Identification of proteins from tuberculin purified protein derivative (PPD) by
LC-MS/MS

Sibele Borsuk^{a#}, Jane Newcombe^{b#}, Tom A. Mendum^b, Odir A. Dellagostin^a
and Johnjoe McFadden^{b*}

^a *Centro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil,*

^b *School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK.*

[#]*contributed equally*

* Corresponding author: School of Biomedical and Life Sciences, University of
Surrey, Guildford, GU2 7XH, Surrey, England, E-mail:
j.mcfadden@surrey.ac.uk

ABSTRACT

The tuberculin purified protein derivative (PPD) is a widely used diagnostic antigen for tuberculosis, however it is poorly defined. Most mycobacterial proteins are extensively denatured by the procedure employed in its preparation, which explains previous difficulties in identifying constituents from PPD to characterize their behaviour in B- and T-cell reactions. We here described a proteomics-based characterization of PPD from several different sources by LC-MS/MS, which combines the solute separation power of HPLC, with the detection power of a mass spectrometer. The technique is able to identify proteins from complex mixtures of peptide fragments. A total of 171 different proteins were identified among the four PPD samples (2 bovine PPD and 2 avium PPD) from Brazil and UK. The majority of the proteins were cytoplasmic (77.9%) and involved in intermediary metabolism and respiration (24.25%) but there was a preponderance of proteins involved in lipid metabolism. We identified a group of 21 proteins that are present in both bovine PPD but were not detected in avium PPD preparation. In addition, four proteins found in bovine PPD are absent in *M. bovis* BCG vaccine strain. This study provides a better understanding of the tuberculin PPD components leading to the identification of additional antigens useful as reagents for specific diagnosis of tuberculosis.

Key words: PPD, LC-MS/MS, proteomic analysis, *Mycobacterium bovis* AN5, *Mycobacterium avium* D4

1. INTRODUCTION

Tuberculosis continues to be a worldwide problem for both humans and animals ¹. Human tuberculosis is predominantly caused by *Mycobacterium tuberculosis*. Bovine tuberculosis, caused mainly by *M. bovis* is an important cause of economic losses and can be a zoonotic infection ². An important control strategy for the prevention of this disease is the use of an effective vaccine. The *M. bovis* bacillus Calmette-Guerin (BCG) vaccine, an attenuated strain of *M. bovis*, has been widely used for control of human tuberculosis despite controversy over its protective efficacy ³. In cattle, BCG has been used in a series of trials with various degrees of protection against *M. bovis* challenge ⁴⁻⁶. However, a major constraint in the use of attenuated mycobacterial vaccines such as BCG is that vaccination of humans or cattle interferes with detection of tuberculosis by tuberculin skin test. The development of tests, which can distinguish between infection with *M. tuberculosis* or *M. bovis* and vaccination with BCG, could greatly assist in the diagnosis of early infection as well as to enhance the use of tuberculosis vaccines on a wider scale.

The tuberculin skin test (Mantoux test) is used in humans and animals to distinguish between infected and uninfected individuals ⁷. The test was originally introduced in the 1890s by Robert Koch, who used a boiled crude preparation of *M. tuberculosis* cultures as the antigen, referred to as old tuberculin. However, this was replaced in the early 1940s by Florence Seibert who introduced and standardized a purified protein derivative of tuberculin (PPD). The tuberculin skin test cannot distinguish between a *M. tuberculosis*

infection and BCG vaccination or exposure to environmental mycobacteria. These cross-reactions are generally attributed to the presence in PPD of antigens shared by other *Mycobacterium* species^{8,9}. PPD is prepared by heat sterilization of 6-week-old *M. tuberculosis*, *M. bovis* or *M. avium* grown in broth medium, followed by filter sterilization and protein concentration using ultrafiltration or ammonium sulphate precipitation^{10,11}. Considering the widespread use of this immunological reagent, it is surprising that little is known about the active components of PPD^{11,12}. Some proteins that are probably present in PPD preparations have been tested as diagnostic reagents. The recombinant proteins MPB59, MPB64, MPB70 and ESAT 6 were evaluated in a differential diagnostic test, but only ESAT 6 was shown to be suitable for differentiating BCG-vaccinated animals from those infected with bovine tuberculosis^{13,14}. Synthetic peptides derived from ESAT6, CFP-10, MPB64, MPB70 and MPB83 were tested alone or in combination, with limited success^{15,16}.

