



HAL
open science

Searching for proteins of subspecies with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculin

Wiebren Santema, Marije Overdijk, Judith Barends, Jeroen Krijgsveld, Victor Rutten, Ad Koets

► **To cite this version:**

Wiebren Santema, Marije Overdijk, Judith Barends, Jeroen Krijgsveld, Victor Rutten, et al.. Searching for proteins of subspecies with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculin. *Veterinary Microbiology*, 2009, 138 (1-2), pp.191. 10.1016/j.vetmic.2009.03.021 . hal-00490555

HAL Id: hal-00490555

<https://hal.science/hal-00490555>

Submitted on 9 Jun 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins

Authors: Wiebren Santema, Marije Overdijk, Judith Barends, Jeroen Krijgsveld, Victor Rutten, Ad Koets

PII: S0378-1135(09)00151-5
DOI: doi:10.1016/j.vetmic.2009.03.021
Reference: VETMIC 4392

To appear in: *VETMIC*

Received date: 8-1-2009
Revised date: 2-3-2009
Accepted date: 6-3-2009

Please cite this article as: Santema, W., Overdijk, M., Barends, J., Krijgsveld, J., Rutten, V., Koets, A., Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.03.021

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins

Authors

Wiebren Santema^{1,2*}, Marije Overdijk¹, Judith Barends³, Jeroen Krijgsveld³, Victor Rutten^{1,4} and Ad Koets^{1,2}

¹ Department of Infectious Diseases and Immunology, Immunology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

² Department of Farm Animal Health, Epidemiology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

³ Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands

⁴ Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

* Yalelaan 1, Postbus 80.165, 3508 TD, Utrecht, the Netherlands

Tel.: +31 (0) 30 2534608, Fax: +31 (0) 30 2533555

Email: w.j.santema@uu.nl

Abstract

Accurate immunodiagnosis of bovine paratuberculosis is among others hampered by the lack of specific antigens. One of the most frequently used antigen preparations is purified protein derivative (PPD), also known as tuberculin. This crude extract has limitations when used in diagnostic assays due to the presence of cross-reactive antigens. The aim of the current study was to systematically analyze the qualitative protein composition of PPD of the major mycobacterial pathogens.

One-dimensional gel electrophoresis followed by tandem mass spectrometry analysis of PPD from *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium bovis* (MB) identified 156, 95 and 132 proteins, respectively. Comparative sequence analysis led to the selection of a MAP-specific protein (MAP1718c), and finally heterologous expression in *E. coli* of this and other diagnostic candidate proteins (MAP3515c and MAP1138c (LprG)) enabled evaluation of their immunogenicity. Lymphocyte proliferation responses did not indicate substantial diagnostic potential of the antigens tested. In contrast serum antibody levels for MAP1138c in paratuberculosis infected cows (N=20) were significantly higher ($p < 0.05$) than in control animals (N=20), despite the conserved nature of this protein.

In conclusion, this study showed that a combination of proteomics and genomics, starting from complex protein mixtures, present in tuberculins, can reveal novel proteins aiding the development of immunodiagnosics for mycobacterial diseases.

Keywords

Tuberculins; Proteomics; Paratuberculosis; Antigen

1. Introduction

Accurate immunodiagnosis of bovine paratuberculosis, a chronic incurable infection of the small intestine caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is among others hampered by the lack of specific antigens (Harris and Barletta, 2001). Purified protein derivative (PPD), also known as tuberculin, produced from heat-processed mycobacterial culture filtrates, is one of the antigen preparations most used in mycobacterial immunodiagnosics, like skin tests in animals and humans, IFN γ assays, and in serum antibody ELISA (Monaghan et al., 1994). The PPD protein mixture likely contains many individual proteins. To limit false positive reactions to antigens shared with other pathogens or innocuous environmental micro-organisms, MAP-specific antigens are needed. Aided by the completion of the prototype mycobacterial genomes of MAP, MAA and *Mycobacterium bovis* (MB) / *Mycobacterium tuberculosis* (MT) we performed a qualitative, comparative proteomic analysis of commercially produced PPDs from MAP, MAA and MB, and evaluated the immunogenicity of three selected MAP proteins.

