyl-CoA dehydrogenase (identified in spots 4 and 5) and fibronectin-binding antigen 85 complex B (spots 11, 12 and 13). In addition, there was more than one protein for some of the spots with a high percentage of protein coverage for each individual protein. For example, three proteins, ribonucleotide-transport ATP-binding protein ABC transporter, citrate synthase II and glyceraldehyde 3-phosphate dehydrogenase were found in spot 6 and 35 kDa protein, phosphoribosylaminomidazole-succinocarboxamide synthase and malate dehydrogenase were both present in spot number 8. Identification of more than one protein in single spots excised from 2D-gels was in agreement with other studies (Hughes et al., 2008; Mehaffy et al., 2010; Thiede et al., 2013).

To assess the relevance of these results, we have cross-referenced our findings with three other studies of the mycobacterial species, the intent of which was to identify total MAP cell surface, cell wall and envelope and MAH cell surface proteins (He and De Buck, 2010; Leite et al., 2015; McNamara et al., 2012). Comparison showed that the proteins that we identified are located on the cell surface, cell wall and cell envelope compartments suggesting that the protein extraction method used in this study was effective in isolating and enriching MAP cell envelope proteins for proteomic analysis. However, some of the proteins that we have identified (i.e. SdhA, Gapdh, FbpB, FadE25_2, FadE3_2 and Mdh) have been reported to exist in multiple cellular locations (ie. in the secretome and cytoplasm) (Facciuolo et al., 2013; Weigoldt et al., 2013). This may be due to undefined translocation mechanisms as explained in earlier studies (Facciuolo et al., 2013) and suggests that these proteins
may have more than one function by acting as so-called “moonlighting proteins” (Kainulainen and Korhonen, 2014). Moonlighting properties of highly conserved glycolytic, metabolic enzymes and bacterial adhesion proteins are well documented and are essential for bacterial survival (Henderson, 2014). For example, antigen 85B (FbpB) is involved in multiple functions such as mycobacterial cell wall synthesis, bacterial adhesion and pathogenesis. However, it is also possible that some proteins may have been released during cell lysis and/or cell death with subsequent attachment to the envelope complexes. There are only few reports available on moonlighting properties of mycobacterial proteins and further studies are warranted.

Because one antigen may not be immunogenic throughout the course of MAP infection (Hughes et al., 2008) serum samples are often pooled for immunoproteomic analysis (Facciuolo et al., 2013; Viale et al., 2014). In view of the fact that the JD-positive cattle and experimentally infected calves from which serum was obtained for this study were at different stages of development of JD, the serum was pooled in order to improve the efficiency of detection of antigenic MAP cell envelope proteins.

Of the 13 antigenic cell envelope proteins that were identified as MAP-specific in this study, we consider six proteins (SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein MAP1233 and DesA2) warrant further investigation as potential targets for the development of novel diagnostic tools or vaccines. The rationale for selecting these six proteins is that they have not been extensively studied to date and knowledge of their utility in diagnostic test development is limited. In an ongoing study, these six proteins were being recombinantly expressed to assess their utility in ELISA analysis of MAP-specific serum antibody levels and as immunogens to generate MAP-specific antibodies for use with immunohistochemistry and immunocapture techniques. Other proteins identified in this study such as MMP and
FbpB, involved in bacterial virulence and survival in the host were not considered for further study as they have been, or are currently being investigated for use in diagnostic test or vaccine development.

Proteins in spot numbers 1 to 3 were identified as succinate dehydrogenase iron-sulfur subunit (SdhA). In general, mycobacterial species have two annotated operons for *sdh* namely *sdh*1 and *sdh*2 with the MAP gene located in the *sdh*2 operon. In *Mycobacterium tuberculosis*, succinate dehydrogenase is essential in maintaining the proper growth rate by fine-tuning the respiratory rate, in adapting to hypoxic environments, in maintaining the membrane potential, in synthesizing ATP and for bacterial persistence (Hartman et al., 2014a; Knapp et al., 2015). Indeed, deletion mutation of *sdh* leads to loss of respiratory control and viability of MTB during the stationary phase of the growth cycle (Hartman et al., 2014a). In *Legionella pneumophila*, SdhA is essential for intracellular survival and replication by blocking host responses or by delaying apoptosis (Zhang et al., 2016). A study of MAP envelope complexes by blue native PAGE analysis also detected SdhA in the cell envelope (Leite et al., 2015). Increased expression of SdhA from MAP isolated from intestinal tissues has been reported suggesting that SdhA is required for energy production and may play a role in the pathogenesis of JD (Weigoldt et al., 2013). Considering the importance of Sdh in the intracellular survival of various pathogenic bacteria, we believe that this protein may represent a useful candidate for diagnostic or vaccine purposes.

Proteins in spot nos. 4 and 5 were identified as acyl-CoA dehydrogenase (FadE25_2), an enzyme that participates in the first dehydrogenase step of the β-oxidation pathway of cholesterol metabolism to produce carbon and energy sources available to mycobacteria (Egan et al., 2008). Studies on proteomic comparisons of *in*
vitro cultures and in vivo isolates of MAP revealed that FadE25_2 expression is upregulated in the latter. These studies suggest that FadE25_2 may be essential for mycobacterial growth, for survival and for replication within macrophages (Egan et al., 2008; Griffin et al., 2011). Immunoblot analysis revealed that serum from cattle with clinical signs of JD reacts strongly with FadE25_2 recombinant protein (Leite et al., 2015) and serum antibodies to this protein have also been reported in animals experimentally infected with MAP (Bannantine et al., 2008a).

Ribonucleotide-transport ATP-binding protein ABC transporter (Mkl) was identified in spot no. 6. In MAP, the Mkl protein is encoded by the MAP4129 gene locus however, little is known about it’s functional role. In MTB, Rv0655ORF encodes Mkl protein (Braibant et al 2000) which is also referred to as mceG based on it role in mammalian cell entry by interacting with YrbEAB proteins to form part of sterol transport systems (Joshi et al., 2006). Under in vitro conditions, mceG is vital for M. tuberculosis to acquire and metabolize cholesterol to produce carbon and energy in cholesterol rich conditions. Therefore, in this sterol importer is essential for M. tuberculosis to survive intracellularly, to maintain bacterial structure and to maintain virulence (Content and Peirs, 2008b; Pandey and Sassetti, 2008). Recently, this immunogenic protein has been isolated from M. tuberculosis pellicles, which could play an important role in biofilm formation (Kerns et al., 2014). In MAP, the Mkl protein is immunogenic and serum from subclinically infected cattle with MAP recognises this protein antigen (Bannantine et al., 2008a; Bannantine et al., 2008b). Considering the importance of Mkl in mycobacterial species, this is an interesting target to explore its utility in the MAP diagnostics.

Spot no. 7 was identified as acyl-CoA dehydrogenase (FadE3_2), an enzyme involved in fatty acid metabolism whose expression is increased under heat stress.
conditions (Gumber and Whittington, 2009; Weigoldt et al., 2011). Proteomic comparison of MAP and MAA by 2-DE showed that this protein is differentially expressed in MAP (Hughes et al., 2008) suggesting that this protein is a potential target in immunodiagnosis and immunocapture of MAP. *In silico* screening of FadE3_2 protein to predict epitopes and immunogenic potential showed that this protein is unique to MAP (Gurung et al., 2012a) and it carries a large number of MHC I T cell epitopes and conformational epitopes for B cells (Gurung et al., 2012a). Moreover, FadE3_2 recombinant protein has been used to detect cell-mediated immune responses and significantly induced INF-γ immune responses in sheep subclinically infected with MAP (Hughes et al., 2013).

Hypothetical protein encoded by the MAP1233 loci was identified in spot no. 10. The amino acid sequence of this protein was analysed by a protein BLAST search to identify a putative functional role and to identify homologous proteins in other mycobacterial species. This analysis revealed protein sequence similarity with the methyltransferase FkbM family group under the super family of S-adenosyl-L-methionine dependent methyltransferases. S-adenosyl-L-methionine dependent methyltransferases play a vital role in biosynthesis, signal transduction, repairing of proteins, regulation of chromatin and silencing of genes by methylation (Schubert et al., 2003; Struck et al., 2012). For example, in *Leptospirillum* FkbM methyltransferases genes are clustered with genes that are involved in the synthesis, export and reconfiguration of polysaccharides (Goltsman et al., 2009). In MAP, this protein may be associated with cell wall biogenesis and remodelling, however, further functional studies are required.
Acyl-ACP desaturase (DesA2) protein, identified in spot no. 13, is involved in the synthesis of diverse mycolic acids (Dyer et al 2005). Functional loss of DesA2 protein by conditional mutations and gene disruptions affects the viability of pathogenic mycobacteria and is necessary for their survival (Wolfe et al., 2013). Transcriptomic analysis revealed that desA2 is up-regulated in MAP and *M. tuberculosis* cultured under acid-nitrosative, heat stress and hypoxic stress conditions (Cossu et al., 2012; Gumber and Whittington, 2009; Wolfe et al., 2013). Moreover, 2-DE proteome profiling of clinical strains of MAP derived from bovine intestinal mucosa showed an increased level of expression for DesA2 (Weigoldt et al., 2013). *In silico* analysis indicates that DesA2 carries several T and conformational B cell epitopes and is an immunogenic protein (Guru et al., 2012a). Indeed, sheep with MAP infection develop lymphoproliferative and IFN-γ responses and serum antibodies to DesA2 (Gurung et al., 2014b). Sheep vaccinated with recombinant DesA2 protein develop a protective immune response to MAP infection suggesting that this protein could be a potential vaccine candidate (Gurung et al., 2015). Considering the constant expression under various stress conditions, localization on the cell surface and immunogenicity, we have selected DesA2 for further studies.

It is apparent that each method of proteomic analysis has certain limitations. *In vitro* culture of MAC bacteria examined in this study may have resulted in protein expression that is different from that which occurs in the host. Moreover, pooling of serum samples from various individual cattle eliminated the opportunity to examine the immunoreactivity of MAP cell envelope proteins in individual cattle. Because our comparison was limited to three MAC bacterial species, it is possible that some of the proteins identified as MAP-specific may share epitopes with other mycobacterial species. In view of this, shot gun proteomic analysis of several mycobacterial
bacterial species, including MAP of different genotypes and pathotypes would complement the gel-based proteomics approach used in this study. It is possible that in addition to our selected list of 13 antigenic MAP cell envelope proteins, other candidates may exist which were not identified in this study and it warrants further investigation.

In conclusion, MAP proteins with species-specific epitopes and candidate immunogenic antigens were identified using a proteomic approach with 2D-DIGE and immunoblotting with sera from JD test-positive and experimentally infected animals. Cell envelope proteins identified in this study may be useful in elucidating the pathogenicity of MAP and developing novel diagnostics tests and vaccines for JD. Our future strategies are focusing on recombinant expression of several of these immunoreactive MAP cell envelope proteins to be used in ELISAs to screen for pathogen-specific serum antibodies in MAP-infected cattle. In addition, combinations of these immunoreactive proteins are being used to generate antibodies for the identification of MAP organisms by immunohistochemistry and immunocapture of from clinical material such as feces and milk for diagnostic purposes.

1.6. References


Gumber, S., Whittington, R.J., 2009, Analysis of the growth pattern, survival and proteome of Mycobacterium avium subsp. paratuberculosis following exposure to heat. Veterinary microbiology136, 82-90.


Gurung, R.B., Purdie, A.C., Begg, D.J., Whittington, R.J., 2012, In silico identification of epitopes in Mycobacterium avium subsp. paratuberculosis proteins that were upregulated under stress conditions. Clinical and Vaccine Immunology19, 855-864.

Gurung, R.B., Purdie, A.C., Whittington, R.J., Begg, D.J., 2015, Cellular and humoral immune responses in sheep vaccinated with candidate antigens MAP2698c
and MAP3567 from *Mycobacterium avium* subspecies *paratuberculosis*. Vaccination Against Mycobacterial Diseases in Animals, 73.


McNamara, M., Tzeng, S.-C., Maier, C., Zhang, L., Bermudez, L.E., 2012, Surface proteome of “Mycobacterium avium” subsp. hominissuis” during the early stages of macrophage infection. Infection and immunity80, 1868-1880.


Chapter 2

Generation of polyclonal antibodies to recombinantly expressed and purified MAP cell envelope proteins for use in clinical detection of MAP bacteria

2.1. Abstract

The aim of the present study was to codon optimize, clone, recombinantly express MAP proteins and using these proteins to generate polyclonal antibodies and test their utility in the diagnosis of MAP infection. Six MAP cell envelope proteins (SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein (MAP1233) and DesA2) were recombinantly expressed in the E. coli BL21 (DE3) strain. Polyclonal antibodies against three of the recombinant proteins (SdhA, FadE25_2 and DesA2) were generated in rats. The specificity analysis of the polyclonal antibodies by 1-DE immunoblotting against MAP, MAH and M. smegmatis total cell envelope protein reacted with single protein bands corresponding to the molecular weights of SdhA, FadE25_2 and DesA2 with very minimal cross-reactivity with MAH and MS cell envelope proteins. In 2-DE immunoblot analysis of polyclonal antibodies to SdhA, FadE25_2 and DesA2 proteins, antibodies reacted specifically with spots correlating with Pl and molecular weight coordinates of these three proteins in the 2-DE proteome map of MAP. In addition, extracts of total MAP cell envelope proteins were used to generate rat anti-MAP polyclonal antibodies and immunoblotting indicated that these polyclonal antibodies were strongly immunoreactive with MAP cell envelope protein extracts with minimal cross-reactivity with extracts of MAH and MS cell envelope proteins. Polyclonal antibodies to total MAP cell envelope extracts were able to identify MAP organisms in the infected tissues using IHC and IF techniques.
Furthermore, polyclonal antibodies to SdhA, FadE25_2 and DesA2 that were coated on protein G magnetic beads immunomagnetically retrieved MAP microorganisms spiked in PBS. Further studies with mixed bacterial cultures and other mycobacterial sub-species will confirm the diagnostic utility of immunomagnetic separation in MAP identification.

2.2. Introduction

Johne's disease is a chronic, progressive, granulomatous enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Britton et al., 2015; Garcia and Shalloo, 2015). JD causes considerable economic losses to the cattle industry due to reduced milk production, body weight and carcass value, premature culling, herd replacement costs and those associated with disease diagnosis (Bhattarai et al., 2013). Currently, JD is diagnosed by fecal culture, PCR, ELISA, gross and histopathological lesions with identification of acid fast bacilli in infected tissues. Culturing of MAP from feces and milk is considered as a gold standard for the diagnosis of JD. During culturing of MAP, use of chemical decontaminants reduces the viability of MAP and affects the sensitivity of the assay (O’Brien et al., 2016). In addition, lower numbers of MAP from low and medium shedders in the milk and feces and lack of efficient methods to concentrate MAP from the samples reduces the sensitivity and specificity of MAP detection by culture. ELISA, the most commonly used diagnostic test, has very low sensitivity and specificity due cross reacting antibodies from environmental exposure to closely related organisms such as *Mycobacterium avium* subspecies *hominissuis* and other non-pathogenic mycobacteria that lead to false positive results. Moreover, seroconversion in MAP infection occurs a few months before the onset of clinical signs and thus leads to false negative results.
Overall, current diagnostic tests for JD lack sensitivity and specificity (Garcia and Shalloo, 2015) mainly during the early phase of the infection. Therefore, there is a need to develop more sensitive and specific diagnostic tests for subclinical JD. One approach is to identify MAP species-specific antigens and associated antibodies and validate their incorporation into new diagnostic tests (Mon et al., 2012; Rosseels and Huygen, 2008). Using this approach, the current project builds on our previous proteomics study in which 15 antigenic cell envelope proteins were identified that are specific to MAP. Six of these proteins (SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein MAP1233 and DesA2) were selected to be recombinantly expressed for use as immunogens to generate MAP-specific antibodies to be used for diagnostic purposes. These proteins are involved in various aspects of energy and lipid metabolism and cell wall biogenesis and are considered to be essential for mycobacterial survival under different stress conditions (Content and Peirs, 2008a; Egan et al., 2008; Hartman et al., 2014b; Wolfe et al., 2013).

Due to the rapid advancement in gene sequencing technologies, complete genome sequences of several C- and S-type MAP strains are available (Li et al., 2005; Wynne et al., 2010). The next generation of gene sequencing gives a platform for post-genomic and proteomics approaches for the identification, production and validation of MAP species-specific antigen candidates for diagnostics as well as for vaccine development purposes (Bannantine et al., 2010a; Leroy et al., 2007; Leroy et al., 2009; Roupie et al., 2012). The first step in this process is to produce sufficient quantities of MAP-specific cell envelope proteins that are of adequate quality for antibody generation and ELISA development. For this purpose, cloning and over-expression of desired MAP gene sequences in heterologous hosts such as *E. coli* cells
is used frequently with subsequent purification of recombinant proteins by resin-based affinity chromatography (Cho et al., 2007; Gumber et al., 2009; Gurung et al., 2014a; Li et al., 2007). However, at times *E. coli* cells fail to express sufficient quantity and quality of recombinant proteins from the genes cloned into a variety of expression vectors for various reasons such as plasmid and protein instability, protein toxicity to cells, poor transcription and/or translation, improper post-translational modifications, physiochemical properties of the expressed proteins, and codon bias (Bannantine et al., 2010b; Cossu et al., 2011; Kashino and Campos-Neto, 2011).

