Proteomic characterisation of bovine and avian purified protein derivatives and identification of specific antigens for serodiagnosis of bovine tuberculosis

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Abstract

Background: Bovine purified protein derivative (bPPD) and avian purified protein derivative (aPPD) are widely used for bovine tuberculosis diagnosis. However, little is known about their qualitative and quantitative characteristics, which makes their standardisation difficult. In addition, bPPD can give false-positive tuberculosis results because of sequence homology between *Mycobacterium bovis* (*M. bovis*) and *M. avium* proteins. Thus, the objective of this study was to carry out a proteomic characterisation of bPPD, aPPD and an immunopurified subcomplex from bPPD called P22 in order to identify proteins contributing to cross-reactivity among these three products in tuberculosis diagnosis.

Methods: Trypsin digests of bPPD, aPPD and P22 were analysed by nanoscale liquid chromatography-electrospray ionization tandem mass spectrometry. Mice were immunised with bPPD or aPPD, and their serum was tested by indirect ELISA for reactivity against these preparations as well as against P22.

Results: A total of 456 proteins were identified in bPPD, 1019 in aPPD and 118 in P22; 146 of these proteins were shared by bPPD and aPPD, and 43 were present in all three preparations. Candidate proteins that may cause cross-reactivity between bPPD and aPPD were identified based on protein abundance and antigenic propensity. Serum reactivity experiments indicated that P22 may provide greater specificity than bPPD with similar sensitivity for ELISA-type detection of antibodies against *M. tuberculosis* complex.

Conclusion: The subpreparation from bPPD called P22 may be an alternative to bPPD for serodiagnosis of bovine tuberculosis, since it shares fewer proteins with aPPD than bPPD does, reducing risk of cross-reactivity with anti-*M. avium* antibodies.

Keywords: Tuberculosis, PPD, P22, Proteome, Cross-reaction

Background

Bovine tuberculosis, caused mainly by *Mycobacterium bovis* (*M. bovis*), is a serious zoonotic infectious disease in cattle that can be also transmitted to humans [1].

Tuberculosis in cattle is subjected to compulsory eradication programmes based on the test-and-slaughter policy and results in a significant economic impact derived from a decreased production, trade restrictions and increased mortality rates in the infected herds [2, 3].

In Spain, the single intradermal tuberculin (SIT) test and interferon-gamma (IFN-γ) assay are used to diagnose bovine tuberculosis in official eradication programmes. Both tests depend on cell-mediated immune responses triggered by Th1 lymphocytes and a bovine purified...
protein derivative (bPPD) obtained from M. bovis. While bPPD is useful, it contains antigens shared by non-tuberculous mycobacteria and mycobacterial disease vaccines [4–7], giving rise to immune cross-reactions that limit its diagnostic specificity. For this reason, vaccination of cattle against mycobacterial diseases such as tuberculosis and paratuberculosis is prohibited in countries running tuberculosis eradication programmes. For the same reason, the diagnostic reliability of recommended tuberculin tests can be improved by comparing reactivity against bPPD with reactivity against avian purified protein derivative (aPPD), derived from M. avium. However, this comparative testing requires additional time, reagents and labor. In addition, it can reduce overall sensitivity, since animals that show greater immunoreactivity against aPPD than bPPD are often judged negative for tuberculosis when in fact they are infected with M. bovis [8, 9].

While standard tuberculin tests focus on cell-mediated immune responses, recent work highlights the importance of humoral responses. Early immune responses in bovine tuberculosis are dominated by cell-mediated immunity. However, some infected animals may have an antibody response in the absence of cell-mediated responses, particularly when the bacterial load is high [10, 11]. Therefore, researchers have been developing serological assays as diagnostic tests to detect infected animals missed by skin tests and the IFN-γ assay [12–15]. Serological tests are simple and inexpensive and can be used to screen many animals in a short time. Preparations of bPPD and P22 have been used as coating antigens in serological immunoassays to detect infected domestic and wild animals [16–20].