Mass Spectrometry (MS) represents an efficient tool to perform analysis due to its high mass accuracy, sensitivity, and ability to deal with complex mixtures. It is a method of choice for characterizing complex proteins samples. Moreover, elucidation of the entire genome sequence makes it possible to analyze the proteome of *M. tuberculosis* and *M. bovis*.

In this work we used the LC-MS/MS which combines the solute separation power of HPLC with the detection power of a mass spectrometer, to characterize the proteins that are present in bovine and avium PPD preparations. Characterization of proteins from PPD preparations will be useful to identify additional antigens to be used in more specific and sensitive

tests that could eventually be able to differentiate BCG vaccinated from infected individuals.

2. MATERIAL AND METHODS

2.1. PPD sample

Commercial bovine and avian PPD samples were obtained from the Instituto Biológico, São Paulo, Brazil and from the Veterinary Laboratories Agency, Weybridge, Surrey, UK. *M. bovis* strain AN5¹⁷ and *M. avium* strain D4¹⁰ were used to prepare the bovine and avium PPD, respectively. In Brazil the strains were cultured in modified Dorset & Henley medium. In UK, however, the strains were cultured in glycerol based Bureau of Animal Industry (BAI) broth medium¹⁸. The Brazilian PPD was prepared by autoclave sterilization and the proteins concentrated using ammonium sulphate precipitation. The UK PPD the culture was heated at 95 °C to inactivate bacilli and left to cool. The proteins were precipitated with 1% trichloroacetic acid from the clarified culture supernatant and resolubilised in PBS. The final product was diluted to a protein concentration of 1 mg/ml in PBS/10% glycerol/0.5% phenol. All preparations were submitted to quality control by the manufacturers and met international recommended standards¹⁹.

2.2 .1-D gel electrophoresis

Fifty micrograms of PPD preparation (avium or bovine) were mixed with 25 µL of SDS loading buffer and boiled for 5 min prior to separation on a 10 cm long, 1 mm thick 18% SDS-polyacrylamide mini-gel (Mini-Protean II,

BioRad.Laboratories, Hemel Hempstead, UK). Protein bands were visualized by Vorum silver staining method²⁰.

2.3. Sample digestion

The entire gel lane was cut into 20 slices. Gel slices were washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate for 15 min at room temperature (RT). The gel pieces were dehydrated by incubating them with 50 μ L 100% ACN for 20 min at RT. Proteins were reduced using 10 mM dithiothreitol, and alkylated with 55 mM iodoacetamide (both in 25 mM ammonium bicarbonate). The gel pieces were dehydrated with ACN as described above, and rehydrated in 25 mM ammonium bicarbonate containing 0.01 mg/mL modified trypsin (Promega, Southampton, UK). Proteins were digested by trypsin for 16–20 h at 37 °C. The tryptic peptides were eluted by incubating the gel pieces with 50 μ L 1% trifluoroacetic acid (TFA) for 20 min at RT. The supernatant containing tryptic peptides was collected. Additional peptides were extracted from gel pieces by incubation with 50 μ L 0.1% TFA in 50% ACN for 20 min at RT, the supernatant was collected and combined with the first extraction. Finally, the extracted digested peptides were concentrated to 10 μ L using Speed-Vac® (Eppendorf UK Ltd, Cambridge, UK.) and stored at -20°C.

2.4. LC-ESI-MS/MS

Samples were analysed by nano-LC-ESI-MS/MS using an Ultimate 3000 nano-HPLC (Dionex, Amsterdam) coupled to a QSTAR™ XL (Applied Biosystems, Foster city, USA), a hybrid quadrupole time of flight instrument.