In general, multiple synonymous codons are used to encode a single amino acid and this is due to the degenerate nature of the genetic code (Boël et al., 2016; Ermolaeva, 2001; Hershberg and Petrov, 2009). In prokaryotes, different codons are used to encode an amino acid with different frequencies by the process of natural selection. For example, fast growing bacteria have large numbers of tRNAs through evolutionary mechanisms and they use a minimal set of anti-codons for translational efficiency and accuracy in comparison with very slow growing bacterial species, and this leads to codon bias among the bacterial species (Ermolaeva, 2001; Hershberg and Petrov, 2009; Johnston et al., 2013). Thus, one of the key issues affecting protein expression levels is due to variations in codon usage, and rare codons in turn affect translation rate and cause translation errors in the recombinant proteins (Elena et al., 2014). Codon bias is highly correlated with whole G+C content of the given genome (Bannantine et al., 1997; Gustafsson, 2009; Muto and Osawa, 1987). This is due to the synonymous substitution of the GC content within the third codon position for most of the amino acids during the process of translation (Palidwor et al., 2010). Overall, GC content for the MAP and *E. coli* is 69.3% and ~50.8%, respectively

93
(Bannantine et al., 2002; Touchon et al., 2009). High GC content affecting the expression of mycobacterial antigenic proteins in many prokaryotic systems due to codon bias has been previously reported (Gvritishvili et al., 2010; Johnston et al., 2013; Lakey et al., 2000; Piubelli et al., 2013).

Statistical approaches through advanced bioinformatics analytical tools and computational biological software are readily available for researchers to calculate codon bias, to optimize codon usage, to calculate the codon adaptation index (Wagner et al., 2005), and to predict the level of protein expression (Komar, 2016; Quax et al., 2015). Furthermore, synthetic gene biology is growing very rapidly and associated costs are progressively being reduced (Elena et al., 2014). These approaches are comparatively easy and less time-consuming, which allows for more efficient recombinant protein production.

In this study, we over expressed six MAP cell envelope proteins for use in polyclonal antibody production for identification of MAP organisms in the infected tissues by immunohistochemistry and immunomagnetic capturing of MAP organisms.

2.3. Materials and methods

Codon optimization

DNA sequences of six MAP genes including MAP3698c (succinate dehydrogenase iron-sulfur subunit-sdhA), MAP0150c (acyl-CoA dehydrogenase-fadE25_2), MAP4129 (ribonucleotide-transport ATP-binding protein ABC transporter- mkl), MAP3651c (acyl-CoA dehydrogenase-fadE3_2), MAP1233 (hypothetical protein) and MAP2698c (acyl-ACP desaturase-desA2) were retrieved from the GenBank database. Nde I (CATATG) and Xho I (CTCGAG) restriction
enzyme sites were added at 5’ and 3’ prime ends of the selected genes respectively. A polyhistidine (6X His) tag was incorporated in the C-terminal region of the ORF to increase the protein solubility and for affinity purification of proteins. Rare codons of the selected genes were then optimized with *E. coli* to maximize their expression. Genes were synthesized as strings of DNA fragments by Invitrogen, Life Technologies (Burlington, ON, Canada). Synthetic DNA fragments were suspended in 10 mM Tris buffer (pH 8.5) at a final concentration of 20 ng/µL, were aliquoted and stored at -20 ºC.

**PCR amplification of synthetic DNA fragments and gel purification**

Synthetic DNA fragments were PCR-amplified using the primers and cycling conditions shown Table 1. All primers were synthesized from laboratory services at the University of Guelph. All of the genes were PCR-amplified by Q5® High-Fidelity DNA Polymerase (New England BioLabs, Whitby, ON, Canada). PCR-amplified products were gel-purified as per standard protocols.
Table 1: List of primers used to amplify DNA fragments and cycling conditions.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers (underline indicates RE sites)</th>
<th>Cycling conditions</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sdhA</td>
<td>Fp: 5’-gaaggagatatacatatggtggaag-3’</td>
<td>Denaturation at 95 °C -4min, 25 cycles of 95 °C-1 min, 63 °C-30 sec, 72 °C-1 min, final ext.</td>
<td>1998</td>
</tr>
<tr>
<td></td>
<td>Rp: 5’-gttgggtgtgtgctgagttttct-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mkls</td>
<td>Fp: 5’-gaaggagatatacatatgaacctta-3’</td>
<td>72 °C-1 min, final ext. at 72 °C-5 min</td>
<td>1050</td>
</tr>
<tr>
<td></td>
<td>Rp: 3’-gttgggtgtgtgctgaggccggaacte-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>desA2</td>
<td>Fp: 5’-gaaggagatatacatatggcagac-3’</td>
<td></td>
<td>865</td>
</tr>
<tr>
<td></td>
<td>Rp: 5’-gttgggtgtgtgctgacggcaata-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fadE25_2</td>
<td>Fp: 5’-gaaggagatatacatatggtggtgtg-3’</td>
<td>Denaturation at 95 °C - 4 min, 25 cycles of 95 °C -1 min, 60 °C - 30 sec, 72 °C -1 min, final ext. 72 °C-5 min</td>
<td>1246</td>
</tr>
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<td></td>
<td>Rp: 3’-gttgggtgtgtgctgagatcaca-3’</td>
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</tr>
<tr>
<td>fadE3_2</td>
<td>Fp: 5’-gaaggagatatacatatgggtgcaaa-3’</td>
<td>Denaturation at 95 °C -4 min followed by 25 cycles of 95 °C-1 min, 65 °C -30 sec, 72 °C-1 min. Final ext. 72 °C -5 min</td>
<td>1248</td>
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<td>Rp: 5’-gttgggtgtgtgctgagacgarracca-3’</td>
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<tr>
<td>Hypothetic</td>
<td>Fp: 5’-gaaggagatatacatatggtgcacttc-3’</td>
<td>Denaturation at 95 °C- 4 min, 25 cycles of 95 °C -1 min, 61 °C- 30 sec, 72 °C for 1 min. Final ext.72 °C- 5 min</td>
<td>753</td>
</tr>
<tr>
<td>alprotein</td>
<td>Rp: 3’-gttgggtgtgtgctgagacgatca-3’</td>
<td></td>
<td></td>
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<tr>
<td>MAP1233</td>
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Restriction digestion of DNA fragments and plasmids, preparation of competent cells, ligation and transformation

Gel-purified DNA fragments and circular pET 30a (+) vector (Novagen Co, Darmstadt, Germany) were double-digested with NdeI and XhoI restriction enzymes (RE) (New England BioLabs, Whitby, ON Canada). The pET 30a (+) vector contains a kanamycin antibiotic resistant and this gene serve as a selection marker. Competent cells of E. coli BL21 (DE3) strain (New England BioLabs, Whitby, ON Canada) were generated using the Inoue method as previously described (Sambrook and Russell, 2006).

For all the ligation reactions, a molar ratio of linearized vector to insert of 1:5 was used. RE-digested and gel-purified DNA fragments were ligated with linearized pET 30a (+) vector using T4 DNA ligase (Invitrogen, 5791 Van Allen Way Carlsbad, CA, USA) overnight at 26 °C. Ligated products were transformed into E. coli BL21 (DE3)-competent cells by heat shock for 90 sec at 42 °C in a water bath and then were incubated on ice for 5 min as previously described (Sambrook and Russell, 2006). One mL of LB broth was added into each tube containing transformed E.coli cells and incubated at 37 °C with vigorous shaking (250) rpm for 1 h. Cells were centrifuged at 17,000g for 2 min at 4 °C. Subsequently, 800 µL of supernatants were discarded and pellets were re-suspended in remaining 200 µL of supernatants. Cells were plated on LB agar plates supplemented with kanamycin (30 µg / mL). Plates were incubated at 37 °C for overnight.
Clone confirmation, PCR and plasmid preparation by alkaline lysis for sequence analysis

To confirm the presence of inserts, four colonies per gene were picked from the plates, and resuspended in 75 µL of nuclease free water, boiled for 10 min, cooled on ice for 5 min and centrifuged at 17,000g for 5 min at 4 ºC. Subsequently, 5 µL of supernatants were used as templates for colony PCR with gene-specific primers and universal T7 primers (promoter and terminator) as described by (Sambrook and Russell, 2006). PCR amplified products were visualized in 1.5% DNA agarose gels. Plasmids were isolated from colonies with PCR-confirmed clones by alkaline lysis (Sambrook and Russell, 2006) and submitted for sequencing at the Advanced Analysis Centre, University of Guelph (Ontario, Canada).

Testing of recombinant protein expression and solubility

Stock cultures (E. coli cells containing) of sequence verified genes stored at 4 ºC were streaked on LB agar plates supplemented with kanamycin (30 µg / mL) and streaked plates were incubated at 37 ºC for overnight. Two colonies per gene were picked from overnight grown plates, suspended in LB broth supplemented with kanamycin (30 µg / mL) and grown for 37 ºC in an orbital shaker (300 rpm) for 4 h. Then, 500 µL of these cultures were added into 20 mL of LB broths supplemented with kanamycin (30 µg / mL) and grown for 37 ºC in an orbital shaker (200 rpm) for overnight. Subsequently, 200 µL of cultures from each sample were aliquoted to check the OD$^{600}$. After reaching desired OD$^{600}$ (0.5 to 0.7), 500 µL of cultures were aliquoted as pre-induction samples and remaining cultures were induced with 0.1 M IPTG. Induced cultures were incubated at 37 ºC with vigorous shaking at 300 rpm for 4 h. One mL of induced cultures from each samples were centrifuged at 12,000g for 3
min at 4 ºC. Pellets were suspended in 80 µL of MQ water and 20 µL of SDS-PAGE loading buffer (6X) and heated 95 ºC for 10 min. 10 µL per sample were loaded into 12.5 % BIS acryl amide gels and ran for 1 h at 180V and gels were stained by rapid coomassie staining procedures to see the expressions. Protein solubility tests were performed as per the standard protocols described (Loughran and Walls, 2011). Different concentrations of glucose were added into the pre-induction culture media containing E. coli cells carrying recombinant clones to control the level of basal expression and cultures were induced with different concentrations of IPTG as previously described (Berrow et al., 2006).

**Purification of recombinant proteins under native conditions**

To obtain large quantities of pure recombinant proteins, E. coli BL21 (DE3) carrying recombinant clones were grown in bulk (250 mL LB broth supplemented with kanamycin (30 µg / mL) and glucose (0.1 to 0.4%) X 4 flasks/gene) for 27 ºC in an orbital shaker (200 rpm) for overnight. Subsequently, 200 µL of cultures from each sample were aliquoted to check the OD$_{600}$. After reaching desired OD$_{600}$ (0.5-0.7), 500 µL of cultures were aliquoted as pre-induction samples and remaining cultures were induced with 0.1 M IPTG (final concentration). Induced cultures were incubated at 37 ºC with vigorous shaking at 300 rpm for 4 h. Induced cultures were harvested at 6500 rpm for 15 min at 4 ºC and bacterial pellets were frozen on dry ice and stored at -80 ºC until further use. Bacterial pellets were resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, 500 mM NaCl, 10 mM imidazole pH 7.4), containing lysozyme (2 mg/mL) (Sigma-Aldrich, 3050 Spruce street, St. Louis, MO, USA) and complete protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, German) and incubated at room temperature for 30 min. Following three freeze (15 min on dry ice)
and thaw (8 min at 37 °C in a water bath) cycles, cells were pulse-sonicated on ice with 90s rests after each sonication. Lysed cells were then centrifuged at 24,000 rpm for 25 min at 4 °C, transferred to new tubes and stored on ice. Up to this stage, the procedure was common for all the soluble recombinant proteins.

FadE25_2 and DesA2 recombinant proteins were purified by PureProteome™ Nickel magnetic beads (EMD Millipore, Temecula, CA, USA) as per manufacturer’s instructions. SdhA and FadE3_2 recombinant proteins were purified by the gravitational flow method using HiTrap™ TALON® crude resins (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) as per the manufacturer’s instructions.

**Purification of recombinant proteins under denatured conditions**

Recombinant MAP gene locus MAP1233 encoding a hypothetical protein when expressed formed insoluble inclusion bodies. Protein purification was performed under denaturing conditions to solubilize the proteins. Bacterial pellets received lysis buffer (50 mM NaH₂PO₄, 500 mM Nacl, pH7.4) and were supplemented with lysozyme (2 mg/mL). Cells were lysed as described above, centrifuged and supernatants were discarded. Pellets were washed twice with 5 ml Tris-HCl (pH 8.0) buffer, centrifuged and supernatants were discarded. Pellets were suspended in PBS (pH 7.4) and disrupted by sonication. To isolate the pure inclusion bodies from cell lysates, the sucrose gradient method was used as previously described (Upadhyay et al., 2012). Proteins in the purified inclusion bodies were dissolved with denaturing urea buffer (8 M urea, 100 mM NaH₂PO₄, 10mM TRIS-HCL pH 8.0) and then further purified with HisPur™ Ni-NTA (Nickel-Nitrilotriacetate) resin (Thermo Scientific, Meridian Rd, Rockford, IL, USA) by gravitational flow as per the previously described protocol (Loughran and Walls, 2011).
**Immunoblot analysis of recombinant proteins with anti-His antibodies**

Purified proteins were electrophoretically separated on 12% bis acrylamide gels, transferred onto nitrocellulose membranes and incubated in blocking buffer [5% skim milk in Tris-buffered saline pH 7.6, contains 0.1% Tween 20 (TBST)] for 1 h at room temperature. Membranes were incubated with monoclonal anti-polyhistidine-alkaline phosphatase antibody (Sigma-Aldrich, St. Louis, MO, USA) (1:2000 in 2% BSA in TBST) for 2 h at RT, washed with TBST buffer and visualized by incubating with NBT/BCIP substrate (Thermo Scientific, Meridian Rd, Rockford, IL, USA) as per manufacturer’s instructions.

**Immunization of rats with recombinant protein antigens**

Six female Sprague-Dawley rats (2 rats/protein) were used to generate polyclonal antibodies against SdhA, FadE25_2 and DesA2 recombinant proteins. In brief, 100 μg (50μg/rat) of purified and desalted proteins were aliquoted into microcentrifuge tubes and mixed with an equal volume of TiterMax gold adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and vortexed 20 min at 4 ºC to prepare an emulsion for the primary immunization. Following collection of pre-immune serum samples, rats were injected intramuscularly with emulsions. For subsequent booster immunization, 50 μg (25μg/rat) of protein antigens were mixed with Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and vortexed to prepare 1:1 emulsions. Three booster immunizations were given on day 14, 28 and 40 respectively. On third day following the last booster immunization, serum was prepared from whole blood collected from terminally anesthetized rats and stored at -80 ºC. Immunization protocols, the use and care of animals in this experiment were approved by the University of Guelph Animal Care and Use Committee.
Immuno blot analysis of rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies against recombinant proteins

For western blot analysis, each one of the purified proteins (SdhA, FadE25_2 and DesA2) were electrophoreically separated on duplicate gels, transferred onto nitrocellulose (NC) membranes and blocked against non-specific protein as described above. One set of membranes was incubated overnight at 4º C with pre-immune serum samples diluted 1:500 with 5% skim milk in TBST. Another set of membranes were incubated overnight at 4º C with the immune serum (1:5000 in 5% skim milk in TBST) from rats immunized with SdhA, FadE25_2 and DesA2 for. NC membranes were incubated with anti-rat IgG HRP-conjugated secondary antibody (Cell signalling Technology, INC, MA01923, USA) (1:5000 in 5% skim milk in TBST) for 1 h at room temperature. After each step, membranes were washed with TBST. The blots were incubated with Clarity\textsuperscript{Tm} Western ECL substrate (Bio-Rad Laboratories, Inc, USA) as per manufacturer’s instructions. Signals were detected using a ChemiDoc\textsuperscript{Tm} imaging system (Bio-Rad Laboratories, Inc, USA).

Immuno blot analysis of polyclonal antibodies against MAP cell envelope proteins

To test the specificity of the rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies to the MAP cell envelope proteins antigens, 1-DE and 2-DE analysis was performed. In brief for 1-DE, 15 µg of MAP, MAH and \textit{M. smegmatis} cell envelope protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific sites were blocked with 5% skim milk in TBST and incubated overnight at 4 ºC with rat anti-SdhA, FadE25_2 and DesA2 antibodies (1:5000 in 2% BSA in TBST). Membranes were washed with TBST and incubated with anti-rat HRP-linked conjugate (1:2000 dilution in 5% skim milk in TBST) for 1
h at RT and then probed with Clarity™ Western ECL substrate (Bio-Rad Laboratories, Inc, USA). For 2-DE western blot analysis, 125 µg of MAP cell envelope proteins underwent isoelectric focusing (IEF) on 24 cm, nonlinear, pH 3–7 Immobiline DryStrips (GE Healthcare) followed by electrophoresis on 12.5% acrylamide gels. Proteins were then identified by the immunoblot procedure as described above using rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies.