Improving diagnostic tests based on bPPD and aPPD requires detailed understanding of their protein composition, which would allow these reagents to be standardised and further optimised to reduce cross-reactivity. However, the composition of both reagents is poorly understood, and available data are to some extent contradictory [21, 22]. Proteomic analysis of bPPD and aPPD used in the UK and Brazil [22] identified 116 proteins in two bPPD preparations and 87 in two aPPD preparations; 32 proteins were shared between bPPD and aPPD. A similar study of a bPPD preparation used in South Korea [21] identified 271 proteins; 33 were also present in the previously analysed preparations from the UK and Brazil, and 15 were T cell antigens that induce cell-mediated immune responses detectable by standard diagnostic tests. These results provide molecular insights into possible false positives due to bPPD, since the T cell antigens showed an average sequence similarity of 78% with M. avium and 74% with M. paratuberculosis proteins.

Given the usefulness of proteomic analysis of bPPD and aPPD, we studied preparations from CZ Veterinaria widely used in European bovine tuberculosis eradication programmes. We also performed proteomic analysis of P22, a novel protein complex affinity-purified from the bPPD preparation for the first time, which may serve as an alternative antigen in tuberculosis immunodiagnosis.

Methods
Ethics statement
All animal experiments in this study were conducted according to Spanish regulations (RD 53/2013) and European regulations (EU Directive 2010/63/EU). All animal procedures were approved by the Ethics Committee of the Instituto de Salud Carlos III (permit CBA22_2014-v2) and by the Community of Madrid (permit PROEX 278/14).

Immunopurification of P22
BALB/c mice were hyperimmunised with bPPD (CZ Veterinaria, Porriño, Spain), giving rise to a hybridoma that secretes a specific monoclonal antibody against an epitope shared by two proteins, MPB70 and MPB83, which form part of a multiprotein complex within bPPD. This monoclonal antibody was conjugated to a HiTrap NHS-activated HP column (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s protocol. The column was loaded with bPPD and the complex containing MPB70 and MPB83, which we named P22, was immunopurified (this process has been patented under patent EP16382579, “Methods and compositions for tuberculosis diagnosis”).

Sample preparation for proteomic analysis
Batches of bPPD prepared from M. bovis strain AN5 and of aPPD prepared from M. avium strain D4 ER were obtained from CZ Veterinaria. P22 was obtained as described above. Each sample (bPPD, aPPD and P22) was analysed in three biological replicates. The protein mixtures were precipitated using trichloroacetic acid/acetone, and total protein concentration was determined using a Pierce protein assay at 660 nm (ThermoFisher Scientific, MA, USA). Protein pellets (20 µg) were resuspended and denatured in 20 µl of 7 M urea/2 M thiourea/100 mM TEAB (pH 7.5), then reduced with 2 µl of 50 mM Tris (2-carboxyethyl) phosphine (pH 8.0, AB SCIEX) at 37 °C for 60 min, and finally incubated for 10 min at room temperature with 2 µl of 200 mM methyl methanethiosulphonate (ThermoFisher Scientific) to block cysteines. To reduce urea concentration, samples were diluted to a final volume of 70 µl using 25 mM TEAB.

Proteins were digested overnight at 37 °C with sequencing-grade modified trypsin (Sigma-Aldrich, MO, USA) added in a trypsin:protein (w/w) ratio of 1:20. Digestion was stopped by adding 1% trifluoroacetic acid,
and reactions were desalted by passage through StageTip C18 Pipette tips (ThermoFisher Scientific) [23]. Desalted eluates were dried-down and stored until proteomic analysis.