2. Material and Methods

2.1 Proteomics

The purified protein derivatives, produced according to the OIE manual for diagnostic tests from MAP strain 3+5/C (PPDP), MAA strain D4 (PPDA) and *M. bovis* strain AN5 (PPDB), were contracted from two producers, CVI-Lelystad (Lelystad, the Netherlands) and CZ Veterinaria (Pontevedra, Spain). Hundred microgram of each PPD was separated by one-dimensional gel electrophoresis in a 13% (w/v) SDS-PAGE gel, followed by in-gel digestion with trypsin. Proteins were reduced with 6.5mM DTT (Roche Diagnostics) followed by alkylation with 54mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) for one hour, and then digested with trypsin at an enzyme: substrate ratio of 1:50 (w/w). Nano-LC-MS/MS was

performed as previously described (van Balkom et al., 2005). Peptides were identified using Mascot software (MatrixScience, version 1.9) using the following settings: trypsin was used as an enzyme with one miscleavage allowed, peptide tolerance was set to 1.2 Da; carbamidomethyl and oxidation of methionine were set as fixed and variable modifications, respectively. Scaffold (version Scaffold_2_00_05, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at greater than 90.0% and 95.0% probability as specified by the Peptide/Protein Prophet algorithms, respectively. Protein identifications were accepted if they contained at least 2 identified peptides. The Geneplot program (available at <http://www.ncbi.nlm.nih.gov/>) was used to identify protein homologues between the sequenced genomes of *Mycobacterium avium* spp. *paratuberculosis* strain K-10, *Mycobacterium avium* 104 and *Mycobacterium bovis* AF2122/97. TBLASTN was used to calculate the homology on protein level.

2.2 Recombinant proteins

Primers were designed from the coding sequence of the MAP strain K10 genome (GenBank: AE016958) using Primer software (Version 1.0). Each forward primer contained the 5' extension CACC to facilitate directional cloning into the pET100/D TOPO vector (Invitrogen, USA). The following primers were used to clone the genes of interest:

MAP1718cF, 5'CACCGTGGCGTTCTCACACATTG3';

MAP1718cR, 5'CTAGCTCTTCCAGACCACCTTG3';

MAP3515cF, 5'CACCATGTATTCCGAGTCGAAG3';

MAP3515cR, 5'TCATGTGCGGCAGCTGATTTCC3';

MAP1138cF, 5'CACCATGCTGGGTATGCAGACCCG3';

MAP1138cR, 5'TCACTTGGTGACCTGGACCTGCTC3'.

Genomic DNA from MAP strain G195 and MAA strain D4 was used as template for the PCR reactions. Amplification was performed on an I-Cycler (BioRad, USA) with Pfu Turbo Cx Hotstart DNA Polymerase (Stratagene, USA). The selected genes were cloned into the pET100/D TOPO vector (Invitrogen, USA), containing an N-terminal His-tag. Expression was performed in BL21 Star (DE3) *E. coli* cells (Invitrogen, USA) using 1.0 mM isopropyl β -D-thiogalactoside induction. Recombinant proteins were affinity-purified on a Ni-column, using Chelating Sepharose Fast Flow (Amersham Bioscience, USA). Purity was checked on 13% (w/v) SDS-PAGE gels and confirmed by Western blot analysis using an anti-His monoclonal antibody (Amersham Bioscience, USA). The purified recombinant proteins were dialyzed against PBS, concentrated using a Vivaspin 10,000 MWCO PES membrane (VivaScience, Germany) and quantified by the BCA kit (Pierce, USA). All procedures were performed according to instructions provided by the manufacturers.

2.3 Immunoassays

Sera were derived from Holstein-Friesian cattle on farms which participated in paratuberculosis field surveys in the Netherlands. The sera were classified based on results from repeated fecal culture and serological responses measured in the absorbed ELISA for paratuberculosis diagnosis (Pourquier, France) and stratified in three groups: frequent shedders (N=20), intermediate shedders (N=20) and control animals (N=20). Serological responses to the recombinant proteins were measured using an ELISA technique as described previously (Koets et al., 2001) with minor modifications. Plates were coated with 1 μ g protein per well, and IgG1 antibody responses were measured. For the proliferation assays, blood samples were used from Holstein-Friesian cattle on dairy farms participating in control programs in the Netherlands. Animals were classified according to their clinical history and laboratory tests as described above. Control animals (N=14) originated from a single dairy farm with no known history of paratuberculosis, participating in the Dutch paratuberculosis

control programs and having obtained the highest herd status based on repeated testing. The infected animals (N=12) originated from different dairy farms and were classified as MAP infected based on repeated positive fecal culture results. Lymphocyte stimulation tests (LST) were performed as described previously (Koets et al., 1999), with the antigens at a concentration of 10 µg/ml. To correct for differences in background proliferation in wells stimulated with culture medium alone, data were expressed as Δ -cpm (cpm in antigen stimulated wells minus cpm in medium control wells). Data were analyzed using SPSS 15 software. ELISA data were normalized by Log transformation and ANOVA was used for comparison of groups. Results of the proliferation assays were compared using a nonparametric Mann-Whitney test on Δ -cpm values. The level of significance was set at $p < 0.05$.