*Extraction of MAP cell envelope proteins and generation of polyclonal antibodies to MAP total cell envelope proteins*

*Bacterial strains and growth conditions*

Three MAP strains (Madonna, gc86 and gd30 strains) isolated from clinical cases from southern Ontario (Canada) were grown in Middlebrook 7H9 broth medium supplemented with glycerol (0.5% v/v), 10% (v/v) oleic acid-albumin-dextrose-catalase and mycobactin-J (2 µg/mL) (Allied Monitor Inc, Fayette, MO, USA) at 37°C for 6 to 8 weeks. Cultures were harvested by centrifugation at 3500 rpm for 30 min at 4°C and washed three times with ice-cold phosphate buffered saline (PBS) (pH 7.4).

*Subcellular fractionation and cell envelope protein extraction*

Subfractionation of MAP was done to obtain cell wall core and cytoplasmic membranes as described (He and De Buck, 2010; Zheng et al., 2012) with a few modifications. Briefly, 2 mL of lysis buffer (0.05 M potassium phosphate, 0.022% (v/v) β- mercaptoethanol, pH 6.5) was added to each gram of frozen bacterial cells (wet cell weight). Lysozyme (final concentration 4.8 mg/mL w/v) (Sigma-Aldrich, Oakville, CA) and complete protease inhibitor cocktail (Roche Diagnostics GmbH,
Mannheim, Germany) were also added to the cells and incubated for 4 h at 37°C with continuous stirring. Bacterial lysates were transferred into 2 mL screw-capped centrifuge tubes containing sterile zirconium-silica beads (0.1mm) (Biospec Products, Inc) and disrupted by 12 cycles of high velocity bead beating with intermittent cooling on ice after each bead beating. Lysates were centrifuged at 1000 g for 15 min at 4°C to remove unbroken cells and debris. Supernatants were centrifuged at 20,000 g for 30 min at 4°C to obtain the pelleted cell walls and the supernatants containing cytoplasmic membrane and cytosolic fractions. Supernatants were centrifuged at 150,000 g for 90 min at 4°C to obtain cytoplasmic membranes and cytosolic fractions. Pellets containing cells wall and membrane fractions [i.e. cell envelope] were then washed twice with PBS (pH 7.4) containing protease inhibitor cocktail to remove cytoplasmic contamination. Cell envelope pellets were re-suspended in standard cell lysis buffer containing 7 M urea (Sigma-Aldrich, St.Louis, MO, USA), 2 M thiourea (GE Healthcare, Montreal, QC, Canada), 30 mM Tris/HCl, 4% CHAPS (pH 8.5) (Affymetrix Inc, Maumee, Ohio, USA), incubated on ice for 30 min with intermittent mixing and centrifuged at 10,000 g for 20 min at 4°C to remove insoluble materials.

Protein extracts were dialysed against descending concentrations of urea, thiourea and CHAPS buffers with a final dialysis with 10 mM PBS (pH 7.2). Following collection of pre-immune serum samples, three female Sprague-Dawley rats were immunized intramuscularly with emulsions of MAP total cell envelope proteins (150 μg/rat) mixed with equal volumes of TiterMax gold adjuvant (Sigma-Aldrich). For subsequent booster immunizations, 75 μg of protein antigens per rat was mixed with Freund's incomplete adjuvant and vortexed to prepare a 1:1 emulsion. Three booster immunizations were given on day 14, 28 and 40 respectively. Rats were
euthanized on the third day following the last booster immunization and serum was prepared from whole blood and stored at -80 °C. Immunization protocols, the use and care of animals in this experiment were approved by the University of Guelph Animal Care and Use Committee.

**Immunoblot analysis of MAP, MAH and MS cell envelope proteins using polyclonal antibodies generated to cell extracts of MAP total cell envelope proteins**

For immunoblot analysis with rat anti-MAP polyclonal antibodies, 25 µg of proteins from MAP, MAH and MS cell envelope proteins were electrophoretically transferred to nitrocellulose membranes as described above, were then incubated with pre-immune (1:1000 dilutions) and post-immune (1:6000 dilutions) serum samples and visualized as described above in immunoblot analysis of polyclonal antibodies against MAP cell envelope proteins section.

**Immunohistochemistry (IHC) and immunofluorescence (IF)**

IHC was performed as previously described (Hemida and Kihal, 2015) with intestinal tissues and lymph nodes from cattle naturally infected with MAP. For negative controls, intestinal tissues and lymph nodes from calves not previously exposed to MAP (kindly provided by Dr. Brandon Plattner, Department of Pathobiology, University of Guelph). Tissue sections (5 µm) were prepared and antigens were retrieved in sodium citrate buffer at 95 °C for 20 min in a water bath and allowed to cool at RT for 30 min. Sections were repeatedly washed with dH₂O and endogenous peroxidase activity was blocked with 3% H₂O₂ in dH₂O for 30 min at RT. Non-specific sites were blocked with goat serum for 1 h in a humidified chamber at RT, washed with TBST and incubated with rat polyclonal antibodies against each
recombinant proteins (SdhA, FadE25_2, DesA2) and the MAP cell envelope proteins diluted to 1:50 in 1% BSA in TBST overnight at 4 °C in a humidified chamber. Sections were then incubated with anti-rat-HRP-linked conjugate (Cell signaling Technology, INC, MA01923, USA) diluted 1:50 in 5% skim milk in TBST and incubated for 1 h at RT in a humidified chamber. Tissue sections were then washed and incubated with 200 µL of ImmPACT NovaRed peroxidase substrate (Vector Lab, Burlingame, CA) in the dark for 5 to 20 min. Slides were washed with dH2O and counter-stained with Harris' haematoxylin solution and mounted with cover slips. Slides were examined under a light microscope for the presence of antigen antibody reactions. For immunofluorescence experiments, tissue sections were processed in a manner similar to that of IHC, except endogenous inactivation of peroxidases and secondary antibodies are labelled with FITC (Abcam, ab97056) and diluted 1:500 in 5% skim milk in TBST. Slides were then mounted with ProLong Gold antifade reagent (Invitrogen, Eugene, Oregon, USA) as per manufacturer’s instructions.

**Immunomagnetic capture of MAP**

Magnetic Protein G Dynabeads (Thermo Fisher Scientific) were aliquoted (10 µL/tube) into 1.5 mL microcentrifuge tubes and washed with PBST (0.1%, pH 7.5), suspended in 200 µL of PBST and loaded with either 3 µg of purified rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies per tube or 5 µL of rat anti-MAP (total cell envelope protein) polyclonal antibodies per tube. Tubes were incubated overnight at 4 °C with gentle mixing and then placed on a magnetic stand where unbound antibodies were removed and beads were washed twice with PBST (0.1% Tween 20, pH 7.4). MAP cultures were harvested by centrifugation at 6500 g for 10 min at RT followed by three washing steps with PBST and bacterial pellets were re-
suspended in PBST. MAP cells were then passed through 25-gauge needles to break the bacterial clumps as mentioned by (Gilardoni et al., 2016). MAP cells were quantified by measuring optical density at 600 nm as mentioned by (Mead, 2013) and an optical density of 0.6 to 0.9 at 600 nm was equivalent to approximately $10^8$ CFU of MAP organisms per mL (Mead, 2013). For exact numbers, optical density adjusted MAP organisms (OD$_{600}$-0.6) were serially diluted from $10^8$ to $10^1$ in one mL of PBST, 100 µL from each dilution plated on Middlebrook 7H11 agar plates supplemented with mycobactin J and OADC medium and plates were incubated at 37°C.

For the IM capture, a volume of 100 µL from each dilution was mixed with 10 µL of antibody-bound protein G beads and incubated at RT for 1 h with mixing on a rotor. For negative controls, beads were coated with anti-Alpha-1-acidglycoprotein or CYP2A5 antibodies and incubated with MAP ($10^7$ CFU) bacteria. Similarly, beads were incubated with PBST containing MAP ($10^7$ CFU) and this also used as a negative control to test for non-specific binding of MAP with beads. Beads were washed 3 times with PBST buffer in a magnetic separator to remove unbound bacteria. Immunomagnetic-captured MAP was then suspended in 50 µL of sterile PBS stored at 4°C until further use.

**PCR assay with immunomagnetically captured MAP**

To test whether IM capture of MAP was successful, a PCR assay was performed using DNA templates prepared from IM-captured MAP and using MAP species-specific (IS900) published primers (Moravkova et al., 2008). In brief, 10 µL of IM-captured MAP from previous steps were transferred into new 1.5 mL microcentrifuge tubes, placed on a magnetic stand and PBS solutions was removed carefully. IM-captured MAP bacteria were re-suspended in 20 µL of TE buffer,
heated at 95 °C in a thermal cycler for 30 min and cooled on ice for 5 min. For the known positive control, 25 µL of MAP culture was boiled in TE buffer for 10 min, cooled on ice followed by quick high speed centrifugation. Four µL of these suspensions were used as a DNA template for PCR and amplification was carried out as per the cycling conditions described by (Moravkova et al., 2008). PCR-amplified products were then visualized in 2% agarose gels.

**Confirmation of MAP attachment to IM beads by culture**

To test the efficiency of MAP recovery from IM captured beads by culture, a volume of 10 µL of beads from each dilution was mixed with 90 µL of sterile MQ water, plated on Middlebrook 7H11 agar plates supplemented with mycobactin J and OADC medium and plates were incubated at 37 °C for 6 to 8 weeks. Colonies were counted and CFU were calculated as per standard procedures.

2.4. Results

**Codon optimization**

In general mycobacterial genomes contain very high level of GC contents (69%) and have disparities in the codon usage with commonly used *E. coli* based prokaryotic expression system. This is a major limiting factor in expression of mycobacterial genes in heterologous expression systems. Therefore, *sdhA, fadE25_2, mkl, fadE3_2, desA2* and the gene for hypothetical protein (MAP1233) were optimized with most frequently used codons of *E. coli*. Details of the CAI, GC and AT percentages of the native and codon optimized genes are given in Table 2.
Table 2. Codon optimized MAP genes in reference with *E. coli*.

<table>
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<tr>
<th>S No.</th>
<th>MAP Genes</th>
<th>GenBank Version</th>
<th>Location in MAP genome sequence (bp)</th>
<th>Native DNA</th>
<th>Optimized DNA</th>
<th>Protein molecular weight (kDa)</th>
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<td>CAI</td>
<td>GC%</td>
<td>AT%</td>
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<td>0.245</td>
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<td>49.44</td>
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**PCR amplification and gel purification of synthetic DNA fragments**

Synthetic gene fragments were used as template in PCR reactions to generate the double strand DNA sequences to clone into pET 30a (+) expression vector. All the six genes were successfully amplified using Q5® high-fidelity DNA polymerase with a low number of amplification cycles (25) to prevent PCR-induced mutational errors. While PCR-amplified genes yielded expected amplicon sizes (fig. 1), non-specific amplification occurred for *sdhA, fadE25_2* and *fadE3_2* necessitating gel purification of these PCR products.

**Figure 1:** PCR amplification of codon optimized fragments of synthetic MAP DNA. All of the genes yielded expected product size (Kbp) as indicated by arrows (*sdhA*-2.0 Kbp, *fadE25_2*-1.25 Kbp, *mkl*-1.050 Kbp, *fadE3_2*-1.250 Kbp, MAP1233- 0.753 Kbp).
Kbp and desA2-0.865 Kbp). Non-specific amplification of products for sdhA, fadE25_2 and fadE3_2 are indicated by asterisks.

Recombinant protein expression

High level of expression of five genes including sdhA, fadE25_2, fadE3_2, desA2 and hypothetical protein was induced with 0.1M IPTG in LB media, however, expression of Mkl protein was not successful due to toxicity to E. coli cells upon induction with IPTG. Further attempts to express this protein under different conditions such as growing cells under low temperatures (23 to 30 °C), addition of glucose into the media, altering plating methods, conducting plasmid stability tests, induction with low concentrations of IPTG (2.5 µM to 10 µM) and expression in BL21(DE3) pLyS cells E. coli cells were unsuccessful.

Protein purification under native and denatured conditions

Recombinant SdhA, FadE25_2, FadE3_2 and DesA2 were soluble, recovered in the supernatants of lysed E. coli cells and MAP1233 hypothetical protein was insoluble and formed inclusion body recovered in the pellet of the lysed cells. The poly-His (C-terminus) tagged recombinant proteins were affinity purified [To avoid co-purification of host cell proteins along with the MAP target proteins], SdhA, FadE25_2, FadE3_2 and DesA2 proteins were purified under native conditions by using Ni-NTA affinity chromatography techniques. Use of nickel magnetic beads yielded pure proteins for FadE25_2 and DesA2, however, SdhA, FadE3_2 and hypothetical protein MAP1233 did not bind to magnetic beads and were purified with cobalt resins. Hypothetical protein MAP 1233 was isolated as inclusion bodies by
sucrose gradients method, denatured with 8 M urea lysis buffer, purified with Ni-NTA resins, and pure proteins were eluted in low pH (4.5) urea denaturation buffer.

**Western blot analysis of recombinant proteins with anti-His antibodies**

Immunoblot analysis of the purified recombinant proteins with monoclonal anti-His antibodies showed expected molecular sizes without any additional protein bands for four recombinant proteins (SdhA, FadE25_2, DesA2 and MAP1233 hypothetical protein) (fig. 2). However, several higher molecular weight bands of 100 kDa and > 150 kDa were observed for FadE3_2 that may be due to dimerization of FadE3_2 protein or co-purification of host cell proteins.

![Image of Western blot analysis](image)

**Figure 2:** Immunoblot analysis of recombinant proteins with anti-His antibodies. Purified recombinant proteins reacted with anti-His monoclonal antibodies at expected molecular sizes (SdhA-2 kDa, FadE25_2-44 kDa, FadE3_2-44 kDa, MAP1233-26 kDa and DesA2-31 kDa). Note: Asterisks indicates co-purified host cell protein or protein dimerization.
**Generation of antibodies against MAP recombinant proteins and immunoblot analysis**

Polyclonal antibodies against three of the recombinant proteins (SdhA, FadE25_2 and DesA2) were generated in rats and immunoreactivity was tested periodically after each immunization as described in Material and Methods. Pre-immune serum samples showed no antigen antibody reactions to the target proteins (data not shown) and post-immune serum samples strongly reacted with SdhA, FadE25_2 and DesA2 recombinant proteins (figs. 3A-C). Antibodies were not generated for three proteins including FadE3_2, Mkl and hypothetical protein MAP1233 for the following reasons. Recombinant Mkl was minimally expressed and was toxic to *E. coli* cells upon induction with IPTG. As a result insufficient amounts of high quality Mkl protein were available for immunization. During the purification of FadE3_2 recombinant protein, *E.coli* host cell proteins were present which may have affected the downstream application of antibodies (i.e. sensitivity and specificity of diagnostic tests). Hypothetic protein MAP1233 was recovered in inclusion bodies. Attempts to refold the protein were unsuccessful; the protein required a minimum of 3M urea to remain soluble and was therefore not used for antibody generation.
Figure 3: Assessment of immunogenicity of recombinant proteins against polyclonal antibodies. Immunoblot analysis of MAP SdhA, FadE25_2 and DesA2 recombinant proteins with rat anti-SdhA (Panel A), anti-FadE25_2 (Panel B) and anti-DesA2 (Panel C) polyclonal antibodies, respectively.

**1-DE and 2-DE immunoblot analysis of polyclonal antibodies against MAP cell envelope proteins**

To determine the specificity of the polyclonal antibodies to the three recombinant MAP cell envelope proteins, 1-DE and 2-DE immunoblotting was performed using total MAP cell wall extracts. Results of 1-D immunoblot analysis revealed single bands of molecular weights corresponding to SdhA, FadE25_2 and DesA2 with very minimal cross-reactivity with MAH and MS cell envelope proteins.
Figure 4: Assessment of specificity of polyclonal antibodies to Mycobacterium avium subs. paratuberculosis and orthologous mycobacterial species. Immunoblot analysis with rat anti-SdhA (Panel A), rat anti-FadE25_2 (Panel B) and rat anti-DesA2 (Panel C) antibodies against MAP, MAH and MS cell envelope proteins. Note: Antibodies reacted with MAP specific proteins with no or minimal cross-reactivity with MAH and M. smegmatis.
In 2-DE immunoblot analysis, polyclonal antibodies reacted specifically with SdhA, FadE25_2 and DesA2 proteins as indicated by a correlation with pI and molecular weight coordinates in the 2-DE proteome map of MAP (fig. 5 A-D). Polyclonal antibodies against SdhA detected several additional MAP protein spots (fig. 5 B) that were subsequently confirmed as SdhA by LC-MS/MS analysis. Similarly, 2-DE immunoblot analysis revealed that polyclonal antibodies against FadE25_2 protein detected one additional MAP protein spot (fig. 5 C) which was confirmed as FadE25_2 by LC-MS/MS. This suggests the existence of post-translational modifications or different isoforms for SdhA and FadE25_2 proteins.

**Figure 5:** Assessment of specificity of polyclonal antibodies to *Mycobacterium avium* supsb. *paratuberculosis*. Western blot (2D-PAGE) of cell envelope proteins of MAP probed with rat anti-SdhA, rat anti-FadE25_2 and rat anti-DesA2 antibodies. Panel A:

**Immunoblot analysis of rat anti-MAP cell envelope proteins polyclonal antibodies**

The specificity of polyclonal antibodies to an extract of total MAP cell envelope proteins was assessed by immunoblotting on nitrocellulose membranes with MAP, MAH and MS cell envelope protein extracts (fig. 6). The results revealed that rat anti-MAP polyclonal antibodies were strongly immunoreactive with MAP cell envelope protein extracts with minimal cross-reactivity with MAH and MS cell envelope proteins.
Figure 6: Assessment of specificity of rat polyclonal antibodies generated against cell envelope proteins from *Mycobacterium avium* subsp. *paratuberculosis*. Western blot (1D-PAGE) analysis of envelope protein extracts from *M. avium* subsp. *paratuberculosis* (MAP), *M. avium* subsp. *hominisuis* (MAH) and *M. smegmatis* (MS).