**Liquid chromatography and mass spectrometry**

Protein digests (1 µg) were analysed by one-dimensional nanoscale liquid chromatography-electrospray ionization tandem mass spectrometry on an Eksigent nanoLC Ultra 1D plus (AB SCIEX) coupled to a 5600 Triple TOF® mass spectrometer (AB SCIEX) equipped with a Nanospray III source. The analytical column was a silica-based reverse-phase Waters Acquity UPLC® M-Class Peptide BEH C18 column (75 µm × 150 mm, 1.7 µm particles, 130 Å pore). The trap column was an Acclaim C18 PepMap™ 100 (100 µm × 2 cm, 5 µm particles, 100 Å pore; ThermoFisher Scientific), which was connected ahead of the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 2 µl/min. The nano-pump provided a flow-rate of 250 nl/min and was operated under gradient elution conditions. Peptides were separated using a gradient ranging from 2 to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). The gradient lasted 250 min in the case of bPPD and aPPD, and 30 min in the case of P22. The injection volume was 5 µl.

Data were acquired on the TripleTOF system using the following operating parameters: ion-spray voltage floating, 2300 V; curtain gas, 35; interface heater temperature, 150; ion source gas 1, 25; and declustering potential, 100 V. Data were acquired in data-dependent acquisition (DDA) mode using Analyst TF 1.7 software (AB SCIEX). A mass spectrometry survey scan (0.25 s) over the mass range 350–1250 Da was followed by 35 tandem mass spectrometry scans (100 ms) over the mass range 100–1800 Da, giving a total cycle time of 4 s. Switching criteria were defined as the presence of ions at an abundance of >90 counts per sec with charges of 2–5 and 350 < m/z < 1250. Former target ions were excluded for 15 s. Collision energy was controlled using a DDA rolling collision energy script.

**Proteomic data analysis**

Data from mass spectrometry and tandem mass spectrometry were processed using Analyst® TF 1.7 software (AB SCIEX). Raw data were converted into mgf files, which were searched against the NCBI RefSeq protein databases for *M. bovis* AN5 in the case of bPPD and P22 (15,834 entries in May 2016) or for *M. avium* subsp. *avium* ATCC 25291 in the case of aPPD (8712 entries in May 2016). These searches were conducted using Mascot Server 2.5.1 (Matrix Science, London, UK), and they included the corresponding reversed entries. Searches were conducted with methylationoliation (C) as a fixed modification and with the following variable modifications: acetyl (Protein N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and Oxidation (M). Peptide mass tolerance was 25 ppm, fragment mass tolerance was 0.05 Da, and 2 missed cleavages were allowed. The false discovery rate (FDR) had to be ≤1% for peptide identification to be considered successful.

The method of spectral counts [24] was used to assess relative abundance of different proteins in bPPD, aPPD and P22. Spectral counts for different proteins were first normalised by dividing them by the total spectral counts of the sample [25]. Relative abundance was represented as the mean of the replicates ± standard deviation (SD). Average antigenic propensity (AAP) of proteins was determined using the Kolaskar and Tongaonkar method [26] (http://imed.med.ucm.es/Tools/antigenic.pl) in order to identify proteins likely to elicit an antibody response. If the entire protein showed AAP >1.0, then all amino acid residues within the protein with AAP >1.0 were considered likely to be antigenic. Sequences of proteins were compared against those in the NCBI database using BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Venn diagrams were prepared using an open-access plotter (http://omics.pnl.gov/software/VennDiagramPlotter.php).

**Indirect ELISA**

Four female BALB/c mice were immunised with three inoculations of bPPD and another four mice with three doses of aPPD. These inoculations were administered at least 2 weeks apart. Sera from the mice were assayed for reactivity against bPPD, aPPD or P22 using an in-house indirect ELISA as follows. Plates were coated with bPPD, aPPD or P22 at 10 µg/ml, then blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS) and washed with PBS containing 0.05% Tween 20 (PBST). Serial twofold dilutions of serum (starting at 1:8) were prepared and assayed in triplicate, with ELISA plates incubated for 1 h at 37 °C. Next, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (SouthernBiotech, AL, USA) was added, and colour was developed by adding o-phenylenediamine substrate (OPD, Sigma–Aldrich). The reaction was stopped using 3 N H2SO4. Optical density was measured at 492 nm. Negative control serum was pooled from four female BALB/c mice that were not immunised.