3. Results

3.1 Protein identification and selection

Analysis of the three Lelystad PPDs by tandem mass spectrometry led to the generation of proteomes for PPDP, PPDA and PPDB, in which 156, 95 and 132 proteins were identified, respectively. Comparison of the generated proteomes by matching protein homologues showed that a large proportion (44-73%) of proteins was shared between the PPDs (figure 1a). In PPDA the total number of proteins identified was markedly less than in both other PPDs, however the ratio of shared proteins with the other PPDs was the highest (73%). In total 25 proteins were shared by PPDP, PPDA and PPDB produced by one manufacturer (CVI-Lelystad). To determine the overlap in proteome within a specific PPD, we next analysed three PPDA's from different manufacturers (two independent batches from CZ Veterinaria and one from CVI-Lelystad). The comparison of the proteomes generated for the PPDA's is depicted in figure 1b. The ratio of shared proteins ranged from 67-81%. In total 39 proteins

were shared between all PPDA preparations, representing 35–43% of the total proteins identified in the PPDA preparations.

Nineteen proteins were identified in each of the PPDs analyzed (table 1). The average homology on protein level as established with TBLASTN analysis for this group of proteins compared to PPDP was 99% (range 99–100%) for PPDA and 87% (range 67–96%) for PPDB homologues. Of these 19 proteins 8 and 5 proteins have been described as immunogenic in bovine paratuberculosis and bovine tuberculosis, respectively.

Next, we selected specific proteins for MAP (table 2). Since MAP and MAA genomes are known to be highly similar, and as a consequence very few MAP-specific proteins exist, we also selected MAP proteins differentially expressed in mycobacteria. To define MAP-specific proteins we selected MAP proteins present in PPDP that were not identified in PPDA, and had no homologues in the sequenced MB genome. Only hypothetical protein MAP1718c had no homologue identified in the sequenced MAA genome and was therefore selected as MAP-specific protein. For the remaining proteins in PPDP homologues are present in the MAA genome with on average over 98% similarity on protein level, which was established with TBLASTN analysis. The hypothetical protein MAP3515c was selected as a first protein, based on differential expression in MAP and MAA, to be screened for immunogenicity. Furthermore, MAP1138c (LprG), a conserved protein present in all PPDs, was selected for evaluation of immunogenicity in paratuberculosis infection, based on immunogenicity of the *M. tuberculosis* homologue. The homology on protein level of MAP1138c as compared with the *M. bovis* LprG protein is 67%.

3.2 Immunoassays

The selected proteins (MAP1718c, MAP3515c and MAP1138c) were successfully cloned and expressed in *E. coli*. The purified proteins were used to evaluate their potential as T cell antigen in a proliferation assay. The results are summarized in figure 2 (A-D). Animals

infected with paratuberculosis showed higher responses to PPDP as compared to control animals ($p < 0.05$). No significant differences between groups were observed for the recombinant MAP1718c, MAP3515c and MAP1138c (LprG) proteins. The purified proteins were also evaluated for immunogenicity in a serum antibody ELISA. The results are summarized in figure 2 (E-H). No significant differences between groups were observed for the recombinant MAP1718c and Map3515c protein. However, high shedders showed significant higher responses than control animals ($p < 0.01$) for the MAP1138c (LprG) protein.

4. Discussion

This study describes a qualitative survey of the proteins present in mycobacterial PPDs. Proteomics has been used to identify proteins in mycobacterial culture filtrates and cell wall extracts (Cho et al., 2006; Xiong et al., 2005). However, we were interested in the protein composition of PPD, the most widely used antigenic preparation in mycobacterial immunodiagnostics. To improve current diagnostic assays, knowledge on the composition of PPD may enable the rational design of a synthetic tuberculin with selected immunogenic non-cross reactive proteins or peptides.