**Immunohistochemistry and immunoflorescence**

Hematoxylin and eosin (H&E) staining of formalin-fixed intestinal tissues from cattle infected with MAP demonstrated mononuclear inflammatory cell infiltrates (fig. 7A) and acid-fast staining indicated the presence of bacilli (fig. 7B). Immunohistochemistry with antibodies generated to MAP whole cell envelope proteins extracts showed strong immunoreactivity to MAP bacteria (fig.7C). Similarly immunofluorescence using FITC-labelled antibodies to MAP whole cell envelope protein extracts also identified the presence of MAP antigens in sections of intestines (fig. 7D) and lymph nodes (fig. 7E). There were no antigen antibody reactions in the control tissue sections using both IHC and IF staining methods (fig. 8B-D). Rat anti-SdhA, anti-FadE25_2 and anti-DesA2 polyclonal antibodies did not show any antigen antibody reactions in these tissues.
**Figure 7:** Immunohistochemical and immunofluorescence staining of tissues sections using anti-MAP cell envelope antibodies. Panel A: H&E stained intestinal tissue section from a MAP-infected cow showing mononuclear inflammatory cell infiltrates. Panel B: Ziehl–Neelsen acid-fast staining of intestinal tissue showing the presence of MAP organisms (red bacilli). Panel C: IHC of intestinal tissue section with antibodies to MAP whole cell envelope protein extract showing strong immunoreactivity (brown colour). Panel D: IF staining of intestinal tissue (Panel D) and lymph node sections (Panel E) with antibodies to total MAP cell envelope protein extract showing strong immunoreactivity with MAP bacteria (bright green fluorescent spots).
Figure 8: Immunohistochemical and immunofluorescence staining of control animal tissues sections with anti-MAP cell envelope antibodies. Panel A: H&E-stained intestinal tissue section from a calf not exposed to MAP. Panel B: IHC of intestinal tissue section from a calf not exposed to MAP probed with antibodies to MAP whole cell envelope protein extracts showed no antigen antibody reactions. IF staining of intestinal tissue (Panel C) and lymph node sections (Panel D) from a calf not exposed to MAP with antibodies to total MAP cell envelope protein extract.

Immunomagnetic capture of MAP

Capturing efficiency of the four polyclonal antibodies was analyzed by PCR targeting MAP species-specific (IS900) primers and culturing of MAP. PCR amplification results revealed that immunomagnetic capture with rat anti-MAP (total
cell envelope) polyclonal antibodies was most efficient, yielded the expected product size of 0.215 Kb for up to $10^2$ CFU of MAP (fig. 9A) and this was confirmed by culture results as well. Immunomagnetic capture with rat anti-SdhA polyclonal antibodies yielded the expected product size for up to $10^3$ CFU of MAP (fig. 9B), whereas immunomagnetic capture with rat anti- FadE25_2 and DesA2 polyclonal antibodies yielded the expected product size for up to $10^5$ CFU of MAP (fig. 10A and B). Negative control samples that included beads without antibodies or antibodies to an unrelated protein (Alpha-1 acid glycoprotein or CYP2A5) failed to produce a PCR product and colonies on the Middlebrook 7H11 agar plates thereby confirming a lack of non-specific binding. This indicates that the magnetic beads coated with polyclonal antibodies to MAP whole cell envelope protein extracts or antibodies to recombinant SdhA, FadE25_2 and DesA2 were able to bind and capture intact MAP bacteria.
**Figure 9:** PCR amplification of MAP microorganisms isolated by immunomagnetic capture using rat anti-MAP polyclonal antibodies. Panel 9A: Antibodies to extract of MAP total cell envelope proteins: PCR amplification using MAP species-specific gene IS900 primers yielded the expected size of 0.215 kbp in MAP ranging from $10^8$ to $10^2$ CFU. Panel 9B: Antibodies to SdhA: PCR amplification using MAP species-specific gene IS900 primers yielded the expected size of 0.215 Kb from $10^8$ to $10^3$ CFU of MAP.
Figure 10: PCR amplification of MAP microorganisms isolated by immunomagnetic capture using rat anti-MAP polyclonal antibodies. Panel 10A: Antibodies to FadE25.2: PCR amplification using MAP species-specific gene *IS900* primers yielded the expected size of 0.215 Kb from $10^8$ to $10^5$ CFU of MAP. Panel 10B: Antibodies to DesA2: PCR amplification yielded the expected size of 0.215 Kb from $10^8$ to $10^5$ CFU of MAP.

2.5. Discussion

This study describes the codon optimization and recombinant expression of several MAP species-specific cell envelope proteins, generation of polyclonal antibodies to these proteins and the use of the antibodies to identify intact tissue MAP bacteria by immunohistochemistry and immunofluorescence and capture of MAP from solution using immunomagnetic beads.

Recombinant protein technology plays a vital role in the development of diagnostic tests and vaccines for various infectious diseases (Souza et al., 2012).
Several studies have focused on the expression of MAP genes in *E. coli* cells to produce recombinant proteins of adequate quantity and quality (Bannantine et al., 2008b; Bannantine et al., 2010b; Cho et al., 2007). However, at times, heterologous expression of genes in *E. coli* is unsuccessful for many reasons, but frequently due to codon bias between MAP and *E. coli* cells. During gene expression, the presence of rare codons in the heterologous host bacteria may adversely affect the translational efficiency of mRNA due to inadequate availability of tRNA isoacceptor pools. This in turn leads to stalling of ribosomes, premature termination of protein synthesis, frameshifting, truncated proteins and overall reduction in the protein level from the heterologous hosts (Johnston et al., 2013). While codon optimization of *Mycobacterium tuberculosis* genes has significantly increased the expression of recombinant proteins in the heterologous host (Kanekiyo et al., 2005; Kashino and Campos-Neto, 2011; Piubelli et al., 2013), few reports are available on codon optimization and maximizing the protein expression of MAP genes in *E. coli*.

In this study, codon optimization of genes for six MAP species-specific cell envelope proteins improved the codon adaptation index, reduced the GC content and increased the AT levels significantly. Codon optimization also helps to decrease mRNA stability, to reduce the tight folding of mRNA and to prevent the formation of mRNA hairpins as well as improve translational efficiency (Johnston et al., 2013). Sequencing of the five PCR-amplified synthetic gene products, revealed three or more addition or/and deletion mutations. Despite the use of a high-fidelity polymerase enzyme with fewer amplification cycles, PCR-induced errors are inevitable with long gene sequences (more than 1 Kbp) (Sharifian 2010).
Of the six proteins that were recombinantly expressed, five were soluble (SdhA, FadE25_2, FadE3_2, DesA2 and Mkl) and one was insoluble hypothetical protein MAP1233). During the affinity purification of the soluble proteins under native conditions with Ni-NTA resin, co-purification of host cell proteins was noticed. Despite making alterations to the purification procedure, co-purification of undesired host proteins persisted for some of the recombinant proteins. It is possible that some of the host cell proteins may contain more histidine amino acids or host cell proteins may bind with recombinant proteins and thus lead to co-purification of host cell proteins. Similar (co-purification) issues have been reported in the literature (Gumber et al., 2009). For FadE25_2 and DesA2 proteins, this problem was overcome by using Pure Proteome nickel beads. However, three proteins (SdhA, FadE3_2 and hypothetical protein MAP1233) did not bind to these beads. Failure of recombinant protein binding to commercial beads has been reported previously, however, an explanation for this was not provided (Kim et al., 2007). Therefore, it is essential to test different resins and purification conditions to maximize the protein purity and one method may not be suitable for all protein.

The codon optimized synthetic gene for Mkl protein had a very low level of expression and was toxic to E. coli cells upon induction with IPTG. Failure of expression of codon-optimized genes has also been reported (Alexeyev and Winkler, 1999) who suggested that the rare codons in the native gene may not be an issue in heterologous expression in E. coli cells. Sometimes synthetic genes may acquire new properties, like unfavourable mRNA secondary structures (Alexeyev and Winkler, 1999) which negatively affect protein expression. Moreover, maximization of codon efficiency by altering rare codons leads to miscoding, frame-shifting errors, premature

125
termination, mistranslations, improper protein folding, functional loss, protein stability, abnormal proteins and toxicity to the host cells (Drummond and Wilke, 2008; Komar, 2016). In the current study, hypothetical protein MAP1233 was isolated as inclusion bodies by sucrose gradient separation and was purified under denaturing conditions. However, we were unable to generate a refolded hypothetical protein as it is highly unstable likely due to some of the factors listed above. Therefore, it is essential to consider other factors such as efficiency of translation, protein folding and biological activity when planning synthetic gene-based recombinant protein production (Komar, 2016). Consequently, we decided that Mkl and hypothetical protein MAP1233 were unsuitable to pursue for antibody generation.

Three MAP species-specific cell envelope recombinant proteins and an extract of total MAP cell envelope proteins were used to generate polyclonal antibodies in rats. Polyclonal antibodies are routinely used in pathogen identification by immunoblot, immunohistochemistry, biosensors and flow cytometry (Frenzel et al., 2013). The advantages of using polyclonal antibodies are that they are relatively quick and easy to generate, less expensive and target multiple epitopes / antigenic determinants on the protein which maximizes the diagnostic sensitivity and specificity of the assays (Shin et al., 2009). In our study, the sensitivity and specificity of polyclonal antibodies were assessed in 1-DE and 2-DE immunoblots that included MAH and M. smegmatis cell envelope proteins. We demonstrated that polyclonal antibodies were specific to MAP cell envelope proteins with very minimal cross reactivity with MAH and M. smegmatis cell envelope proteins. Others have shown that polyclonal antibodies generated against MAP cell wall proteins represent very specific, sensitive and useful tools in the identification MAP organisms (Stabel et al.,
1996). While antibodies specific to MAP are useful in the diagnosis of JD, generation of these antibodies is difficult due to the genetic similarity of the MAP with other closely related mycobacterial species (Bannantine et al., 2011). Several previous studies have used different methods to generate antibodies against MAP. The following aspects differentiate our antibodies with other studies: i) antigen targets were selected based on 2-D DIGE proteomic and 2-DE immunoblot analysis in an effort to identify individual cell envelope proteins that were MAP-specific and antigenic and ii) only MAP cell envelope proteins, either recombinantly expressed or as an extraction of total cell envelope proteins were used as antigens without other cellular protein components.

IHC and IF analysis of formalin-fixed intestinal and lymph node sections infected with MAP using rat polyclonal antibodies to total MAP cell envelope proteins revealed strong antigen-antibody reactions. Previous studies using different commercial and in-house antibodies for IHC and IFC showed variable sensitivity when compared to various gold standard diagnostic approaches (Martinson et al., 2008; Pedersen et al., 2011; Shin et al., 2009). For example, Martinson reported very low sensitivity for IHC compared to fecal culture. In contrast, others found that IHC was more sensitive in identification of MAP organisms in tissues sections compared to acid-fast staining (Brees et al., 2000; Hemida and Kihal, 2015). Also, Shin et al. (2009) showed that polyclonal antibodies from chickens (IgY) were more specific and sensitive in MAP capturing by magnetic separation. However, we determined that our rat polyclonal antibodies to SdhA, FadE25_2 and DesA2 antibodies were not able to identify MAP organisms in tissue sections. This may be due to irreversible damage to these antigens by formalin fixation in tissue sections used in these experiments or
Localization / accessibility of the antigens in *in vivo* MAP. Importantly, antigens of membrane origin are more susceptible to decay in formalin fixative than cytoplasmic antigens (Grillo et al., 2015). Other possible reasons could be masking of epitopes, protein-protein interactions and change in protein conformation by cross-linking of proteins by the formalin fixatives (Grillo et al., 2015). Therefore, further studies with other antigen retrieval methods, frozen tissue sections or tissues fixed in formalin for shorter periods are necessary to test the use of anti-SdhA, FadE-25_2 and DesA2 antibodies in IHC and IF.

In experiments on immunomagnetic capture of MAP, we show that polyclonal antibodies to extracts of MAP cell wall proteins that were coated on Dyna protein G beads are capable of capturing MAP organism’s suspended in PBS solution. This was confirmed by culture and conventional PCR with MAP species-specific *IS 900* primers. We also found that polyclonal antibodies to all three recombinant MAP cell wall proteins (SdhA, FadE25_2 and DesA2) were able to bind and retrieve MAP organisms. Recent studies found that magnetic nanoparticles coated with anti-MAP polyclonal or monoclonal antibodies were effective in the identification of MAP from clinical samples (Gilardoni et al., 2016). The advantages of immunomagnetic capture methods is that they concentrate the target bacterium from the non-specific bacterial pool and inhibitory substances, thereby facilitating rapid and enhance sensitivity of identification of MAP by the downstream diagnostic applications such as PCR, culture, and acid-fast staining of MAP (Foddai et al., 2010; Khare et al., 2004; Shin et al., 2009).

In summary, we have cloned, expressed and purified MAP species-specific cell envelope proteins, and have generated polyclonal antibodies to recombinant
SdhA, FadE25_2 and DesA2 proteins and shown their specificity to MAP by immunoblot analysis. We have also generated polyclonal antibodies to an extract of MAP cell envelope proteins that were able to identify MAP organisms in infected tissues using IHC and IF techniques. IHC techniques are more sensitive than acid-fast staining of bacilli and therefore anti-MAP cell envelope antibodies could be used in diagnostic laboratories. Studies involving more tissue samples are necessary to further assess the sensitivity and specificity of these antibodies in identifying MAP by IHC. Furthermore, the use of these polyclonal antibodies in immunomagnetic capture of MAP suspended in PBS provides proof in principle for this approach as a rapid, sensitive and specific tool in the diagnosis of MAP. Further studies with mixed bacterial communities e.g as in feces, and other mycobacterial cultures are needed to assess the diagnostic accuracy of the IMS in MAP identification. Considering the chronic nature of JD, MAP shedding patterns and variable host immunological responses, it is essential to develop new sensitive and specific diagnostic tests to detect subclinical MAP infection in order to prevent and control and spread of this disease.

2.6. References


combination of immunomagnetic bead separation-conventional PCR and real-time PCR. Journal of clinical microbiology42, 1075-1081.


Chapter 3

Development and validation of novel ELISAs with *Mycobacterium avium* subspecies *paratuberculosis* cell envelope protein and MAP specific recombinant protein antigens for the serodiagnosis of Johne’s disease

3. 1. Abstract

We have previously shown that the antigenicity of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) cell envelope proteome is different from closely related mycobacterial species such as *Mycobacterium avium* subspecies *hominisuis* (MAH) and the environmental *Mycobacterium smegmatis* (MS) suggesting that this finding may be exploited for the diagnosis of Johne’s Disease (JD) in dairy cattle. The objective of this study was to evaluate the potential of an ELISA to detect anti-MAP serum antibodies using MAP cell envelope protein antigens including six MAP-specific recombinant proteins (SdhA, FadE25_2, FadE3_2, Mkl, DesA2 and hypothetical protein (MAP1233)) as well as an extract of MAP total cell envelope proteins. At a selected cut-off value 0.611 (OD_{450}), the sensitivity and specificity of the ELISA coated with MAP total cell envelope protein extract without serum pre-absorption was 72 and 90%, respectively. The calculated area under the Receiver Operating Characteristic (ROC) curve was 0.808. Serum pre-absorption significantly increased the ELISA performance characteristics with a sensitivity of 75% and a specificity of 96% at a selected cut-off value of 0.384 (OD_{450}) and an area under the ROC curve of 0.960. By comparison on the same set of samples, results from a commercial IDEXX serum ELISA showed a sensitivity of 56% and specificity of 99%. Regarding ELISA with recombinant proteins, SdhA protein resulted in a higher
diagnostic sensitivity of 94% and lower diagnostic specificity of 79% at a selected cut-off criterion of >0.483 (OD$_{450}$). The hypothetical protein (MAP1233) yielded a lower sensitivity of 67% and higher specificity of 95% at the selected cut-off of >0.543 (OD$_{450}$). Specificities of the ELISA with recombinant proteins reported in this study were less than that of the commercial IDEXX Serum ELISA test. Future ELISA experiments with partial MAP proteins and peptides or epitopes unique to MAP might improve test specificity in the early diagnosis of JD.