**Results and discussion**

Bovine tuberculosis is a serious problem for public health and animal health, and the relative lack of specificity and sensitivity of current standard diagnostic tests based on
bPPD and aPPD means that some animals showing false positive results for tuberculosis infection are needlessly slaughtered, while animals showing false negative results are spared and may pose a threat to disease control and eradication. Improving bPPD as a diagnostic agent has become even more important as serological tuberculosis tests become more attractive than SIT and IFN-γ assays.

Here we provide detailed proteomic descriptions of bPPD and aPPD and identify components common to both preparations. These results may help guide efforts to improve the diagnostic performance of bPPD and, in particular, reduce cross-reactivity. Finally, we propose a less complex bPPD-derived immunoproduct more amenable to standardisation and less likely to cross-react with antibodies against *M. avium* proteins, which may serve as a substitute for bPPD in immunodiagnostic tests against tuberculosis.

**Proteins in bPPD, aPPD and P22**

We identified 2678 peptides in bPPD, 6465 in aPPD and 492 in P22. We then reconstructed the proteins present in each preparation and we included only proteins that (1) were represented by at least two peptides in each replicate where they were present, and (2) were present in at least two of the three replicates. In this way, our study identified 456 proteins in bPPD (Additional file 1), 1019 in aPPD (Additional file 2) and 118 in P22 (Additional file 3). The proteins identified in bPPD correspond to at least 8.5% of the coding sequences in the *M. bovis* genome; the proteins in aPPD, to at least 23.3% of the *M. avium* subsp. *avium* coding sequences; and the proteins in P22, to 1.5% of *M. bovis* coding sequences.

We were able to identify far greater numbers of proteins in bPPD and aPPD than previous studies, even though we used the same source strains for bPPD and aPPD as those studies. Studies of bPPD in UK, Brazil and South Korea identified only 104, 49 and 271 proteins, respectively [21, 22]. Studies of aPPD in UK and Brazil found only 63 and 57 proteins, respectively [22]. These discrepancies may reflect differences in how the PPDs were obtained from bacterial culture, as well as hardware and procedural differences in how proteomics data were obtained (e.g. mass spectrometer model, liquid chromatography flow rate, in-gel versus in-solution digestion), and how proteomics data were searched against the databases.

Tables 1, 2 and 3 show the 10 most abundant proteins in bPPD, aPPD and P22, respectively. These account for approximately 50% of total proteins determined in bPPD and P22 but no more than 15% in aPPD. These results imply that the vast majority of antibodies induced by *M. bovis* infection should be detectable in a serodiagnostic test based on bPPD or P22. Our estimates of relative protein abundances in the three preparations were obtained without labelling, which means that they are influenced by the proteomics methodology, particularly the duration of the dynamic exclusion process [25, 27]. Label-free abundances will also underestimate actual abundance in mixtures in which one or a few proteins dominate strongly. Thus, our abundance estimates are likely to be relatively accurate in the case of aPPD, in which no protein accounts for more than 2.7% of total protein, but they are likely to be underestimated in the case of bPPD, in which the ESAT-6-like protein EsxB (CFP-10) accounts for 12.2% of total protein, and in the case of P22, in which MPB70 accounts for 26.0% of total protein. The high abundance of MPB70 in *M. bovis* and its immunological properties suggest that replacing bPPD with P22 may increase the sensitivity of bovine tuberculosis immunodiagnostic test [28].