The peptides were separated on a 75 μ m, 15 cm, C18 pepmap column (Dionex-LC packing, Amsterdam) maintained at 30 °C. The flow rate was set at 350 nl/min using a solvent gradient of 2-50% B (90% ACN, 0.1% formic acid, solvent A was 2% ACN, 0.1% formic acid), over 30 min and 90% B for 5 min, under the control of Chromeleon software. The MS data was acquired using Independent Data Acquisition with Analyst[®] QS 1.1 software. A one second survey scan was acquired from m/z 350 to 1600 with the charge states 2-4. The two most intense product ions with a m/z of between 65- 1600 amu were automatically selected for MS/MS. The electrospray voltage was set at 1600 V and the curtain gas at 11 arbitrary units.

2.5. Database Analysis

The peak lists of MS/MS spectra from all the gel slices for any given sample were pooled and submitted to MASCOT (v2.2.04, Matrix Science, London, UK) and analysed using the MS/MS ion search program. Protein identifications were made by searching against the National Center for Biotechnology Information non-redundant (NCBI nr) data base with the taxonomy set for the *M. tuberculosis* complex (NCBI taxonomy ID code 77643) or the *M. avium* (NCBI taxonomy ID code 1764) complex for the bovine and avium samples, respectively. Up to one missing trypsin cleavage was allowed, with fixed modification of carbamidomethyl (C) and variable modification of oxidation (M). The search parameters were set with a peptide and MS/MS fragment tolerance of 1.2 and 0.6 Da respectively. MH_2^{2+} and MH_3^{3+} were selected as the precursor charge states. Due to the denatured condition of the sample, protein identifications were considered significant if

there was a single peptide with a minimum of 4 consecutive 'b' or 'y' ions. An automatic decoy database search using MASCOT was performed for each sample. The MASCOT data files were re-analysed using Scaffold (version Scaffold_2_03_01, Proteome Software Inc. Portland, OR). This was used to validate MS/MS based peptide and protein identifications. The identifications were assigned using Protein Prophet algorithm ²¹. Data on the protein identification probability, number of unique peptides and % coverage is included in supplementary table 1. Where the protein identification was made using a single peptide, the sequence, peptide modifications, MASCOT ion and identity score, charge state, observed m/z, expected mass, delta AMU and % coverage has been included in supplementary table 2. NCBI nr accession numbers were converted to RV numbers using <http://genolist.pasteur.fr/TubercuList/index.html>. To determine the protein class, (C) cytoplasmic protein; (TMHMM) membrane protein; (TATP) Tat signal peptide; (SIGNALP) Sec signal peptide; (SECRETOME) Non-classical secretion, the tools available at <http://www.cbs.dtu.dk/> and <http://tbsp.phri.org> websites were used.

3. RESULTS

3.1. Identification of proteins in PPD samples using LC-MS/MS

Both bovine PPD and avium PPD samples were examined from two different sources: Brazil (PPD_{bov} BR and PPD_{avi} BR) and the UK (PPD_{bov} UK and PPD_{avi} UK). Proteins were initially separated by one-dimensional SDS-PAGE which showed a smear of mostly low molecular weight protein fragments (Figure 1). The gel was then cut into slices and the proteins subjected to in-gel

trypsin digestion before LC-MS/MS. A total of 171 unique proteins were identified by LC-MS/MS analysis based on at least one identified peptide per protein with a MASCOT confidence level above 95% (table 1). Because the aim of this study was to identify proteins that might be useful for diagnosis of bovine tuberculosis, all *M. avium* proteins with a *M. tuberculosis* orthologue are referred to as their *M. tuberculosis* orthologue. The majority of the proteins were found in the range between 10 and 50 kDa, similar to the distribution of proteins encoded by the genome. The protein with the lowest molecular mass in this study was 7.2 kDa (Rv0787A - Conserved hypothetical protein). The probable polyketide synthase PKS7 (Rv1661), with a molecular mass of 220 kDa observed by LC-MS/MS represented the largest identified protein.

The Decoy function on MASCOT was used to perform an automatic decoy database search for each of the samples. During the search, every time a protein sequence from the target database is tested, a random sequence of the same length is automatically generated and tested generating a false discovery rate (FDR). The FDR for the peptide matches above the identity threshold were as follows, PPDbov Uk: 0.98%, PPDbovBR: 4.24%, PPDavi UK:1.19%, PPDavi BR:2.11%.