The proteome analysis showed that the PPDs have a high range of shared proteins most likely partly responsible for the limited specificity of PPDs. In this group well-characterized and evolutionary conserved antigens are present like the 85 A, B, C complex proteins, heat shock protein 70 and Antigen D (bacterioferritin). The protein homology for these proteins is high, especially between the closely related species MAP and MAA, and likely also non-pathogenic environmental species. However, based on the range of protein homology, we speculate that specifically when MAP/MAA and MB proteins are compared, there could be peptides or protein regions within these conserved proteins that elicit pathogen-specific immune responses.

The majority of proteins identified in PPDB have been previously identified in culture filtrates or cell membrane analysis (Xiong et al., 2005). Tuberculosis-specific diagnostic antigens, like ESAT-6 and CFP-10, (Cockle et al., 2002) are present in PPDB. Andersen et al. described 30 antigens of *M. tuberculosis* recognized by T cells or effective as vaccine in animal models (Andersen and Doherty, 2005). Sixteen of these proteins were identified in PPDB in this study.

The hypothetical protein MAP1718c was identified as MAP-specific protein in PPDP, because it was absent from the sequenced genome MAA strain 104. However, in the current study a 99% identical homologue of the MAP1718c gene could also be amplified from MAA strain D4 and it was shown that a large sequence polymorphism containing MAP1718c was present in 44 of the 96 non-MAP isolates (Semret et al., 2005). Apparently strain variation within mycobacterial species may further complicate the search for diagnostic candidates.

The other potential MAP-specific proteins we have selected are based on differential expression of these proteins in the context of the tuberculin production process, in PPDP as compared to PPDA, rather than on the absence of the homologous gene in MAA. The immunoassay data showed that MAP1718c and MAP3515c are not immunodominant paratuberculosis antigens, unlike MAP1138c (LprG) that is recognized by antibodies during the course of paratuberculosis infection. LprG was recognized by high shedders to a larger extend than by intermediate shedders which may be related to the stage of the disease these animals are in. This phenomenon was also observed in other serodiagnostic studies using crude antigens (Koets et al., 2001). Recently, MAP LprG was also identified by screening of a MAP gene fusion library and found to be immunogenic in bovine paratuberculosis (Dupont et al., 2005). However, the sensitivity of the LprG ELISA appears to be much lower than the Pourquier ELISA.

Genomic approaches have led to the identification of MAP-specific ORFs by comparative sequence analysis followed by heterologous expression and immunogenicity evaluation (Bannantine et al., 2004). This has not yet resulted in novel specific antigens useful for diagnostic approaches. A disadvantage of this approach is the lack of knowledge on transcription of the identified ORFs. Proteome analysis identifies proteins which are actually transcribed under the conditions studied. Future work should also aim to identify differentially expressed antigens, as this could be a key in detecting novel diagnostic antigens. If the induced proteome of MAP during infection is different from related environmental mycobacteria, resulting in a differential induction of the immune response, we propose that conserved antigens can be useful as diagnostic targets. An interesting approach in this respect is the recent study by Hughes et al., who compared the proteome of *in vitro* cultured MAP and MAA bacteria and selected 32 differentially expressed MAP proteins (Hughes et al., 2008). Eight proteins were also identified in the current study, of which only MAP3627 was found to be immunogenic in ovine paratuberculosis.

In conclusion, we have used proteomics as a tool to qualitatively analyze tuberculin. Three MAP proteins (MAP1718c, MAP3515c and MAP1138c) were tested in immunodiagnostic assays. The evolutionary conserved recombinant MAP1138c protein is best recognized of these three proteins by paratuberculosis infected cows in a serum antibody ELISA. These results support the line of thought that there could be evolutionary conserved proteins or parts thereof that elicit pathogen and/or infection-specific immune responses. They warrant further study as many of these proteins are important targets for the host immune system.

Acknowledgement

The authors express their thanks to Jolanda Meijlis (CVI-Lelystad) and Eugenia Puentes (CZ Veterinaria) for the gifts of tuberculins. This work was supported by the Netherlands Proteomics Centre.