**Proteins shared among bPPD, aPPD and P22**

A total of 146 proteins were found to be common to bPPD and aPPD, as those studies. Studies of bPPD in UK, Brazil and South Korea

| No. | NCBI accession | Name                             | Relative abundance (%) | AAP       |
|-----|----------------|----------------------------------|------------------------|-----------|
| 1   | 489495023      | ESAT-6-like protein EsxB         | 12.2 ± 1.1             | 0.9934    |
| 2   | 489495046      | 6 kDa early secretory antigen target | 11.4 ± 2.0          | 0.9935    |
| 3   | 489509783      | Cell surface protein MPB70       | 8.5 ± 0.9              | 1.0478    |
| 4   | 489513185      | Molecular chaperone GroES        | 3.7 ± 0.1              | 1.0245    |
| 5   | 489509769      | Cell surface protein MPB83       | 3.4 ± 0.1              | 1.0336    |
| 6   | 489497323      | Molecular chaperone GroEL        | 3.0 ± 0.2              | 1.025     |
| 7   | 489516779      | ESAT-6-like protein EsxL         | 2.21 ± 0.1             | 1.0118    |
| 8   | 489503953      | ESAT-6-like protein EsxN         | 2.1 ± 0.0              | 1.0193    |
| 9   | 489504801      | Hypothetical protein MPB63       | 2.0 ± 0.0              | 1.0332    |
| 10  | 489505308      | Alpha-crystallin                 | 1.6 ± 0.3              | 1.0135    |

AAP average antigenic propensity
proteins in the respective preparations. Of these 146 proteins, only 43 were present in P22 (Fig. 1). The numbers of these proteins with relative abundances > 0.1% based on spectral counting were 61 in bPPD, 92 in aPPD and 43 in P22 (Additional file 4). Estimation of the ability of these proteins to induce a B cell-mediated immune response [26] identified 32 that we predict contribute to cross-reactivity (Table 4), either because they show an abundance ≥ 0.5% in at least one of the two PPDs or because they have AAP ≥ 1.04. Only 21 of these 32 proteins are present in P22 (Table 4), suggesting that it may provide greater diagnostic specificity than bPPD.

### Experimental comparison of the immunogenicity of bPPD, aPPD and P22

We hypothesised that P22 could offer greater specificity than bPPD in serodiagnosis because P22 shares far fewer proteins with aPPD, while still offering comparable sensitivity since P22 and bPPD share seven highly abundant proteins, five of which are predicted to be immunogenic (Tables 1, 3).