Unique proteins were identified in each PPD preparation but many proteins were identified in several samples, as illustrated in the Venn diagram, Figure 2. There was clearly a greater degree of overlap between Brazilian and UK bovine PPD preparations (37 common proteins) and similarly between Brazilian and UK avium PPD preparations (33 common proteins), than there was between avium and bovine PPD preparations from the same country (22

common proteins between UK avium and bovine PPD; 16 common proteins between Brazilian avium and bovine PPD). Nine proteins (Rv0216, Rv0350, Rv0440, Rv0652, Rv0685, Rv1436, Rv1980c, Rv2145c, and Rv2244) were detected in all PPD preparations (avium and bovine). Eleven were unique to PPDbov BR and 52 to PPDbov UK. A group of 21 proteins (Rv0242c, Rv0248c, Rv0467, Rv0632c, Rv0831c, Rv0896, Rv1093, Rv1133c, Rv1916, Rv1926c, , Rv2031c, Rv2626c, Rv2873, Rv2875, Rv2940c, Rv3001c, Rv3028c, Rv3417c, Rv3716c, Rv3841, Rv3846 and Rv3874) was identified in both bovine PPD preparations, but not in avium preparations (Table 1 and Figure 2).

Genes encoding four of the proteins identified in bovine PPD preparations are deleted in the genome of *M. bovis* BCG vaccine strain ²²: Rv1980c (MPT64), Rv1984c (CFP21), and Rv3874 (CFP-10), Rv3875 (ESAT-6). The major established secreted proteins, such as antigen 85 components, MPT32, MPT64, MPT83, MPT53 and MPT70 were present in PPD preparations.

3.2. Predicted location of PPD protein

The identified proteins were classified according to the *M. tuberculosis* annotation (Table 1). As can be seen, the predominant class are predicted cytoplasmic proteins, accounting for 77.9%. Membrane or secreted proteins with different signal peptides (Tat, Sec and Non-Classical secretion) accounted for only 22.4% of the proteins present (Table 1). The most common protein class among the avium PPD was also cytoplasmic proteins. Twenty

one proteins were classified as secreted. Of this, Rv0685, Rv1174c, Rv1411c, Rv1860, Rv1886c, Rv1980c are also found in bovine preparations.

3.3. Functional annotation of PPD proteins

The annotated *M. tuberculosis* H37Rv proteins have been classified into 12 distinct groups (<http://genolist.pasteur.fr/TubercuList/index.html>). The 171 proteins identified by LC-MS/MS in this study were distributed across ten functional groups (Table 1). The largest fraction (24.25%) of proteins identified are predicted to be involved in intermediary metabolism and respiration (functional group 7) but prokaryotic cell wall and cell processes (functional group 3) also occupy a large fraction (21.1%) of the identifications. The only functional group that is significantly enriched in the PPD preparations is lipid metabolism proteins which occupy 12.3% of PPD identifications but only 5.9% of genome annotations.

3.5. Most frequently detected PPD components

Although mass spectrometry is not an inherently quantitative tool, the abundance of a protein in a complex sample will be reflected in the frequency of peptides detected from that protein by mass spectrometry of that sample. Several methods have recently been developed to utilize protein coverage by detected peptides to generate approximate relative quantitation of proteins in complex mixtures. Using MASCOT each detected PPD protein was therefore assigned a Exponentially Modified Protein Abundance Index (emPAI) score²³ based on its relative coverage and the results were ranked to generate an

estimate of relative protein abundance. The ten proteins from each of the samples with the highest emPAI values are shown in Table 2. The EsxB protein (ESAT-6-like protein EsxB - Rv3874) and the 10 kDa chaperone protein (Rv3418c) were had the highest emPAI score in the UK bovine PPD; whereas EF-Tu (elongation factor EF-Tu - Rv0685) and Rv0865 (probable molybdopterin biosynthesis Mog protein) were predicted to be the most abundant in the Brazilian bovine PPD. The proteins EF-Tu (Rv0685), DnaK (70 kDa heat shock protein - Rv0350), groEL2 (60 kDa chaperonin 2 - Rv0440), Mpt83 protein (Rv2873) and the acyl carrier protein (Rv2244) appeared to be abundant in both UK and Brazilian bovine PPD. The acyl carrier protein (Rv2244) was abundant in all PPD samples.