Literature

- Andersen, P., Doherty, T.M., 2005, TB subunit vaccines--putting the pieces together. *Microbes Infect* 7, 911-921.
- van Balkom, B.W., van Gestel, R.A., Brouwers, J.F., Krijgsveld, J., Tielens, A.G., Heck, A.J., van Hellemond, J.J., 2005, Mass spectrometric analysis of the *Schistosoma mansoni* tegumental sub-proteome. *J Proteome Res* 4, 958-966.
- Bannantine, J.P., Hansen, J.K., Paustian, M.L., Amonsin, A., Li, L.L., Stabel, J.R., Kapur, V., 2004, Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. *J Clin Microbiol* 42, 106-114.
- Cho, D., Sung, N., Collins, M.T., 2006, Identification of proteins of potential diagnostic value for bovine paratuberculosis. *Proteomics* 6, 5785-5794.
- Cho, D., Shin, S.J., Talaat, A.M., Collins, M.T., 2007, Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. *Protein Expr Purif* 53, 411-420.
- Cockle, P.J., Gordon, S.V., Lalvani, A., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2002, Identification of Novel *Mycobacterium tuberculosis* Antigens with Potential as Diagnostic Reagents or Subunit Vaccine Candidates by Comparative Genomics. *Infect Immun* 70, 6996-7003.
- Dupont, C., Thompson, K., Heuer, C., Gicquel, B., Murray, A., 2005, Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *J Med Microbiol* 54, 1083-1092.
- Harris, N.B., Barletta, R.G., 2001, *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. *Clin Microbiol Rev* 14, 489-512.
- Hughes, V., Bannantine, J.P., Denham, S., Smith, S., Garcia-Sanchez, A., Sales, J., Paustian, M.L., McLean, K., Stevenson, K., 2008, Proteome-determined *Mycobacterium avium* subspecies *paratuberculosis*-specific proteins: immunogenicity in ovine paratuberculosis. *Clin Vaccine Immunol*.
- Koets, A.P., Rutten, V.P., Hoek, A., Bakker, D., van Zijderveld, F., Muller, K.E., van Eden, W., 1999, Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. *Vet Immunol Immunopathol* 70, 105-115.
- Koets, A.P., Rutten, V.P., de Boer, M., Bakker, D., Valentin-Weigand, P., van Eden, W., 2001, Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect Immun* 69, 1492-1498.
- Monaghan, M.L., Doherty, M.L., Collins, J.D., Kazda, J.F., Quinn, P.J., 1994, The tuberculin test. *Vet Microbiol* 40, 111-124.
- Rhodes, S.G., Gavier-Widen, D., Buddle, B.M., Whelan, A.O., Singh, M., Hewinson, R.G., Vordermeier, H.M., 2000, Antigen specificity in experimental bovine tuberculosis. *Infect Immun* 68, 2573-2578.
- Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K., Huygen, K., 2006, Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infect Immun* 74, 202-212.
- Semret, M., Alexander, D.C., Turenne, C.Y., de Haas, P., Overduin, P., van Soolingen, D., Cousins, D., Behr, M.A., 2005, Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. *J Clin Microbiol* 43, 3704-3712.

- Sugden, E.A., Brooks, B.W., Young, N.M., Watson, D.C., Nielsen, K.H., Corner, A.H., Turcotte, C., Michaelides, A., Stewart, R.B., 1991, Chromatographic purification and characterization of antigens A and D from *Mycobacterium paratuberculosis* and their use in enzyme-linked immunosorbent assays for diagnosis of paratuberculosis in sheep. *J Clin Microbiol* 29, 1659-1664.
- Xiong, Y., Chalmers, M.J., Gao, F.P., Cross, T.A., Marshall, A.G., 2005, Identification of *Mycobacterium tuberculosis* H37Rv integral membrane proteins by one-dimensional gel electrophoresis and liquid chromatography electrospray ionization tandem mass spectrometry. *J Proteome Res* 4, 855-861.

Figure captions

Figure 1

Panel A. Venn diagram showing the overlap of the proteins identified in PPDP, PPDA and PPDB from CVI-Lelystad. **Panel B.** Venn diagram showing the overlap of the proteins identified in three PPDA batches, from CZ Veterinaria (CZV 1 and CZV 2) and CVI-Lelystad. Numbers of proteins per section are indicated.

Figure 2

Panel A-D: proliferative responses of PBMC to (A) PPDP, (B) MAP1138c, (C) MAP1718c and (D) MAP3515c expressed as delta CPM for control animals (Neg, N=14), and MAP shedding animals (Pos, N=12). **Panel E-H:** serum antibody responses. Results of the commercial Pourquier absorbed ELISA are depicted in (E). Results for (F) MAP1138c, (G) MAP1718c and (H) MAP3515c expressed as the logarithmic optical density corrected for medium value for high shedding (High), intermediate shedding (Inter) infected animals and control animals (Neg) (N=20 for each group). The horizontal line marks the group average value. Statistically significant differences ($p < 0.05$) between the different groups are indicated.