We compared the ability of immunosera from mice hyperimmunised with bPPD or aPPD to recognise bPPD,
| No. | NCBI accession | Description | Relative abundance in % Mean ± SD | AAP | Ident/query coverage |
|-----|----------------|-------------|-----------------------------------|-----|---------------------|
|     |                |             | bPPD | aPPD | P22 | bPPD | aPPD |
| 1   | 489513185      | Molecular chaperone GroES | 3.6 ± 0.0 | 1.1 ± 0.1 | 1.0 ± 0.3 | 1.0243 | 1.0261 | 97/100 |
|     | 4566238        | 10 kDa heat shock protein |          |      |      |       |       |      |
| 2   | 489497323      | Molecular chaperone GroEL | 3.0 ± 0.2 | 1.6 ± 0.2 | 2.5 ± 0.6 | 1.0251 | 1.0244 | 94/100 |
|     | 497665169      | Molecular chaperone GroEL |          |      |      |       |       |      |
| 3   | 489516779      | ESAT-6-like protein EsxL | 2.2 ± 0.1 | 0.7 ± 0.2 | 1.4 ± 0.3 | 1.0124 | 1.0238 | 87/100 |
|     | 489971186      | Hypothetical protein |          |      |      |       |       |      |
| 4   | 489503953      | ESAT-6-like protein EsxN | 2.1 ± 0.0 | 0.7 ± 0.2 | 1.2 ± 0.4 | 1.0193 | 1.0238 | 94/100 |
|     | 489971186      | Hypothetical protein |          |      |      |       |       |      |
| 5   | 489496900      | Molecular chaperone DnaK | 1.5 ± 0.1 | 0.8 ± 0.2 | 0.9 ± 0.1 | 1.0157 | 1.0149 | 95/94  |
|     | 497665296      | Molecular chaperone DnaK |          |      |      |       |       |      |
| 6   | 489500428      | ESAT-6-like protein EsxI | 1.4 ± 0.1 | 0.7 ± 0.2 | –      | 1.0154 | 1.0238 | 87/100 |
|     | 489971186      | Hypothetical protein |          |      |      |       |       |      |
| 7   | 489498552      | Elongation factor Tu | 1.2 ± 0.0 | 1.3 ± 0.4 | 2.0 ± 0.2 | 1.0225 | 1.0226 | 97/100 |
|     | 489970203      | Elongation factor Tu |          |      |      |       |       |      |
| 8   | 489506691      | Meromycolate extension acyl carrier protein | 1.0 ± 0.1 | 0.7 ± 0.3 | –      | 1.0236 | 1.0243 | 92/100 |
|     | 489969124      | Acyl carrier protein |          |      |      |       |       |      |
| 9   | 4992513161     | Secretion protein | 0.7 ± 0.2 | 0.7 ± 0.2 | –      | 1.0113 | 1.0238 | 93/100 |
|     | 489971186      | Hypothetical protein |          |      |      |       |       |      |
| 10  | 489506256      | Cell wall synthesis protein Wag31 | 0.7 ± 0.1 | 0.7 ± 0.2 | 1.0 ± 0.4 | 0.9988 | 1.0052 | 86/100 |
|     | 489968974      | DivIVA domain-containing protein |          |      |      |       |       |      |
| 11  | 489501012      | 5-Methyltetrahydropteroylglutamate synthase | 0.6 ± 0.0 | 0.1 ± 0.0 | 1.8 ± 0.5 | 1.0388 | 1.0374 | 86/99  |
|     | 497661234      | 5-Methyltetrahydropteroylglutamate synthase |          |      |      |       |       |      |
| 12  | 489500440      | Peptidase M22 | 0.5 ± 0.0 | 0.7 ± 0.3 | 0.6 ± 0.2 | 0.9816 | 0.9844 | 87/100 |
|     | 489971185      | Peptidase M22 |          |      |      |       |       |      |
| 13  | 489498442      | SOS ribosomal protein L7/L12 | 0.5 ± 0.0 | 1.4 ± 0.0 | –      | 1.0392 | 1.0327 | 93/100 |
|     | 489970202      | SOS ribosomal protein L7/L12 |          |      |      |       |       |      |
| 14  | 489504514      | Bacterioferritin | 0.5 ± 0.1 | 2.7 ± 1.6 | 0.8 ± 0.1 | 1.0193 | 1.0226 | 88/100 |
|     | 489973362      | Bacterioferritin |          |      |      |       |       |      |
| 15  | 489495992      | Diacylglycerol acyltransferase | 0.5 ± 0.1 | 0.6 ± 0.0 | 0.9 ± 0.3 | 1.0142 | 1.0141 | 85/95  |
|     | 497665638      | Diacylglycerol acyltransferase |          |      |      |       |       |      |
| 16  | 489504572      | Diacylglycerol acyltransferase | 0.5 ± 0.0 | 0.9 ± 0.1 | 1.0 ± 0.1 | 1.0166 | 1.0192 | 81/95  |
|     | 500042691      | Diacylglycerol acyltransferase |          |      |      |       |       |      |
| 17  | 489998054      | Serine protease | 0.5 ± 0.0 | 0.9 ± 0.2 | –      | 1.0357 | 1.0264 | 73/96  |
|     | 656316315      | Peptidase S1 |          |      |      |       |       |      |
| 18  | 489503193      | Universal stress protein | 0.4 ± 0.0 | 0.6 ± 0.0 | 0.4 ± 0.0 | 1.0434 | 1.0431 | 88/100 |
|     | 497663153      | Universal stress protein |          |      |      |       |       |      |
| 19  | 489513178      | Molecular chaperone GroEL | 0.4 ± 0.0 | 0.7 ± 0.1 | 0.6 ± 0.1 | 1.0416 | 1.0399 | 84/98  |
|     | 497664719      | Molecular chaperone GroEL |          |      |      |       |       |      |
| 20  | 554796584      | Aconitate hydratase | 0.3 ± 1.0 | 0.6 ± 0.2 | 1.0 ± 0.2 | 1.0252 | 1.0245 | 89/99  |
|     | 656316442      | Aconitate hydratase |          |      |      |       |       |      |
| 21  | 489509166      | Hypothetical protein | 0.3 ± 0.0 | 0.7 ± 0.2 | –      | 1.0146 | 1.0152 | 87/100 |
|     | 497663814      | Hypothetical protein |          |      |      |       |       |      |
| 22  | 489997754      | Diacylglycerol acyltransferase | 0.2 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 | 1.0225 | 1.0188 | 83/96  |
|     | 500042691      | Diacylglycerol acyltransferase |          |      |      |       |       |      |
| 23  | 489501803      | ATP synthase subunit beta | 0.2 ± 0.0 | 0.6 ± 0.1 | 0.3 ± 0.3 | 1.0239 | 1.0238 | 95/98  |
|     | 497661528      | ATP synthase subunit beta |          |      |      |       |       |      |
| 24  | 489496308      | Acyl dehydratase | 0.1 ± 0.1 | 1.4 ± 0.7 | 0.7 ± 0.0 | 1.0414 | 1.0457 | 80/99  |
|     | 564987547      | Acyl dehydratase |          |      |      |       |       |      |
| 25  | 489509098      | Iron-dependent repressor IdeR | 0.1 ± 0.0 | 0.2 ± 0.0 | –      | 1.0362 | 1.0409 | 93/100 |
|     | 489972034      | Dihydrofolate reductase |          |      |      |       |       |      |
aPPD and P22. Serum from mice immunised with aPPD reacted more strongly against aPPD and less strongly against bPPD and P22, indicating substantial cross-reaction (Fig. 2a). Similarly, serum from mice immunised with bPPD reacted against bPPD and P22, but it also cross-reacted with aPPD (Fig. 2b). These results suggest that, in the field, animals reacting against both aPPD and bPPD, particularly those reacting more strongly against aPPD, may be incorrectly categorised as negative.