4. DISCUSSION

PPD is an immunological reagent widely used for diagnosis of tuberculosis, but knowledge about the components of this heat-inactivated culture filtrate of mycobacteria is very limited ^{11,12,24-27}. In this study a proteomic analysis of tuberculin purified protein derivative (PPD) samples used for diagnosis of animal tuberculosis in Brazil and UK was carried out in order to identify the proteins present in this immunological reagent.

BCG vaccination has limited use in bovine tuberculosis since it generates tuberculin positivity and thereby interferes with the skin test used to control TB in cattle²⁸. Seibert *et al.* in 1926 and 1941^{29,30} were the first to attempt to characterize PPD components but identification of specific antigens in the preparation was not attempted until 1974 by Kuwabara & Tsumita ³¹. Remarkably little has been done since that time to identify the components of

PPD. Although whole-blood gamma interferon assays have been developed for diagnostic of bovine tuberculosis ^{32,33}, and depending on the antigens used, these tests can effectively differentiate vaccinated from infected animals ^{13,14,16}, they are more expensive and difficult to implement in remote places. It would be desirable to have an alternative test with similar features to the current tuberculin test, that is, easy to perform under field conditions, inexpensive and that does not require sophisticated equipments and laboratory infrastructure.

Examination of the one-dimensional PAGE of PPD samples (Figure 1) indicated that most if not all of the proteins in PPD were partially or completely degraded which is consistent with previous studies which have failed to isolate defined constituents from PPD by 1D or 2D-SDS-PAGE ^{12,27}. Nevertheless, it was possible to detect many protein fragments by in-gel trypsin digestion and LC-MS/MS. Clearly, although the proteins are largely degraded, the degradation is incomplete so that some peptide fragments remain intact for identification by LC-MS/MS. It seems likely that the immunological components of PPD are some fraction of these intact peptide fragments. Identification of those intact peptides present in PPD is thereby a route towards elucidating the immunologically active components of PPD. This supposition is supported by the finding that, although only a fraction of the proteins encoded by *M. bovis* were detected in the assay, this fraction included most of the previously characterized immunodominant antigens of *M. bovis* including MPT53, MPT64, MPT70, MPT83, GroEL, GroES, HspX, ESAT-6 CFP-10 and CFP-21 (Table 1). The MPB70 and MPB83 have been demonstrated to be resistant to heat degradation ³⁴⁻³⁶. The Rv0350 and

Rv0440 proteins present in all four PPD samples, had previously been identified from PPD and old tuberculin preparations using polyclonal and polyvalent antibodies in crossed immunoelectrophoresis and monoclonal antibodies L7 and TB78, respectively^{37,38}. Many of the PPD proteins identified have previously been identified culture filtrates of *M. tuberculosis* H37Rv³⁹.

Proteomic analysis of bovine PPD from two different sources gave similar results in terms of the number of proteins detected. However, it should be borne in mind that LC-MS/MS is, at best, a semi-quantitative tool. If a protein is detected then it clearly is present in the sample, but the converse is not true. There are many reasons for why a protein that may be present in a protein preparation may not be detected (e.g. abundance of the protein, level of degradation, efficiency of the trypsin digest, peptide ionization and ion suppression). Of 153 proteins identified in either the Brazilian or UK PPD 37 proteins were identified in both samples. The number of proteins identified in the avium PPD preparations tested in our study was lower compared to the bovine PPD preparations. This may have been a consequence of greater protein degradation as no clear bands were visible in the avium preparations examined by 1D-SDS-PAGE. The differences between the BR and UK PPD could also be because of differences in preparation methods. The strains used to prepare the bovine and avium PPD are the same in Brazil and in UK, However the media used to grow the bacteria, the inactivation procedure and precipitation method are different.