3. 2. Introduction

Johne’s disease (JD) is a non-treatable chronic granulomatous enteritis of cattle and small ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Speer et al., 2006). JD is associated with profuse diarrhea, emaciation, submandibular oedema and eventually death of infected animals due to poor nutrient absorption. JD is endemic in North America, prevalent worldwide and poses considerable economic burden to the cattle industry due to production losses and herd replacement costs (Barkema et al., 2010; Garcia and Shalloo, 2015). There are four stages in JD. In silent stage, infected animals are healthy without shedding of MAP in the feces. In stage II, the disease is subclinical and infected animals appear healthy and can intermittently shed MAP in the feces thereby contaminating the environment and acting as a source of infection to herd-mates. Current diagnostic tests have very limited sensitivity in the diagnosis animals at stage I and II of infection and cattle may remain undiagnosed for several years. In stage III (clinical disease) and IV (advanced clinical disease), infected animals exhibit typical clinical signs of JD such as intermittent to continuous diarrhea, weight loss and emaciation and shed large numbers of MAP in the feces (Whitlock and Buergelt, 1996).
Currently, JD is diagnosed by several methods including: culturing of MAP in the feces and tissues, identification of MAP DNA by PCR from clinical samples, serum and milk and detection of humoral immune responses by ELISA (Britton et al., 2016). While fecal culture is considered to be the gold standard diagnostic test, there are several deficiencies as the test has limited sensitivity, is time consuming (5-16 weeks to visualize colonies), expensive and labor-intensive. Detection of MAP DNA in the feces is also used in JD diagnosis but the presence of PCR inhibitors and extraction of DNA from fecal samples is challenging. Lack of an accurate and reliable diagnostic test to identify animals in early stages of MAP infection is an impediment to the control of JD (Garcia and Shalloo, 2015).

Among the various JD diagnostic tests, ELISA is commonly used due to its simplicity and cost-effectiveness. Overall, sensitivity and specificity of the commercial ELISA kits varies from 45 to 57% and 85 to 99%, respectively, for fecal culture-positive cases (Collins et al., 2005; Speer et al., 2006). To minimize false positive cases, serum absorption with *M. phlei* is a common practice as it improves specificity but reduces sensitivity (Cho et al., 2006). ELISA sensitivity also varies due to fluctuations in the antibody titer depending on the stage of infection (Toft et al., 2005). Moreover, there is variability between kits with samples showing seropositivity in one ELISA kit and seronegativity in another (McKenna et al., 2006a). This variability is due to the different crude antigen preparations and specific components of commercial kits which is proprietary information. Indeed, none of the commercial ELISA kits can be used as a single test to diagnose early stage MAP infection in dairy cattle (Khol et al., 2012). Several attempts have been made to identify suitable antigen candidates for the serodiagnosis of subclinical JD infections.
(Mikkelsen et al., 2011b). However, selection and incorporation of MAP-specific and sensitive antigens in ELISA is a challenging task due to genetic similarity of MAP with other *M. avium* species and sharing of antigenic epitopes with other mycobacterial and non-mycobacterial species. Exposure of animals to these related bacterial species may generate antibodies that cross-react with MAP antigens affecting the specificity of MAP ELISAs (Facciuolo et al., 2013).

During MAP infection, the cell-mediated immune response is dominant in the early stages of infection with seroconversion occurring in the later stages of infection. Therefore, humoral immune responses may not be detectable during the early stages of infection (Speer et al., 2006; Stabel, 2000) using undefined antigens but is entirely possible using appropriate defined MAP antigens. Others have shown MAP-specific humoral immune responses in calves as early as 134 days after experimental infection with MAP (Waters et al., 2003). Identification of MAP-specific antigens that could be incorporated into ELISAs could be valuable in JD diagnosis. Indeed, flow cytometry analysis of antibody binding to mycobacteria showed that MAP cell surface antigens are particularly sensitive and subspecies-specific (Eda et al., 2006).

In Chapter 1 of this thesis, we have shown that the antigenicity of some MAP cell envelope proteins is different from its genetically close relative *M. avium* subsp. *hominisuis* (MAH) and the environmental mycobacterium *M. smegmatis* using a 2-DE immunoblot analysis approach. The aim of this study was to test the serodiagnostic potential of ELISA using an extract of total MAP cell envelope proteins as well as six MAP-specific recombinant proteins i.e. SdhA, FadE25_2, FadE3_2, Mkl, DesA2 and hypothetical protein MAP1233.
3.3. Materials and methods

**Extraction of MAP cell envelope proteins**

*Bacterial strains, media and growth conditions*

Three MAP strains (Madonna, gc86 and gd30 strains) isolated from clinical cases from southern Ontario (Canada) were graciously provided by Dr. L. Mutharia, University of Guelph. MAP strains were grown in Middlebrook 7H9 broth medium supplemented with glycerol (0.5% v/v), 10% (v/v) oleic acid-albumin-dextrose-catalase and mycobactin-J (2 μg/mL) (Allied Monitor Inc, Fayette, MO, USA) at 37°C for 6 to 8 weeks. Cultures were harvested by centrifugation at 3500 rpm for 30 min at 4°C and washed three times with ice-cold phosphate buffered saline (PBS) (pH 7.4). Bacterial pellets were then washed with 0.16 M sodium chloride solution.

*Subcellular fractionation and cell envelope protein extraction*

Subfractionation of MAP was done to obtain cell wall core and cytoplasmic membranes as described (He and De Buck, 2010; Zheng et al., 2012) with a few modifications. Briefly, 2 mL of lysis buffer (0.05 M potassium phosphate, 0.022% (v/v) β- mercaptoethanol, pH 6.5) was added to each gram of frozen bacterial cells (wet cell weight). Lysozyme (final concentration 4.8 mg/mL w/v) (Sigma-Aldrich, Oakville, CA) and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were also added to the cells and incubated for 4 h at 37 °C with continuous stirring. Bacterial lysates were transferred into 2 mL screw-capped centrifuge tubes containing sterile zirconium-silica beads (0.1mm) (Biospec Products, Inc) and disrupted by 12 cycles of high velocity bead beating with intermittent cooling on ice after each bead beating. Lysates were centrifuged at 1000 g for 15 min
at 4 °C to remove unbroken cells and debris. Supernatants were centrifuged at 20,000 g for 30 min at 4 °C to obtain the pelleted cell walls and the supernatants containing cytoplasmic membrane and cytosolic fractions. Supernatants were centrifuged at 150,000 g for 90 min at 4 °C to obtain cytoplasmic membranes and cytosolic fractions. Pellets containing cells wall and membrane fractions [i.e. cell envelope] were then washed twice with PBS (pH 7.4) containing protease inhibitor cocktail to remove cytoplasmic contamination. Cell envelope pellets were re-suspended in standard cell lysis buffer containing 7 M urea (Sigma-Aldrich, St.Louis, MO, USA), 2 M thiourea (GE Healthcare, Montreal, QC, Canada), 30 mM Tris/HCl, 4% CHAPS (pH 8.5) (Affymetrix Inc, Maumee, Ohio, USA), incubated on ice for 30 min with intermittent mixing and centrifuged at 10,000 g for 20 min at 4 °C to remove insoluble materials. Supernatants containing soluble proteins were quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Inc, USA), aliquoted and stored at -20 °C.

**Recombinant protein antigen purification**

Five recombinant proteins (SdhA, FadE25_2, FadE3_2, Mkl and DesA2) were purified under native conditions with immobilized metal affinity chromatography (IMAC) techniques using HiTrap™ TALON®crude resins (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) as per the manufacturer’s instructions and hypothetical protein was purified under denaturing conditions using HisPur™ Ni-NTA (Nickel-Nitrilotriacetate) resin (Thermo Scientific, Meridian Rd, Rockford, IL, USA) as per the manufacturer’s instructions. The level of protein purities was tested on SDS-PAGE and coomassie blue staining (Simpson, 2010). Proteins purified under native conditions were dialyzed in PBS (10 mM, pH 7.4) and 10-20% glycerol was added. Protein concentrations were quantified by BCA assay, and samples were
aliquoted and stored at -80 °C until further use. We were not able to refold hypothetical protein and the denatured form was subsequently used for ELISA experiments.

**Serum samples**

A total of 150 serum samples from dairy cows that had been tested for JD based on fecal culture and IDEXX serum ELISA (see Table 1) were graciously provided by Dr. David Kelton (University of Guelph).

**Table 1**: Summary JD diagnostic test results of cattle from which serum samples were obtained.

<table>
<thead>
<tr>
<th>Name of the diagnostic test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal culture</td>
<td>36</td>
<td>114</td>
<td>150</td>
</tr>
<tr>
<td>IDEXX serum</td>
<td>21</td>
<td>129</td>
<td></td>
</tr>
</tbody>
</table>

**Optimization of ELISA with MAP cell envelope proteins**

The checkerboard titration method was used to optimize the indirect ELISA components such as coating buffer, blocking buffer, antigen concentrations, primary antibody dilutions and conjugate dilutions as previously described (Crowther, 2000). In brief, antigens (200 to 6.25 ng/well) were diluted in PBS (10 mM, pH7.4) and bicarbonate coating buffer (0.1M, pH9.6). Diluted antigens (100µL/well) were added into the 96 well microplates (F-bottom, clear, high binding) (Greiner bio-one, Germany), covered and incubated overnight at 4°C on a shaker platform (45rpm). Plates were washed three times with PBS in an automatic plate washer. To select a suitable blocking buffer, plates were incubated with different blocking buffers such as superbloc (PBS) blocking buffer (Thermo Scientific, Rockford, IL, USA), 3% skim milk in PBS, 2% BSA in PBS (Roche Diagnostics, GmbH, Germany) and 2% BSA in
PBS (Santa Cruz Biotechnology, Dallas, USA) for 2 h at RT on a shaker (85 rpm) and plates were washed three times with PBS. To optimize the primary antibody dilutions, MAP test-positive (n=10) bovine serum (Fecal culture and IDEXX serum ELISA) samples were pooled and served as a positive control. Similarly, ten MAP test negative (n=10) bovine serum samples (Fecal culture and IDEXX serum ELISA) were pooled and served as negative controls. Positive and negative serum samples were serially diluted (1:500 to 1:8000) in the same blocking buffers with the addition of 0.5% Tween 20. Serially diluted serum samples (100 µL) were added in duplicate wells and plates were incubated at RT for 2 h on an orbital shaker (85 rpm). Plates were then washed 6 times with PBST on an automatic plate washer. Conjugated detection antibody (affinity-purified rabbit anti-bovine IgG) (Jackson Immunoresearch Laboratories Inc., West Gove, PA) was serially diluted (1:5000 to 1:3200000 or 3.2x10^-6) in the same buffers used for blocking with the addition of 0.5% Tween20. Diluted conjugate (100 µL) was added to wells and plates were incubated at RT for 2 h on a shaker (85 rpm). Plates were washed six times with PBST. During optimization, different controls (without antigens, without blocking and without serum) were used to measure signal to noise ratio due to non-specific binding of reagents (serum and non-serum factors).

**Serum absorption**

Three mycobacterial species i.e. *M. avium* subsp. *hominisuis* (MAH), *M. smegmatis* and *M. phlei* were used for serum absorption. In brief, frozen glycerol stock cultures were streaked on Middlebrook7H11 agar plates and incubated at 37 °C. From these plates, single colonies for each bacterial species were picked and sub-cultured in 15 mL of 7H9 media at 37 °C. From this, 4 mL of culture from each
bacterial species was aseptically aliquoted and subcultured into 7H9 culture media (250 mL x 2 flasks/bacteria) and cultures were harvested separately by centrifugation at 4000 rpm for 20 min at 4 °C and washed twice with PBST. One group was heat-killed at 100°C for 15 min, cooled at RT and washed three times with PBS. Another group of bacterial pellets received neutral buffered formalin (0.5% final concentrations) and incubated at RT for 2 h on a rotor followed by repeated washing (3 times) with PBS. Heat and formalin-killed bacterial pellets were suspended in PBS, pooled and stored at 4 °C until further process. Serum samples were diluted (1:100) in 2% BSA PBST diluents containing killed bacterial (10% v/v) culture. Serum samples were incubated at 4 °C overnight on a rotor. Absorbed serum samples were centrifuged at 13,000 g for 20 min at 4 °C. Supernatants were transferred into new microcentrifuge tubes and stored at -20 °C until further processing.

Validation of ELISA with MAP cell envelope proteins and recombinant proteins

After optimization of ELISA components, subsequent ELISAs were performed with single dilutions of antigens and antibodies. A total of 150 serum samples from cows with known status for MAP based on fecal culture results were used so that relative sensitivities and specificities could be calculated in order to validate the new assay. In brief, MAP cell envelope proteins were diluted in bicarbonate coating buffer to a final concentration of 250 ng/mL and 100 µL of diluted antigen was added to each well of 96 well microplates. Plates were covered with lids, incubated at 4 °C on a shaker (45 rpm) overnight and washed three times with PBS by an automatic plate washer. Wells were blocked with 2% BSA (IgG free) (Santa Cruz Biotechnology, Dallas, USA) in PBS, incubated for 2 h at RT on a shaker (85 rpm) and washed three times with PBS using an automatic plate washer.
Absorbed serum samples were diluted to 1:1000 in 2% BSA in PBST (0.5% Tween 20) and each sample was added into duplicate wells. For non-absorbed serum, samples were diluted 1:1000 in 2% BSA in PBST (0.5% Tween 20) and each sample was added into duplicate wells. The remaining procedures were common for both the absorbed and non-absorbed samples. Plates were incubated at RT for 2 h on a shaker (85 rpm) and washed 6 times with PBST. Wells were incubated with HRP-linked conjugate antibody (affinity-purified rabbit anti-bovine IgG) (Jackson Immunoresearch Laboratories Inc., West Gove, PA), diluted (1:7500) in 2% BSA in PBST for 2 h at RT on a shaker and washed six times with PBST. Each well received 100 µL of highly sensitive TMB substrate (Bio legend, USA), incubated for 20 min at RT and reactions were stopped with 100 µL of 2 N H₂SO₄. Readings were measured at OD₄₅₀ using a microplate reader. Experiments were repeated twice to test repeatability and reproducibility.

**ELISA with recombinant proteins**

All the components for recombinant protein-based ELISA were optimized using a checker-board titration method. After optimization of ELISA components, subsequent ELISAs were performed with single dilutions of antigens (250 ng/ mL) in bicarbonate buffer. Absorbed serum samples were diluted 1:500 in 2% BSA in PBST (0.5% Tween 20) and each sample was added into duplicate wells. The remaining steps were the same as stated above in the validation section for ELISA with MAP total cell envelope proteins.
**Statistical analysis**

Fecal culture results were chosen as the gold standard of JD diagnosis in order to compare serum samples and to calculate the sensitivity and specificity of the ELISAs. Diagnostic sensitivity and specificity of the MAP cell envelope protein ELISA and five recombinant proteins ELISA including confidence intervals of 95% (CI-95%) were calculated from MAP-positive and -negative serum samples. The ability of the tested antigens to discriminate between MAP fecal culture -positive and -negative cows was assessed by plotting the area under the receiver operating characteristic curve (AUC<sub>ROC</sub>) using Medcalc 10.3.0.0Software (Mariakerke, Belgium). Diagnostic sensitivities and specificities were estimated based on maximum Youden index J.

3.4. Results

**Diagnostic sensitivity and specificity of MAP cell envelope protein ELISA**

Based on the preliminary absorbance values, bicarbonate buffer was more efficient than PBS. Blocking with 2% BSA (IgG free) in PBS produced the least amount of background signal, and the same buffer was used in primary and secondary antibody dilutions. Based on data from checkerboard titrations, 25 ng of antigens/well was chosen. Similarly, based on the checkerboard titrations, a dilution of 1:1000 was chosen for serum dilution and 1:7500 was selected for the HRP-conjugated secondary antibody.

After determining the optimal conditions for the MAP total cell envelope protein ELISA, validation of the assay with serum samples from 36 cows that were fecal culture positive and serum samples from 114 cows that were fecal culture
negative for MAP were assessed. This assessment was undertaken without serum absorption with related mycobacterial species in order to minimize the number of ELISA steps, to reduce time, to reduce costs and labour that are associated with culturing, and maintenance and preparation of lysates of mycobacterial species required for the absorption. Fecal culture (FC) results were used as the gold standard to compare and calculate the sensitivity and specificity of the newly developed ELISA. Sensitivities and specificities were determined at different cut-off points. At a cut-off value of 0.611 (OD\textsubscript{450}), the diagnostic sensitivity and specificity of the MAP cell envelope protein ELISA without serum absorption was 72 (95% CI – 54.8 – 85.8) and 90% (95% CI-83.4 – 95.1) respectively. The calculated area under the ROC curve was 0.808 (95% CI-0.735 to 0.867, P <0.0001) (Figure 1A).

![Figure 1: Receiver operator curves for ELISA. A: Receiver operator curve (ROC) for MAP envelope protein ELISA using unabsorbed (A) and absorbed (B) serum samples. Note: Small circles indicates Youden index J cut-off criterion for each ELISA.](image)

Figure 1: Receiver operator curves for ELISA. A: Receiver operator curve (ROC) for MAP envelope protein ELISA using unabsorbed (A) and absorbed (B) serum samples. Note: Small circles indicates Youden index J cut-off criterion for each ELISA.
In order to evaluate the level of cross-reactivity of MAP ELISA test-positive (n=6) and -negative (n=14) serum samples with other mycobacterial species, ELISA was performed with plates coated with *M. smegmatis* and MAH cell envelope proteins. The results revealed that MAP test-positive serum samples showed significant OD$_{450}$ values of > 0.2 to <0.38 for MAH and > 0.4 to < 1.0 for MS cell envelope proteins. Similarly, MAP test-negative serum samples showed significant OD$_{450}$ values of > 0.1 to <0.548 for MAH and > 0.2 to < 0.7 for *M. smegmatis* cell envelope proteins. In view of these results, we included a serum absorption step with MAH and *M. smegmatis* in addition to the routine serum absorption that was performed with *M. phlei* in order to improve the specificity. Using a cut-off value of 0.384 (OD$_{450}$), the diagnostic sensitivity and specificity of the MAP envelope protein ELISA after serum absorption was 75 and 95% respectively (Table 2). The calculated area under the ROC curve was 0.896 (Table 2) (Figure 1B). Results revealed that serum absorption has significantly improved the sensitivity and specificity of the ELISA.