1 **Tables**

2

3 **Table 1** Proteins identified in all PPDs analyzed (N=19). The locus tag of each protein for all three mycobacterial species analyzed is depicted.

4 Antigenicity of each protein within bovine paratuberculosis and bovine tuberculosis as described in literature is shown. MW: molecular weight in

5 Da, ND: not described.

Protein name	Locus tag MAP	Locus tag MAA	Locus tag MB	MW (Da)	ParaTB antigen	TB antigen	Reference
35kd antigen	MAP_2855c	MAV_3635	Mb2765c	29.442	ND	ND	
Aconitate hydratase 1	MAP_1201c	MAV_3303	Mb1511c	101.097	ND	ND	
Acyl carrier protein	MAP_1997	MAV_2193	Mb2268	12.467	ND	ND	
Adenosylhomocysteinase	MAP_3362c	MAV_4211	Mb3276c	54.476	ND	ND	
Antigen 85-A	MAP_0216	MAV_0214	Mb3834c	36.078	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
Antigen 85-B	MAP_1609c	MAV_2816	Mb1918c	34.676	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
Antigen 85-C	MAP_3531c	MAV_5183	Mb0134c	38.035	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
ATP synthase F1, beta subunit	MAP_2451c	MAV_1527	Mb1342	53.080	ND	ND	
Bacterioferritin	MAP_1595	MAV_2833	Mb1907	18.462	Yes	ND	(Sugden et al., 1991)
Chaperone protein DnaK	MAP_3840	MAV_4808	Mb0358	66.510	Yes	Yes	(Koets et al., 1999; Rhodes et al., 2000)
60 kDa chaperonin 2	MAP_3936	MAV_4707	Mb0448	56.597	Yes	Yes	(Koets et al., 1999; Rhodes et al., 2000)
LprG protein	MAP_1138c	MAV_3367	Mb1446c	24.401	Yes	ND	(Dupont et al., 2005)
Malate dehydrogenase	MAP_2541c	MAV_1380	Mb1272	34.587	ND	ND	
ModD protein	MAP_1569	MAV_2859	Mb1891	37.512	Yes	ND	(Cho et al., 2007)
Ribosomal protein L7/L12	MAP_4126	MAV_4507	Mb0671	13.413	ND	ND	
Translation elongation factor Tu	MAP_4143	MAV_4489	Mb0704	43.752	ND	ND	
Trypsin	MAP_3527	MAV_5187	Mb0130	36.698	No	ND	(Cho et al., 2007)
Universal stress protein family protein	MAP_1339	MAV_3137	Mb1662	13.708	ND	ND	
Wag31 protein	MAP_1889c	MAV_2345	Mb2169c	26.988	ND	ND	

6

7

8

9

10 **Table 2** Candidate MAP-specific proteins. Proteins identified in PPDP, which were not identified in PPDA, and do not have a homologue in the
 11 sequenced *M. bovis* genome as established with Geneplot comparison (N=14). MW: molecular weight in Da.

Protein name	Locus Tag MAP	Locus tag MAA	MW (Da)
Hypothetical protein	MAP_0143	MAV_0139	11.617
Hypothetical protein	MAP_0151c	MAV_0146	16.518
Hypothetical protein	MAP_0494	MAV_0588	32.672
Hypothetical protein	MAP_1718c		15.518
Hypothetical protein	MAP_1754c	MAV_2494	31.093
FdxC_1	MAP_2039	MAV_2150	12.245
Probable cysteine desulfurase	MAP_2120c	MAV_2055	69.971
Hypothetical protein	MAP_2677c	MAV_1244	14.559
Hypothetical protein	MAP_2770	MAV_3539	19.488
Hypothetical protein	MAP_2786c	MAV_3558	11.708
Hypothetical protein	MAP_3515c	MAV_5200	22.240
Hypothetical protein	MAP_3560	MAV_5153	20.058
Hypothetical protein	MAP_3659	MAV_4950	53.808
Hypothetical protein	MAP_3804	MAV_4844	30.298

12

13

Figure 1