A key aim of this work was to identify proteins that could be used to generate an immunological reagent that could be used to differentiate *M. bovis* infection from infection with environmental mycobacteria, such as *M.*

avium. Twenty-one proteins were identified in both bovine PPD preparations, but not in *avium* preparations (Table 1). BLAST analysis shows that 10 of these proteins are not present in *M. avium* genome (Table 1) and are therefore definitely not present in *avium* PPD. The genes encoding the remaining 11 proteins are present in *M. avium* genome so their absence could be due to technical factors (as discussed above) or they may be present at lower levels than in *M. bovis*⁴⁰. These proteins are therefore candidates for further investigation towards development a skin test that can differentiate exposure of the animal to environmental mycobacteria from bovine tuberculosis. The use of a PPD composed exclusively with antigens absent in environmental mycobacterial species would be a significant improvement in the specificity of the test. Although, we do not yet know whether the *M. bovis*-specific proteins we have detected in bovine PPD are immunologically active in the test, the results clearly point the way forward towards development of a specific defined PPD.

Antigens that are present in PPD yet absent in the BCG strain of *M. bovis* are also of great interest, as their use could result in a test able to differentiate infected from vaccinated animals⁴¹. We detected four such antigens in bovine PPD but all of them (MPT64, CFP21, CFP10 and ESAT-6) have previously been investigated as antigens for development of diagnostic tests^{13,16,42} capable of differentiating BCG-vaccinated animals from those infected with bovine tuberculosis¹³⁻¹⁶. Although no novel BCG-absent antigens were detected in the current analysis the fact that the study did pick up nearly all the known *M. bovis*-specific antigens demonstrates the power of this approach. It is likely that further proteomics analysis will detect additional

antigens in the PPD preparations that could possibly include unknown *M. bovis*-specific antigens absent in BCG.

In conclusion, this study provides a proteomic analysis of the most important immunological reagent in TB control in animals and man: PPD. We identified several proteins in four different preparations, some of which may be potential targets for development of a more specific skin test capable of distinguishing between *M. bovis* infection and infection with environmental mycobacteria. We are currently evaluating selected recombinant antigens regarding their potential in skin tests for the diagnosis of tuberculosis.

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6. REFERENCES

1. WHO Global Tuberculosis Control. WHO/CDS/TB/2002: 2002.
2. Daborn, C. J.; Grange, J. M. HIV/AIDS and its implications for the control of animal tuberculosis. *Br. Vet. J.* 1993, **149** , 405-417.
3. Bloom, B. R.; Fine, P. E. M. The BCG experience: implications for future vaccines against tuberculosis. In *Tuberculosis: Pathogenesis, Protection, and Control*, Bloom, B. R., Ed.; ASM Press: Washington, DC, 1994; 531-558.
4. Buddle, B. M.; de Lisle, G. W.; Pfeffer, A.; Aldwell, F. E. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. *Vaccine* 1995, **13**, 1123-1130.
5. Buddle, B. M.; Keen, D.; Thomson, A.; Jowett, G.; McCarthy, A. R.; Heslop, J.; de Lisle, G. W.; Stanford, J. L.; Aldwell, F. E. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae*. *Res. Vet. Sci.* 1995, **59** , 10-16.
6. O'Reilly, L. M.; Daborn, C. J. The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. *Tuber. Lung Dis.* 1995, **76 Suppl 1**, 1-46.
7. Coler, R. N.; Skeiky, Y. A.; Ovendale, P. J.; Vedvick, T. S.; Gervassi, L.; Guderian, J.; Jen, S.; Reed, S. G.; Campos-Neto, A. Cloning of a *Mycobacterium tuberculosis* gene encoding a purified protein derivative protein that elicits strong tuberculosis-specific delayed-type hypersensitivity. *J. Infect. Dis.* 2000, **182** , 224-233.
8. Daniel, T. M.; Janicki, B. W. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. *Microbiol. Rev.* 1978, **42** , 84-113.
9. Young, D. B. Heat-shock proteins: immunity and autoimmunity. *Curr. Opin. Immunol.* 1992, **4** , 396-400.
10. Dall'Stella, R.; Krieger, M. A.; Burger, M.; Agottani, J. B.; Chahad-Ehlers, S.; Thomaz-Soccol, V. Development of bioprocess for the production of purified protein derivative with Brazilian strains of *Mycobacterium tuberculosis* for diagnosis use. *J. Biotechnol.* 2007, **127**, 278-287.
11. Klausen, J.; Magnusson, M.; Andersen, A. B.; Koch, C. Characterization of purified protein derivative of tuberculin by use of monoclonal antibodies: isolation of a delayed-type hypersensitivity reactive component from *M. tuberculosis* culture filtrate. *Scand. J. Immunol.* 1994, **40**, 345-349.