**Table 2**: Calculated sensitivities, specificities and ROC$_{(AUC)}$ for the MAP total cell envelope protein and IDEXX serum ELISAs.

<table>
<thead>
<tr>
<th>ELISA type</th>
<th>Serum absorption</th>
<th>Se%</th>
<th>95% CI</th>
<th>Sp%</th>
<th>95% CI</th>
<th>ROC$_{(AUC)}$</th>
<th>95% CI</th>
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<td>MAP ENV ELISA</td>
<td>No</td>
<td>72.22</td>
<td>54.8 – 85.8</td>
<td>90.35</td>
<td>83.4 – 95.1</td>
<td>0.808</td>
<td>0.735</td>
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<td>MAP ENV ELISA</td>
<td>Yes</td>
<td>75.00</td>
<td>57.8 – 87.9</td>
<td>95.61</td>
<td>90.1 – 98.6</td>
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</table>
In order to compare the efficiency of the MAP cell envelope ELISA to the IDEXX commercial ELISA, results were compared with fecal culture as a gold standard. In relation to fecal culture, IDEXX serum ELISA results as per the cut-off value given by the manufacturer recommendations are also shown in Table 2. Of the 150 cattle serum samples, 36 cows were fecal culture positive, of which 20 cows were JD test-positive by both fecal culture and IDEXX serum ELISA (Table 1). The calculated area under the ROC curve for the IDEXX serum ELISA was 0.833 (Table 2). Comparison of ROC(AUC) for the three ELISAs revealed that the MAP total cell envelope protein ELISA using absorbed serum samples had the highest ROC(AUC) value (Figure 2).
**Figure 2:** Receiver operator curves for various ELISAs. ROC curves comparing the performance of MAP envelope protein ELISA with and without serum absorption and IDEXX serum ELISA. Note: Small circles indicates Youden index J cut-off criterion for each ELISAs.

**Optimization of ELISA with recombinant proteins**

For ELISA with recombinant proteins, based on the preliminary absorbance values, bicarbonate buffer was more efficient than PBS and 2% BSA (IgG-free) was the most efficient blocking agent. Based on data from checkerboard titrations, 25 ng
of antigens/well was chosen. Similarly, based on the checkerboard titrations, a dilution of 1:500 was chosen for serum dilution and 1:7500 was selected for the HRP-conjugated secondary antibody.

**Seroreactivity of recombinant protein antigens**

For all six recombinant protein antigens OD$_{450}$ values for MAP fecal culture test-negative animals were less than for MAP fecal culture test-positive animals (Table 3). ROC$_{AUC}$ analyses were performed to measure the discriminatory power of the MAP recombinant protein ELISA assay to differentiate positive and negative animals. ROC$_{(AUC)}$ was above 0.7 for all recombinant protein antigens used in this study (Figure 3: A to F). SdhA had the highest ROC$_{(AUC)}$ of 0.921 (95% CI –0.851 to 0.965) and FadE3_2 had the lowest ROC$_{(AUC)}$ of 0.787 (95% CI-0.713 to 0.849) (Table 4). Youden index J analysis was performed to measure the trade-off between sensitivity and specificity at different cut-off values in order to assess the performance of a diagnostic test. ELISA results for SdhA recombinant protein revealed a high Youden index J of 0.735 and FadE3_2 showed the lowest value of 0.522. Sensitivities and specificities at the selected cut-off points for individual recombinant proteins are given the Table 4. SdhA protein resulted in the highest diagnostic sensitivity of 94% (95% CI-80.3 - 99.3) and the lowest diagnostic specificity of 79 (95% CI-67.9 - 88.3) at a selected cut-off criterion of >0.483. Hypothetical protein MAP1233 produced the lowest sensitivity of 67% (95% CI-49.0 - 81.4) and highest specificity of 95% (95% CI-87.6 - 98.2) at a selected cut-off criterion of >0.543. Overall, ELISA results for the six recombinant proteins showed higher sensitivity and lower specificity than the IDEXX serum ELISA results.
Table 3: Mean OD$_{450}$ value difference between MAP fecal culture positive and negative serum samples with recombinant protein antigens.

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Mean OD$_{450}$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC –ve</td>
</tr>
<tr>
<td>SdhA</td>
<td>0.400</td>
</tr>
<tr>
<td>FadE25_2</td>
<td>0.383</td>
</tr>
<tr>
<td>FadE3_2</td>
<td>0.330</td>
</tr>
<tr>
<td>Mkl</td>
<td>0.447</td>
</tr>
<tr>
<td>Hypothetical protein MAP1233</td>
<td>0.350</td>
</tr>
<tr>
<td>DesA2</td>
<td>0.368</td>
</tr>
</tbody>
</table>
Figure 3: Receiver Operating Characteristic curves for ELISAs with recombinant protein antigens: A: SdhA, B: FadE25_2, C: FadE3_2, D: Mkl, E: Hypothetical protein MAP1233, F: DesA2. Note: Small circles indicate Youden index J cut-off criterion for each recombinant protein antigen.

Table 4: Calculated sensitivities and specificities at the selected cut-off points, ROC\textsubscript{AUC} and Youden index J for the six recombinant protein antigens.

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Se%</th>
<th>95% CI</th>
<th>Sp%</th>
<th>95% CI</th>
<th>ROC\textsubscript{(AUC)}</th>
<th>95% CI</th>
<th>Youden index J</th>
<th>Cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>SdhA</td>
<td>94.12</td>
<td>80.3 - 99.3</td>
<td>79.41</td>
<td>67.9 - 88.3</td>
<td>0.921</td>
<td>0.851 - 0.965</td>
<td>0.735</td>
<td>&gt;0.483</td>
</tr>
<tr>
<td>FadE25_2</td>
<td>70.59</td>
<td>52.5 - 84.9</td>
<td>89.66</td>
<td>82.6 - 94.5</td>
<td>0.845</td>
<td>0.777 - 0.899</td>
<td>0.602</td>
<td>&gt;0.533</td>
</tr>
<tr>
<td>FadE3_2</td>
<td>69.44</td>
<td>51.9 - 83.7</td>
<td>82.76</td>
<td>74.6 - 89.1</td>
<td>0.787</td>
<td>0.713 - 0.849</td>
<td>0.522</td>
<td>&gt;0.456</td>
</tr>
<tr>
<td>Mkl</td>
<td>83.33</td>
<td>67.2 - 93.6</td>
<td>89.25</td>
<td>81.1 - 94.7</td>
<td>0.895</td>
<td>0.829 - 0.942</td>
<td>0.725</td>
<td>&gt;0.579</td>
</tr>
<tr>
<td>hypothetical protein MAP1233</td>
<td>66.67</td>
<td>49.0 - 81.4</td>
<td>94.51</td>
<td>87.6 - 98.2</td>
<td>0.859</td>
<td>0.786 - 0.915</td>
<td>0.611</td>
<td>&gt;0.543</td>
</tr>
<tr>
<td>DesA2</td>
<td>69.44</td>
<td>51.9 - 83.7</td>
<td>92.17</td>
<td>85.7 - 96.4</td>
<td>0.861</td>
<td>0.796 - 0.912</td>
<td>0.616</td>
<td>&gt;0.546</td>
</tr>
</tbody>
</table>
3.5. Discussion

In this study we have investigated the use of total MAP cell envelope antigens and six recombinantly expressed MAP cell envelope protein antigens for the development of ELISA tests for the identification of serum antibodies to MAP bacteria during infection. Our finding that MAP cell envelope proteins are antigenically distinct from proteins of the closely related MAH and the environmental *M. smegmatis* suggested that MAP cell envelope proteins would be useful in detecting MAP-specific serum antibodies in MAP-infected cattle. Commercial ELISAs are most commonly used in the serodiagnosis of JD, however, test specificity is limited by the use of crude antigen preparations such PPD, protoplasmic antigens (PPA) and LAM that contain epitopes shared / conserved in other mycobacterial and non-mycobacterial species. This leads to false positive diagnoses of JD due cross-reacting antibodies that are not related to MAP exposure (Eda et al., 2006). For instance estimated specificities and sensitivities of five commercial ELISA tests for the diagnosis JD varied from 87.4 to 99.8% and 27.8 to 44.5% respectively in comparison to fecal culture (Collins et al., 2005). Since current ELISAs are unable to accurately identify animals with subclinical infection, incorporation of MAP-specific proteins in ELISA-based JD diagnostic tests may improve sensitivity and specificity (Eda et al., 2006).

We hypothesized that inclusion of extracts of MAP total cell envelope proteins and recombinantly expressed individual MAP species-specific antigenic proteins in an ELISA format would improve the diagnostic sensitivity and specificity. Cell envelope proteins play critical roles in bacterial pathophysiology when infecting target tissues and are often accessible to the host immune system and are therefore often
immunogenic (Leite et al., 2015). Coating of ELISA plates with formalin-treated whole MAP cells or cell surface proteins extracted from formalin-treated, sonicated MAP cells revealed a diagnostic sensitivity and specificity of more than 95% in the serodiagnosis of JD (Speer et al., 2006). Another study used flow cytometry to detect MAP subspecies-specific IgG antibodies against MAP cell surface from cattle subclinically infected with MAP. Results revealed the presence MAP subspecies-specific components on the cell surface that could be used in the serodiagnosis of JD (Eda et al., 2005). Our approach is unique in that we used clinical strains of MAP isolated from the same geographical region. Use of local MAP strains in diagnostic kit development is often advantageous and could improve JD diagnosis. For example, protein extracted from MAP cell surface antigens from the USA strains had a sensitivity of 97.1% when tested in the USA and 21.8% when tested in India (Scott et al., 2010; Singh, 2015).

In the present study, MAP cell envelope proteins were extracted under denaturing conditions using urea, thiourea and CHPAS detergent. Denaturation helps to expose hydrophobic regions of proteins and favours protein binding with solid phase ELISA plates (Crowther, 2000). In addition, denaturation minimizes proteolysis, reduces secondary structures and exposes more linear epitopes for antigen and antibody interactions. However, it is possible that conformational epitopes could have been damaged in this assay.

ELISA with unabsorbed serum samples resulted in a sensitivity and specificity of 72% and 89% respectively. ELISA with MAP cell envelope proteins without serum absorption showed less specificity and this may be due to the presence of cross-reacting antibodies, which were generated due to environmental exposure of cattle to
other mycobacterial species. ELISA with unabsorbed serum samples is sensitive but less specific and affects test accuracy (McKenna et al., 2005a). Exposure to shared epitopes in environmental mycobacteria leads to a high proportion of false positive reactions as has been reported in some herds (Osterstock et al., 2007). For serum absorption, we included MAH and *M. smegmatis* in addition to the traditional absorption with *M. pheli*. Absorbent bacterial species used in this study were autoclaved to prevent contamination and introduction of proteases that may degrade and affect the quality of antibodies during absorption. Autoclaving may also expose a greater variety of cross-reacting epitopes than with traditional lysis of bacteria for serum absorption (Gruber and Zingales, 1995). In addition, absorbent cells were killed with formalin to preserve surface antigens. It has been shown that formalin-treated whole MAP bacilli retain antigenic integrity, specific antibody reactivity and react more strongly (> 3.8 times) with MAP test-positive serum than with MAP test-negative serum (Speer et al., 2006).

In our study, serum absorption significantly improved sensitivity and specificity. This is in contrast to an earlier study in which two commercial ELISA with absorbed serum samples revealed low sensitivities of 13.9% and 16.6% and specificities of 95.9% and 97.1% in comparison to fecal culture. In comparison, an ELISA with unabsorbed serum showed a sensitivity of 27.8% and specificity of 90% when compared to fecal culture (McKenna et al., 2005a). The possible reasons for the improved sensitivity in our study may be due to the use of MAP species-specific cell envelope proteins, indigenous MAP strains, large numbers of MAP-specific epitopes and serum absorption with MAH and *M. smegmatis*. However, in the present study, five animals that were negative by fecal culture and IDEXX serum
ELISA showed high OD$_{450}$ values with MAP cell envelope protein ELISA. The presence of MAP-specific antibodies suggests that these animals may be in the early stages of MAP infection and were not detected by fecal culture and commercial ELISA. A follow-up study might be useful to determine whether these animals have subsequently tested positive by IDEXX serum ELISA so that the accuracy of the new MAP ELISA assays can be assessed. Similarly, nine animals were positive by fecal culture but negative by MAP envelope protein ELISA. It is possible that these animals are infected with MAP but their MAP-specific antibody levels are below the detection limit. Another possible reason may be due to passive shedding of MAP rather than infection (Sweeney et al., 1992). Nonetheless, a definitive explanation awaits re-testing of these animals to gain further epidemiological information regarding their JD status.

A number of studies have assessed the efficacy of different MAP components in ELISA such as MAP culture filtrate antigens (Shin et al., 2008), whole MAP bacilli (Speer et al., 2006), MAP surface proteins (Scott et al., 2010), PPA (Adji et al., 2015), MAP-specific recombinant proteins (Kawaji et al., 2012) and showed that in general in-house ELISAs have better diagnostic sensitivity and specificity than that of commercial ELISA kits. The diagnostic specificity and sensitivity of our assay is less than that of other studies that use MAP cell surface antigens. For example, flow cytometry analysis and ethanol vortex ELISA with MAP surface antigens revealed a serodiagnostic specificity of 96.7% and 100% and sensitivity of 95.2% and 97.4% respectively (Eda et al., 2006; Eda et al., 2005). However, serum samples used in these two studies were from herds with a known JD status (JD-free or -positive) over a period of time and animals with a known shedding pattern (mild, moderate and
heavy fecal shedding from high prevalence herds) and this could lead to bias in the calculation of the sensitivity and specificity (Nielsen and Toft, 2008). For instance, ethanol vortex ELISA was repeated with other serum samples (n=38) and results revealed that 70% of the animals were false positive for JD (Scott et al., 2010). Indeed, comparison of test accuracies between various JD diagnostic tests was not possible (Nielsen and Toft, 2008).

We also assessed an ELISA with six MAP cell envelope recombinant protein antigens (SdhA, FadE25_2, FadE3_2, Mkl, DesA2 and hypothetical protein MAP1233) that were identified as MAP species-specific based on our earlier 2D-DIGE comparative proteomic analysis and 2-DE immunoblot analysis. The rationale for this approach is that ELISA with one or more MAP-specific immunogenic antigens could improve the sensitivity and specificity of JD serodiagnosis. In the development of our recombinant protein ELISAs, all the components were optimized as reported in earlier studies. Recombinant protein antigens used in our study were purified from E. coli and therefore serum samples were initially pre-absorbed with E. coli cell lysates (heat-killed and formalin-inactivated). However, we did not observe a significant difference with or without pre-absorption. This was contrast to earlier studies wherein serum absorption with E. coli reduced background noise in ELISA (Gumber et al., 2009; Gurung et al., 2012b). It is possible that MAP cell envelope recombinant protein antigens selected in the present study were more specific to MAP and thus had reduced cross reactivity against E. coli cell proteins.

Our ELISAs with recombinant protein antigens were able to differentiate MAP fecal culture-positive and -negative serum samples. This finding was in agreement with earlier studies that used similar approaches to evaluate the
serodiagnostic potential of recombinant MAP protein antigens (Bannantine et al., 2008c; Leroy et al., 2009). SdhA and MAP1233 hypothetical recombinant protein ELISAs showed the highest and lowest sensitivity of 94% and 67%, respectively. The low sensitivity of the recombinant protein ELISA is not surprising in view of the complex nature of MAP infection. It has been shown that one antigen may not be sufficiently sensitive and specific during the entire course of infection and therefore future experiments with cocktails of MAP-specific recombinant protein antigens might improve the test sensitivity and enable detection of animals at different stages of JD.

Among the six recombinant proteins, hypothetical protein and DesA2 showed a high specificity of 95% and 92% respectively. DesA2 recombinant protein ELISA had a ROC\(_{(AUC)}\) value of 0.84. Earlier studies with DesA2 recombinant protein ELISA showed ROC\(_{(AUC)}\) values of 0.69 and 0.70 (Gurung et al., 2013; Gurung et al., 2012b), however, these studies used refolded recombinant proteins that could have altered the protein properties such as structure, orientation and antigenicity resulting in low ROC\(_{(AUC)}\) values. Four recombinant proteins including SdhA, FadE25_2, FadE3_2 and Mkl showed less specificity. In general, specificities of ELISAs with recombinant proteins reported in this study were less than that of the commercial ELISA tests. Indeed, false positivity with recombinant protein-based ELISAs has been reported earlier (Gurung et al., 2012b). Considerable numbers of animals in the false positive and false negative category is typically expected in JD diagnosis (Köhler et al., 2007). In addition to the MAP species-specific epitopes, it is possible that the antigens used in this study may contain other epitopes that may be present in other mycobacterial or non-mycobacterial species and environmental exposure of cattle to these
microorganisms led to false positives. Future experiments with partial proteins or peptides could improve test specificity.