12. Rowland, S. S.; Ruckert, J. L.; Cummings, P. J. Low molecular mass protein patterns in mycobacterial culture filtrates and purified protein derivatives. *FEMS Immunol. Med. Microbiol.* 1999, **23**, 21-25.
13. Buddle, B. M.; Parlane, N. A.; Keen, D. L.; Aldwell, F. E.; Pollock, J. M.; Lightbody, K.; Andersen, P. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. *Clin. Diagn. Lab Immunol.* 1999, **6**, 1-5.
14. Buddle, B. M.; Ryan, T. J.; Pollock, J. M.; Andersen, P.; de Lisle, G. W. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. *Vet. Microbiol.* 2001, **80**, 37-46.
15. Vordermeier, H. M.; Cockle, P. C.; Whelan, A.; Rhodes, S.; Palmer, N.; Bakker, D.; Hewinson, R. G. Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. *Clin. Diagn. Lab Immunol.* 1999, **6**, 675-682.
16. Vordermeier, H. M.; Whelan, A.; Cockle, P. J.; Farrant, L.; Palmer, N.; Hewinson, R. G. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clin. Diagn. Lab Immunol.* 2001, **8**, 571-578.
17. Paterson, A. B. The production of bovine tuberculo-protein. *J. Comp Pathol. Ther.* 1948, **58**, 302-313.
18. Keating, L. A.; Wheeler, P. R.; Mansoor, H.; Inwald, J. K.; Dale, J.; Hewinson, R. G.; Gordon, S. V. The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for in vivo growth. *Mol. Microbiol.* 2005, **56**, 163-174.
19. World Health Organization International standard for purified protein derivative of bovine tuberculin. 760:22 ed.; 1986.
20. Mortz, E.; Krogh, T. N.; Vorum, H.; Gorg, A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics.* 2001, **1**, 1359-1363.
21. Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* 2003, **75**, 4646-4658.
22. Cole ST; Brosch R; Parkhill J, e. a. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998, 393-537.
23. Ishihama, Y.; Oda, Y.; Tabata, T.; Sato, T.; Nagasu, T.; Rappsilber, J.; Mann, M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of

sequenced peptides per protein. *Mol. Cell Proteomics*. 2005, **4**, 1265-1272.