There were certain limitations to our experimental approach. In this study, we used serum samples collected from cattle from MAP-positive herds some of which were likely exposed to different levels of MAP bacteria. A lack of samples from JD free herds could result in a degree of bias in the calculation of sensitivity and specificity. Therefore, testing of serum samples from additional true negative and true positive samples might yield a more definitive assessment of sensitivity and specificity. In addition, serum samples from *M. bovis*-positive cattle could be used to further assess the level of cross-reactivity. While serum absorption is common practice for the removal of cross-reactive antibodies, the efficiency of this procedure is unclear. Finally, an increase in sample size would increase the statistical power of the study.

In conclusion, this study assessed the utility of various MAP cell envelope antigens in the development of ELISA tests to detect serum antibodies to MAP. We showed that the use of extracts of MAP total cell envelope proteins with serum that was absorbed with MAH, *M. smegmatis* and *M. phlei* produced ELISA results with higher sensitivity and specificity than the commercial IDEXX serum ELISA. ELISAs that used six recombinant MAP cell envelope protein antigens were able to detect MAP species-specific serum antibodies and differentiate MAP fecal culture -positive and -negative serum samples with reasonably high sensitivities. However, specificities were comparatively less than the commercial IDEXX serum ELISA and therefore future ELISA experiments with partial proteins and peptides or epitopes unique to MAP could improve test specificity. The potential use of these recombinant
proteins ELISAs for early diagnosis and control of JD requires further investigation and validation.

3.6. References


SINGH, S.V., 2015, Comparison of ‘Indigenous ELISA kit’ with ‘Ethanol Vortex ELISA kit (USA)’ for detection of Mycobacterium avium paratuberculosis infection.


Johne's disease (JD) is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). JD is global in distribution and endemic in many countries (Geraghty et al., 2014; Stevenson, 2015a). Young animals of less than 6 to 12 months of age are more susceptible to MAP infection than adult animals (Lombard, 2011; Marcé et al., 2011). While the fecal-oral route is the major route in disease transmission, young calves are also infected through consumption of milk and colostrum containing viable MAP from infected cattle (Donat et al., 2016; Whittington and Sergeant, 2001). MAP infection incurs significant economic losses to the cattle industry (Garcia and Shalloo, 2015). In addition to the economic burden, MAP has been associated with Crohn’s disease (inflammatory bowel disease), type 1 diabetes, multiple sclerosis and autism in humans and these are growing concerns to public health (Sechi and Dow, 2015a). The absence of effective treatments and vaccines for MAP infection has become a major challenge in the effective management of JD. Considering these facts, early detection and isolation of the infected animal from the herd is an effective way to control the JD.

Currently, JD is diagnosed by isolation of MAP from the tissues and feces, molecular detection MAP DNA in clinical samples like feces or tissues and measuring of CMI and humoral immune responses (Salem et al., 2013). Each method has its advantages and disadvantages. For instance, fecal culture is considered to be the gold standard for the diagnosis of MAP, but the sensitivity of fecal culture for subclinically infected animals varies between 23 and 49% and for clinical cases 70%
(Bosward et al., 2010). Other disadvantages are that it is time-consuming (6 to 15 weeks for fecal culture), costly, laborious and requires a battery of tests to confirm the infection. In PCR, sensitivity of 4.1% for low to moderate fecal shedders and sensitivity of 76% for heavy shedders has been reported (Hasonova and Pavlik, 2006). For ELISA, overall sensitivity and specificity varies from 7 to 94% and 41 to 100% respectively (Khol et al., 2012). In general, current diagnostic tests are low in sensitivity and specificity for animals in the subclinical stages of infection and a major barrier in the control and eradication of JD. It is believed that every single animal with the clinical form of JD will infect 15 to 20 animals, only half of which are detectable by current diagnostic tests (Whitlock et al., 2000).

An ideal diagnostic test with high sensitivity and specificity to identify subclinical cases of JD is preferable (Mikkelsen et al., 2011b). Identification and characterization of MAP species-specific antigens and testing of their utility in the diagnosis and vaccine formulas is a major focus in MAP research (Olsen et al., 2002). However, it is well known that the development and validation of a novel diagnostic test for MAP is a challenging task. This is due to the complex nature of MAP infection which has characteristics such as prolonged subclinical infection without any sign of illness, unpredicted nature and timing of disease progression, close genetic similarity of MAP with other MAC members and sharing of antigenic epitopes with other pathogenic and non-pathogenic mycobacterial and with non-mycobacterial species (Bannantine et al., 2003a; Britton et al., 2016; Gilardoni et al., 2012; Mikkelsen et al., 2011b; Osterstock et al., 2007). Selection of MAP-specific and -sensitive antigens and their incorporation into diagnostic assays for the accurate detection in clinical samples is one of the major challenges in the management of this disease (Faccioulo et al., 2013).
The aim of this thesis work was to use proteomic approaches to identify MAP species-specific protein antigens from cell envelope components and to test these antigens for their use in the diagnosis of MAP infection as a proof-of-principle assessment. The first objective was to identify the MAP species-specific proteins from the cell envelope components by comparing migration and pI profiles of MAP membrane proteins extracted from two other mycobacterial species i.e. MAH and *M. smegmatis* which are ubiquitous in the environment by 2D-DIGE comparative proteomic analysis. Subsequent assessment of the antigenicity of MAP species-specific proteins was achieved by 2-DE western blot analysis with serum samples from naturally MAP-infected cattle (or JD-test positive cattle). MAP shares genetic similarity with other members of the *Mycobacterium avium* complex (MAC) at their nucleotide level. However, MAP differs from other MAC members in many ways e.g. i) MAP is very slow growing and fastidious, ii) MAP is an obligatory intracellular pathogen affecting intestinal tissues and associated lymph nodes of ruminants and iii) MAP infections have a prolonged subclinical infection (Bannantine et al., 2003a). Therefore, we hypothesized that the MAP cell envelope protein profiles or their expression levels differ from other MAC members and other mycobacterial species thereby providing uniqueness to MAP. The cell envelope is the first cellular component to interact with environment, host and their immune system upon infection and has a major role in bacterial survival in adverse environmental conditions and within the host (Wolfe et al., 2010). Thus, knowledge about the MAP envelope protein profile is useful in understanding the pathophysiology of MAP infection and to identify MAP species-specific antigens for the development of novel diagnostics, therapeutics and vaccines.
Comparison of MAP, MAH and *M. smegmatis* revealed that proteomic profiles of cell envelopes of these three bacterial species are significantly different. These differences are likely due to the post-translational modifications which may result in a slight shift in the coordinates of the spots on the 2-DE gel. Alternatively, the differentially expressed protein spots might be MAP species-specific proteo forms of conserved proteins. Indeed, our 2-DE western blot assay showed that many of these proteins are antigenically unique and immunogenic. This could be an important factor for the existence of phenotypic and pathogenic differences between mycobacterial species. However, several strings of protein spot distribution patterns were parallel in the three mycobacterial species suggesting that some proteins are common to these species but differ in isoforms, post-translational modifications (PTM) and levels of expression. These results indicate that the proteome profile of a given organism is very complex and dynamic in nature and a unidirectional relationship with genomics is not always present (Hochstrasser and Sanchez, 2000). Undoubtedly, core genomes of closely related organisms may be the same but their expression levels in one organism may differ from other organisms due to evolutionary changes and environmental pressures (Callister et al., 2008).

Thirteen immunogenic protein spots from a 2-DE gel were selected for their identification by LC-MS/MS and a total of 15 proteins were identified. LC-MS/MS results also suggested the presence of PTM or different isoforms for MAP cell envelope proteins represented by multiple spots by 2D-DIGE. For example the iron-sulfur subunit of succinate dehydrogenase, acyl-CoA dehydrogenase and fibronectin-binding antigen 85 complex B were identified in separate strings of adjacent spots. Proteome analysis showed that some of the envelope proteins are conserved among the pathogenic and non-pathogenic mycobacterial species. For instance, antigen 85B
is highly conserved in the mycobacterial species. However, based on 2D-DIGE analysis, we speculate that there could be peptide regions or parts of protein regions of these conserved proteins might be specific to MAP. These peptide regions could elicit pathogen-specific immune responses and might represent regions that could be used in the development of MAP-sensitive and -specific diagnostics. Proteins identified in this study are involved with intermediary and lipid metabolism, cell wall biogenesis and bacterial virulence. Our findings are in agreement with other studies on *M. tuberculosis* and MAP cell envelope proteomic analysis that reported that these proteins are involved in intermediary and lipid metabolism, cellular respiration, cell wall biogenesis and virulence as major proteins components of the mycobacterial cell envelope (Gu et al., 2003; He and De Buck, 2010; Målen et al., 2010). In conclusion, our comparative proteomic analysis of three bacterial species results showed that protein profiles of MAP are different from MAH and *M. smegmatis*. MAP-specific proteins are also immunogenic. Our results suggest the existence of MAP protein isoforms and PTMs, however, further studies are required to prove this.

Future studies focusing on entire proteome profiling of three bacterial species using modern mass spectrometry-based proteomics technique like the front-end basic pH reversed phase fractionation combined with LC/MS/MS analysis could be used to generate more comprehensive proteome profiles and their expression levels. Comprehensive comparative proteomics studies with the use of isobaric tag labelling (e.g., iTRAQ or TMT) could be used to discover differentially expressed antigenic proteins. Proteins with high pI values i.e. above pH 7.0 were not studied, therefore, future experiments using different pH ranges and longer strip length could be useful to identify more MAP species-specific proteins.
Surface labelling of MAP, MAH, *M. smegmatis* and other mycobacterial species proteins with membrane-impermeable and hydrophilic reagents such as biotinylating agents and subsequent identification with spectrometry analysis of labeled proteins and their comparative analysis could represent an alternate approach to explore more MAP species-specific, surface-exposed proteins and epitopes. Studies focussing on protein isoforms and post-translational modifications of MAP cell envelope proteins are other interesting areas through which to identify MAP species-specific proteins. In addition to cell envelope proteins, research focused on other molecules such as lipoglycans and lipoproteins antigens is also a viable option to identify MAP species-specific antigens, since lipid components constitute a major component in the mycobacterial cell wall.

The MAP-specific protein antigens identified in this study have the potential to be incorporated into JD diagnostics to detect MAP antigens and MAP-specific antibodies from serum and milk samples. In order to pursue this objective we need to have MAP antigens in sufficient quality and quantity to proceed. Therefore, our second study focussed extraction of total MAP cell envelop proteins and on recombinant expression of six MAP species-specific proteins and generation of polyclonal antibodies against these proteins. Typically, expression of mycobacterial genes in heterologous host bacteria like *E. coli* is a challenging task due to a high percentage of G+C content in their genome leading to codon biasness (Piubelli et al., 2013). Codon optimization improved the expression of recombinant proteins, which is in agreement with other studies (Kanekiyo et al., 2005; Kashino and Campos-Neto, 2011). Upon purification, FadE3_2 soluble recombinant protein showed co-purification of host bacterial cell protein. The presence of impurities may affect the
sensitivity and specificity of the polyclonal antibodies and therefore FadE3_2 was not used for antibody generation.

Hypothetical protein MAP1233 was expressed as inclusion bodies and purified under denaturing conditions. Inclusion bodies (IBs) may lose tertiary structure, conformation and immunogenicity in comparison to the soluble forms of native antigens and thus may not be suitable for immunization and antibody generation (Piubelli et al., 2013). Our attempts to refold the IBs into soluble forms with different refolding buffers and refolding techniques were unsuccessful. Moreover, hypothetical protein MAP1233 required a minimal concentration of 3M urea or 250 mM of arginine hydrochloride in order to be solubilised. The presence of denaturants and additives is not suitable for immunization and therefore hypothetical protein (MAP1233) was not pursued for the antibody generation. The mkl gene was recombinantly expressed at very low levels and was toxic to E. coli cells upon induction with IPTG resulting in insufficient quantities for immunization. It has been reported that synonymous codon substitution could leads to frame shift errors, truncated proteins, mistranslations, improper protein folding, protein stability and toxicity to bacterial expression hosts (Angov, 2011; Komar, 2016). Finally, for comparison purposes, MAP total cell envelope proteins were also extracted and used to generate rat anti-MAP polyclonal antibodies.

Polyclonal antibodies generated in this study had very minimal cross reactivity with MAH and M. smegmatis cell envelope proteins and were specific to MAP cell envelope proteins. These results revealed that the MAP proteins identified in chapter 1 were antigenically distinct. Antibodies are powerful tools in life science and clinical research to identify and purify antigens, and to diagnose and treat infections (Lipman et al., 2005). In Chapter 2, immunohistochemical (IHC) and immunoflorescence (IF)
analysis of MAP-infected tissues showed that rat polyclonal antibodies generated against total cell envelope protein extracts were able to detect MAP microorganisms in the infected tissues. At present, there are no MAP species-specific antibodies available commercially and most of the reported studies used anti-\emph{M. bovis} antibodies for IHC assays (Brees et al., 2000; Martinson et al., 2008). IHC analysis is more sensitive and specific than traditional acid fast staining and histopathological examination (Pedersen et al., 2011). Our results showed that anti-MAP polyclonal antibodies can be used for the routine diagnosis of MAP antigens in tissue samples. However, rat polyclonal antibodies against SdhA, FadE25_2 and DesA2 were not able to detect MAP antigens in the tissues samples by IHC and IF analysis. It is possible that prolonged fixation of tissues in formalin fixatives resulted in irreversible damage to these antigens. Variation in the intensity of tissue immunoreactivity depending upon the fixatives rather than subcellular location of target antigens has been reported by earlier studies (Paavilainen et al., 2010). Therefore, further studies with frozen tissues sections, optimization of other conditions like tissue fixation with different fixatives to preserve antigens, testing with different antigen retrieval methods and optimization of other reagents used in IHC and IF assays are necessary. Also, antibodies generated from recombinant proteins that may not retain the necessary tertiary structure required for antibody recognition of epitopes found on native proteins.

Our use of polyclonal antibodies for immunomagnetic separation of MAP showed the potential to isolate MAP from feces, milk and tissue for MAP diagnosis. To isolate MAP from clinical samples, decontamination is required in order to kill contaminating organisms and thus could affect the viability of MAP bacteria and lowers the sensitivity of the culture-based assays. In addition, during the subclinical
phase the number of organisms shed in the feces and in the milk (2 to 8 CFU) is low (Grant et al., 1998). Currently, challenges to PCR-based diagnostics targeting MAP-specific DNA sequences includes meticulous extraction of good quality MAP DNA and reduction in test sensitivity by PCR inhibitors in the samples. Therefore, concentration of MAP from clinical samples prior to culture and PCR assays could improve the sensitivity of these assays (Foddaï et al., 2010; Ricchi et al., 2016). Our results illustrate that four polyclonal antibodies against the following antigens anti-MAP total cell envelope, SdhA, FadE25_2, and DesA2 were able to retrieve MAP cells spiked in PBS. Moreover, antibodies to MAP total cell envelope proteins were able to retrieve as low as $10^2$ CFU of MAP. Earlier studies identified $10^3$ to $10^5$ CFU of MAP in IMS-PCR based assays (Chui et al., 2010; Grant et al., 2000). While previous studies used antibodies generated against inactivated whole and heat-killed MAP cells (Grant et al., 1998; Kim et al., 2016), the use of polyclonal antibodies to MAP cell envelope proteins of our study proved to be more specific and sensitive in the capture of MAP bacteria. However, recombinant protein antibodies showed variable sensitivity in the recovery of MAP. This may be because antibodies to recombinant proteins target single antigens with possibly reduced level of expression and abundance leading reduced recovery of MAP. Other possible reasons are lack of PTMs and tertiary conformation in the recombinant proteins thereby affecting the affinity and avidity of antibody recognition of native antigens on the MAP cell surface.

Our preliminary study on IHC and IF analysis showed promising results. Testing of more tissue samples and comparison of IHC and IF with other JD diagnostics such as acid fast staining, histopathology, tissue and fecal culture might be useful to assess the sensitivity and specificity of anti-MAP antibodies in JD
diagnosis. In addition, testing tissue samples infected with other mycobacterial species like *M. bovis* and MAH would help in estimating the level of cross-reactivity. Generation of monoclonal antibodies using MAP cell envelope protein components could help to improve the sensitivity and specificity and to develop novel antibody-based proteomic assays for JD diagnosis. Immunomagnetic capturing experiments with different concentrations of mixed bacterial cultures would also be useful in measuring the specificity of the polyclonal antibodies. In addition, immunomagnetic capturing experiments with milk and fecal samples from naturally infected animals are needed to calculate sensitivity and specificity of IMS at the field level. Finally, recombinant expression of proteins in mycobacterial species would mimic the natural protein synthesis mechanisms such as protein folding, maturation and PTMS and helps to retain biological activity.

In our third study, MAP cell envelope proteins and recombinant proteins were used to detect MAP specific antibodies in cattle serum samples in the ELISA format. ELISA is most commonly used in JD diagnosis due its simplicity and minimal cost. However, ELISA has low sensitivity and specificity for early and subclinical stages of MAP infection. Identification and testing of MAP-specific antigens or components for serodiagnostic evaluation is a central theme in MAP research.

We hypothesized that whole cell envelope protein antigens used in this study can improve sensitivity and specificity in the serodiagnosis of JD. Results with MAP cell envelope protein ELISA using pre-absorbed serum with MAH, *M. smegmatis* and *M. phlei* resulted in increased detection of fecal culture-positive animals than commercial ELISA. It is possible that some of the animals were in the early stages of infection and were missed by the commercial ELISA but were detected by our in-house ELISA. Commercial ELISAs use crude antigens the specific components of
which are unknown and are protected as trade secrets. Moreover, in MAP infection one antigen may not be expressed throughout the disease and different antigens may be expressed at different disease stages depending on the cross-talk between host and MAP. Therefore, we speculated that the defined cocktails of antigens used in this study were able to more accurately detect MAP-specific serum antibodies than by commercial ELISA. An additional advantage is that we used three MAP strains isolated in Southern Ontario with the premise that local strains would have better sensitivity in ELISA detection of MAP. The MAP strains and their isolation regions are not known in the commercial ELISAs. It is possible that use of the same strains to prepare ELISA antigens rather than using different strains may explain the low sensitivity of MAP IDEXX serum ELISA.

Some of the animals were positive by fecal culture and negative by our in-house ELISA. It has been reported that during the subclinical stage the Th1 immune response is predominant with minimal humoral response and therefore fecal culture may be more effective in identifying the presence of MAP infection than ELISA (Nielsen and Toft, 2008). However, ingestion of MAP from contaminated environments and passive shedding of MAP without actual infection leads to false positive diagnosis of infection (Sweeney et al., 1992). In the present study, some of the animals were negative by fecal culture but showed high OD450 values with MAP envelope protein ELISA and it is possible these animals were positive but missed by fecal culture. This may due to be the absence of shedding on the sample collection day and/or that very few MAP organisms were present in the feces and were below the detection level by fecal culture. The relationship between fecal culture and seroconversion has been reported based on experimental and natural MAP infections. However, temporal relationships between fecal shedding and seroconversion has not
been completely investigated (Nielsen and Toft, 2008) and thus complicates the comparison between fecal culture and ELISA. Reported discrepancies between JD diagnostic tests is explained by multi-factorial aspects such as exposure dose, age, MAP prevalence, MAP shedding pattern, time of testing, interpretation of test results, antigen preparations used in ELISA, environmental microbial populations and geographical variations. This further complicates the direct comparison between various diagnostic tests employed in JD diagnosis.

Our ELISA using recombinant protein antigens (SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein MAP1233 and DesA2) detected MAP-specific antibodies and differentiated MAP by fecal culture-positive and -negative animals. In this study, diagnostic sensitivities for recombinant protein antigens varied between 67% and 94%, results that are comparable with earlier studies using ELISA for serodiagnosis of JD (Collins, 2002; Dargatz et al., 2001a). Thus, it is feasible to use these recombinant proteins for the serodiagnosis of MAP in the ELISA format. However, reported specificities in this study were lower than that of earlier studies using recombinant antigens in the serodiagnosis of JD. Reporting of false positivity in JD diagnosis is a well-known phenomenon due to the complex nature of JD and sharing of antigenic components between related and non-mycobacterial species. Considering the endemic nature of JD, it would be feasible to initially screen animals with less specific diagnostic tests to identify all the suspicious animals in the herd followed by further testing of responders with more specific tests to confirm infection and to control disease spread. Herd level prevalence of MAP is another factor that needs to be considered while interpreting the ELISA tests results. For instance, herds with a high prevalence of MAP may lead to more false positives due to higher exposure to
MAP than low prevalence herds. Retesting of false positive cases with more sensitive tests would improve the accuracy of the diagnosis.

Our study with a limited number of serum samples showed that ELISA with MAP envelope proteins is more sensitive than commercial ELISA, however, further validation with more samples is needed. In addition, antigen stability and shelf life of antigen-coated plates at different temperatures and time points needs to be evaluated to scale up the ELISA for screening of large numbers of samples. In this study, we used denatured MAP total cell envelope proteins and this could lead to loss of conformational epitopes. Therefore, in future experiments comparison with MAP native protein antigens would complement the ELISA results. For recombinant proteins, use of partial proteins or peptide sequences instead of entire antigens may yield results with optimal sensitivity and specificity. During the early stages of JD, infection is mostly localised in the intestines and measuring of the peripheral immune response may not accurately reflect disease status. Therefore diagnostics that target localized immune responses such as mucosal immunity could be an alternate approach.

In conclusion, in this thesis we have shown that the MAP cell envelope protein profile is antigenically distinct from MAH, a MAC subspecies and many of these proteins are immunogenic and have considerable potential in the diagnosis of JD. Antibodies generated with MAP cell envelope proteins can be useful in the detection of MAP antigens in infected tissues by IHC and IF. Polyclonal antibodies generated to three MAP-specific recombinant proteins (SdhA, FadE25_2 and DesA2) were more specific for MAP with very minimal cross-reactivity to MAH and MS cell envelope proteins. Protein G magnetic beads coated with polyclonal antibodies generated to
MAP cell envelope proteins were able to retrieve MAP spiked in PBS and may be useful for concentration of MAP in diluted clinical samples. ELISA developed using MAP cell envelope protein antigens is more sensitive than commercial IDEXX serum ELISA suggesting feasibility for large scale screening of JD. ELISA with recombinant proteins showed very good sensitivity but lower specificity than with the commercial ELISA. ELISA using partial proteins or peptides of the recombinant proteins will assist with further refinement of this ELISA to improve specificity and sensitivity in the serodiagnosis of JD.
References


Grant, I., Pope, C., O’Riordan, L., Ball, H., Rowe, M., 2000, Improved detection of Mycobacterium avium subsp. paratuberculosis in milk by immunomagnetic PCR. Veterinary microbiology77, 369-378.


Stevenson, K., 2015, Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* and the influence of strain type on infection and pathogenesis: a review. Veterinary research 46, 1.

Summary and conclusion

Our first study focused on identifying antigenic MAP species-specific proteins from the MAP cell envelope by comparing MAP, MAH and *M. smegmatis* cell envelope protein profiles using 2D-DIGE, 2-DE immunoblot and LC-MS/MS proteomic approaches. 2D-DIGE showed that only a few protein spots are shared by all three bacterial species. More importantly, we observed that many protein spots were specific to the MAP cell envelope. However, several strings of protein spots with the same mass but with different pI values were observed in the 2-DE map of all three bacterial species, and some of these strings were located in close proximity to each other. This finding suggests subtle differences in amino acid sequences or post-translational modifications of homologous proteins across species that cause slight shifts in DIGE coordinates. Structural and functional relationships of proteins, protein-protein interactions, catalytic functions of enzymes, formation of protein complexes and antigenic epitopes are often modified by PTM.

2-DE immunoblot analysis with serum from MAP-infected cattle revealed reactivity with many MAP-specific proteins. A pattern of 13 of these spots aligned with the MAP-specific protein spots identified on the composite 2D-DIGE image of the three mycobacterial cell envelope proteins and the spots that were selected for protein identification by LC-MS/MS analysis. A total of fifteen proteins were positively identified. LC-MS/MS results were also suggestive of different isoforms or PTM for proteins that were represented by multiple spots on 2D-DIGE. These include iron-sulfur subunit of succinate dehydrogenase (SdhA) identified in spots 1, 2 and 3 and fibronectin-binding antigen 85 complex B (FbpB) in spots 11, 12 and 13. In addition, there was more than one protein for some of the spots with a high percentage
of protein coverage for each individual protein. Cell envelope proteins identified in this study may be useful in elucidating the pathogenicity of MAP and developing novel diagnostics tests and vaccines for JD.

In the second study six of the fifteen antigenic MAP cell envelope proteins (SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein MAP1233 and DesA2) were selected for recombinant expression as these proteins are involved in various aspects of energy and lipid metabolism and cell wall biogenesis and are essential for mycobacterial survival. Ultimately, three recombinant proteins (SdhA, FadE25_2 and DesA2) were suitable for generation of rat polyclonal antibodies and 1-DE and 2-DE immunoblotting confirmed that the reactivity of these antibodies was specific to MAP proteins SdhA, FadE25_2 and DesA2 with very minimal cross-reactivity with MAH and M. smegmatis cell envelope proteins. An extract of total MAP cell envelope proteins was also used to generate rat anti-MAP polyclonal antibodies and immunoblot analysis revealed specificity to MAP with minimal cross-reactivity with MAH and M. smegmatis cell envelope proteins.

IHC and IF using antibodies generated to MAP whole cell envelope proteins extracts showed strong immunoreactivity to MAP bacteria in sections of formalin-fixed intestinal tissues and lymph nodes from cattle infected with MAP. However, rat anti-SdhA, anti-FadE25_2 and anti-DesA2 polyclonal antibodies were not able to detect MAP microorganisms in these tissues.

Polyclonal antibodies were then coated on Dyna protein G magnetic beads for immunomagnetic separation (IMS) of MAP microorganisms. Our results illustrate that polyclonal antibodies extract of MAP total cell envelope, and recombinant SdhA, FadE25_2, and DesA2 are able to retrieve MAP cells spiked in PBS. Anti-MAP (total
cell envelope) polyclonal antibodies were the most efficient by retrieving as little as $10^2$ CFU of MAP. Recombinant protein antibodies showed variable sensitivity in the immunomagnetic recovery of MAP ranging from $10^5$ to $10^8$. This may be because antibodies to recombinant proteins were against single antigens and their level of expression and abundance may be less and thus would lead to low recovery of MAP. Other possible reasons are lack of PTMs and conformational changes in the recombinant proteins expressed in *E. coli* subsequently affecting the affinity and avidity of the polyclonal antibodies against their native antigens on the MAP cell surface. Further studies with mixed bacterial and other mycobacterial cultures are needed to assess the diagnostic accuracy of the IMS in MAP identification.

The third study focused on serodiagnostic evaluation of serum antibodies to MAP using the ELISA format with various MAP target antigens including MAP total cell envelope protein extract and six recombinant proteins i.e. SdhA, FadE25_2, FadE3_2, Mkl, DesA2 and hypothetical protein (MAP1233). The use ELISA microtitre plates coated with an extract of MAP envelope proteins to detect antibodies in unabsorbed serum samples resulted in a sensitivity of 72% and specificity of 89%. However, the diagnostic sensitivity and specificity of the MAP envelope protein ELISA after serum absorption was 75% and 96% respectively. In our study, serum absorption significantly improved sensitivity and specificity. In comparison, ELISA with the commercial IDEXX kit showed a sensitivity of 56% and specificity of 99%.

ELISAs with recombinant protein antigens differentiated between MAP-fecal culture positive and -negative serum samples with reasonably high sensitivities. However, specificities were comparatively lower than the commercial IDEXX ELISA. Future ELISA experiments with partial proteins and peptides or epitopes unique to MAP might improve test specificity. The potential use of these recombinant
proteins ELISAs for early diagnosis and control of JD requires further investigation and validation.
Appendix I

2-DE buffers

Cell lysis buffer with urea and thiourea

- Tris (1M, not pH’d) 30 mM 3.0 mL
- Thiourea (2 M) 15.22 g
- Urea (7 M) 42.0 g
- CHAPS (4% w/v) 4.0 g
pH to 8.5* and make up to 100 mL with distilled water
Adjust to pH 8.5 on ice with dilute HCL. Small aliquots can be stored at –20 °C for up to 3 months.

Rehydration buffer stock solution with urea and thiourea

- Urea (7 M) 10.5 g
- Thiourea (2M) 3.8 g
- CHAPS (2% w/v) 0.5 g
Make up to 25 mL with deionized water, aliquot (1.5 ml) and store at -20 °C. Add DTT and IPG buffer just prior to use.

SDS equilibration buffer

- Tris-Cl (1.5M, pH 8.8) (75 mM) 15.0 mL
- Urea (6M) 108.11 g
- Glycerol (30% v/v) 90 mL
- SDS (2% w/v) 6.0 g
Make up to 300 mL with deionized water, separate in 10, 30 mL aliquots and store at –20 °C.
Agarose sealing solution

- 1x SDS electrophoresis buffer
- Agarose 0.5% 125 mg
- Bromophenol blue Trace A few grains

Combine in 250 mL Erlenmeyer flask, disperse by swirling, heat in a microwave oven until the agarose is completely melted and allow the agarose to cool slightly before using. Do not adjust pH and can be stored in flask at 4 °C up to one month.

GE Healthcare DALT Six - 400 mL (six gels, 1 mm thickness – for DIGE)

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<th>Volume required for (mL)</th>
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<tbody>
<tr>
<td>Final %</td>
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<tr>
<td>Acrylamide stock (40%)</td>
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<tr>
<td>125</td>
</tr>
<tr>
<td>1.5 M Tris-Cl, pH 8.8</td>
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<tr>
<td>100</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>167</td>
</tr>
<tr>
<td>10% SDS</td>
</tr>
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<tr>
<td>TEMED</td>
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<tr>
<td>225 μL</td>
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<tr>
<td>10% APS</td>
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Hoefer SE 600 Ruby – 75 mL (two gels)

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<td>Acrylamide stock (40%)</td>
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<tr>
<td>1.5 M Tris-Cl, pH 8.8</td>
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<td>18.75</td>
</tr>
<tr>
<td>Water</td>
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<tr>
<td>32</td>
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<tr>
<td>0.75</td>
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<td>TEMED</td>
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<tr>
<td>40 μL</td>
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<td>10% APS (add last)</td>
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<td>375 μL</td>
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SYPRO Ruby gel fix/destain

- Methanol (10%) 100 mL
- Acetic acid (7%)- 70 mL

Make up to 1000 mL with deionized water and store at RT for up to 3 months.

Bind-silane solutions

- Ethanol 4 mL
- Glacial acetic acid 100 µL
- Bind saline 5.0 µL
- Deionized water 900 µL

Add 1 mL/plate (large plates), 0.5 mL/plate (small plates), wipe until dry with lint free tissue, cover the plate with a lint free tissue to prevent dust contamination and leave on the bench for 1.5 hr (min 1 hr) for excess bind saline to evaporate.

50X TAE Stock Solution for each litre of solution (1 L)

- Tris Base 242 g
- Glacial acetic acid 57.1 mL
- 0.5 M EDTA 100 mL

Dissolve in Tris base in about 500 mL of MQH₂O, add EDTA and acetic acid, bring final volume of 1L with MQH₂O, autoclave it and store at RT.

Final (1x) working concentration

- Tris-Acetate 0.04 M
- EDTA 0.001 M

Phosphate-buffered saline (PBS), pH 7.4

- NaCl 8 g
- KCl 0.2 g
- Na₂HPO₄·7H₂O 2.68 g
- KH₂PO₄ 0.24 g
- MilliQ water 900 mL
- pH to 7.4 and adjust volume to 1 L

**Immunoblotting**

12.5% separating gel

- Milli-Q water 3.5 mL
- 1.5M tris, pH 8.8 2.5 mL
- 10% SDS 100 μL
- 30% acrylamide 4.0 mL
- 10% ammonium persulfate* 100 μL
- TEMED 5 μL

4% stacking gel

- Milli-Q water 3.1 mL
- 0.5M Tris, pH 6.8 1.25 mL
- 10% SDS 50 μL
- 30% acrylamide 665 μL
- 10% ammonium persulfate* 50 μL
- TEMED 5 μL

1.5 M Tris-HCl, pH 8.8

Tris base 18 g

Milli-Q water 80 mL

*pH to 8.8 and adjust volume to 100 mL

0.5 M Tris-HCl, pH 6.8

Tris base 6 g

Milli-Q water 80 mL

pH to 6.8 and adjust volume to 100 mL
10% SDS
SDS 10 g
Milli-Q water 90 mL
Adjust volume to 100 mL

10% ammonium persulfate (APS)*
APS* 10 mg
Milli-Q water 1 mL
*Prepare fresh

5X Laemmli Buffer (order of addition is important)
- 2.0 M Tris-HCl, pH 6.8 3.125 mL
- β-mercaptoethanol 5 mL
- SDS 2 g (dissolve completely before proceeding)
- Glycerol 10 mL (add slowly)
- 1% bromophenol blue 200 μL
-Bring to final volume 20 mL with MilliQ water
Aliquot in amber tubes as β-mercaptoethanol is light sensitive and store at -20 °C

1% Bromophenol blue
- bromophenol blue 100 mg
- MilliQ water 10 mL

5X SDS-PAGE running buffer, pH 8.3
- Tris Base 60 g
- Glycine 288 g
- SDS 20 g
- MilliQ water 3 L
pH to 8.3 and adjust volume to 4 L
Transfer buffer (pH 8.1-8.4)

- Tris Base 12.12 g
- Glycine 57.6 g
- Methanol 800 mL
- MilliQ water 3 L

Adjust volume to 4 L and store at 4°C

Tris buffered saline (TBS), pH 7.6

- Tris Base 9.6 g
- NaCl 32 g
- MilliQ water 3 L

pH to 7.6 and adjust volume to 4 L

TBS-Tween 20 (TBS-T), 0.1% tween

- TBS 999 mL
- Tween 20 1 mL

0.5 M EDTA, pH 8.0

- EDTA 9.3 g
- Milli-Q water 80 mL

Adjust pH to 8.0 with NaOH and bring to final volume of 100 mL.
## Appendix II

### Source of materials

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<td>0.22 μM bottle top vacuum filter</td>
<td>Corning Inc, Corning, NY</td>
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<tr>
<td>1.0 N NaOH</td>
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