24. Affronti, L. F.; Lind, A.; Ouchterlony, O.; Ridell, M.; Wisingerova, E. An evaluation of the polyacrylamide gel electrophoresis fractionation method for the production of *Mycobacterium tuberculosis* skin test preparations. I. Production, physiochemical characterization and serological analyses. *J. Biol. Stand.* 1986, **14**, 177-186.
25. Bardarov, S. S.; Kriakov, J.; Karakashyan, A.; Sirakova, T. D.; Markov, K. Characterization of PPD protein antigens in whole cell lysates of *Mycobacterium bovis* BCG. *FEMS Microbiol. Lett.* 1990, **59**, 89-93.
26. Huebner, R. E.; Schein, M. F.; Bass, J. B., Jr. The tuberculin skin test. *Clin. Infect. Dis.* 1993, **17**, 968-975.
27. Kitaura, H.; Kinomoto, M.; Yamada, T. Ribosomal protein L7 included in tuberculin purified protein derivative (PPD) is a major heat-resistant protein inducing strong delayed-type hypersensitivity. *Scand. J. Immunol.* 1999, **50**, 580-587.
28. Hewinson, R. G.; Vordermeier, H. M.; Buddle, B. M. Use of the bovine model of tuberculosis for the development of improved vaccines and diagnostics. *Tuberculosis*. 2003, **83**, 119-130.
29. Seibert, F. B. The isolation of a crystalline protein with tuberculin activity. *Science* 1926, **63**, 619c-620c.
30. Seibert, F. B. The chemistry of the proteins of the acid-fast bacilli. *Bacteriol. Rev.* 1941, **5**, 69-95.
31. Kuwabara, S.; Tsumita, T. Letter: Primary structure of tuberculin-active protein from tubercule bacilli. *Jpn. J. Exp. Med.* 1974, **44**, 129-132.
32. Denis, M.; Wedlock, D. N.; McCarthy, A. R.; Parlane, N. A.; Cockle, P. J.; Vordermeier, H. M.; Hewinson, R. G.; Buddle, B. M. Enhancement of the sensitivity of the whole-blood gamma interferon assay for diagnosis of *Mycobacterium bovis* infections in cattle. *Clin. Vaccine Immunol.* 2007, **14**, 1483-1489.
33. Palmer, M. V.; Waters, W. R.; Thacker, T. C.; Greenwald, R.; Esfandiari, J.; Lyashchenko, K. P. Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with *Mycobacterium bovis*. *Clin. Vaccine Immunol.* 2006, **13**, 387-394.
34. Harboe, M.; Wiker, H. G.; Lachmann, P. J. Carrier effect of concanavalin A-reactive and -non-reactive material in tuberculin PPD. *Scand. J. Immunol.* 1990, **32**, 263-271.

35. Harboe, M.; Nagai, S.; Wiker, H. G.; Sletten, K.; Haga, S. Homology between the MPB70 and MPB83 proteins of *Mycobacterium bovis* BCG. *Scand. J. Immunol.* 1995, **42**, 46-51.
36. Wiker, H. G. Unmatched sequences in public databases - exemplified by tuberculin-active protein. *Scand. J. Immunol.* 2004, **59**, 607-608.
37. Harboe, M. Antigens of PPD, old tuberculin, and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. *Am. Rev. Respir. Dis.* 1981, **124**, 80-87.
38. Harboe, M.; Nagai, S. MPB70, a unique antigen of *Mycobacterium bovis* BCG. *Am. Rev. Respir. Dis.* 1984, **129**, 444-452.
39. Malen, H.; Berven, F. S.; Fladmark, K. E.; Wiker, H. G. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics*. 2007, **7**, 1702-1718.
40. Garcia Pelayo, M. C.; Garcia, J. N.; Golby, P.; Pirson, C.; Ewer, K.; Vordermeier, M.; Hewinson, R. G.; Gordon, S. V. Gene expression profiling and antigen mining of the tuberculin production strain *Mycobacterium bovis* AN5. *Vet. Microbiol.* 2009, **133**, 272-277.
41. Hope, J. C.; Thom, M. L.; Villarreal-Ramos, B.; Vordermeier, H. M.; Hewinson, R. G.; Howard, C. J. Exposure to *Mycobacterium avium* induces low-level protection from *Mycobacterium bovis* infection but compromises diagnosis of disease in cattle. *Clin. Exp. Immunol.* 2005, **141**, 432-439.
42. Campos-Neto, A.; Rodrigues-Junior, V.; Pedral-Sampaio, D. B.; Netto, E. M.; Ovendale, P. J.; Coler, R. N.; Skeiky, Y. A.; Badaro, R.; Reed, S. G. Evaluation of DPPD, a single recombinant *Mycobacterium tuberculosis* protein as an alternative antigen for the Mantoux test. *Tuberculosis. (Edinb.)* 2001, **81**, 353-358.

FIGURE CAPTIONS

Figure 1. 1D-SDS-PAGE of PPD samples. 1, Protein Weight Marker (Promega, Southampton, Uk); 2, Avium PPD sample (PPDavi BR); 3, Bovine PPD sample (PPDbov BR); 4, Protein Weight Marker : 5, Bovine PPD (PPDbov UK; 6, Avium PPD (PPDavi UK).

Figure 2: Venn diagram with the unique and shared proteins between avium and bovine PPD samples. The number of proteins unique to each sample is shown under the name. Proteins present in more than one sample are identified by the corresponding Rv number.