Using P22 in ELISA-type assays may avoid this problem, offering greater specificity and comparable or greater sensitivity than bPPD for tuberculosis serodiagnosis. Proof-of-concept surveys testing P22 for serodiagnosis of animal tuberculosis are ongoing. Casal et al. [20] showed that an antibody detection test significantly improved the sensitivity of in vivo bovine tuberculosis diagnosis. Hence, diagnostic techniques detecting both cellular and humoral responses may be an alternative for controlling 

| No. | NCBI accession | Description | Relative abundance (%) ± SD | AAP aPPD | aPPD bPPD | AAP aPPD |
|-----|----------------|-------------|-----------------------------|---------|-----------|---------|
| 26  | 489996564, 497664056 | D-3-phosphoglycerate dehydrogenase | 0.1 ± 0.0 0.3 ± 0.0 - 1.0603 1.0551 89/100 | aPPD | bPPD | aPPD |
| 27  | 489500222, 495294442 | Molybdenum cofactor biosynthesis protein | 0.1 ± 0.0 0.1 ± 0.0 0.3 ± 0.4 1.0536 1.0476 96/91 | aPPD | bPPD | aPPD |
| 28  | 554793963, 497660570 | Hypothetical protein O217_19000 | 0.1 ± 0.0 0.4 ± 0.0 0.2 ± 0.2 1.0490 1.0422 85/89 | aPPD | bPPD | aPPD |
| 29  | 489497388, 489970521 | Dihydrolipoyl dehydrogenase | 0.1 ± 0.0 0.1 ± 0.0 0.3 ± 0.3 1.0408 1.0343 90/99 | aPPD | bPPD | aPPD |
| 30  | 489497373, 489970531 | Hypothetical protein | 0.1 ± 0.0 1.2 ± 0.2 - 1.0212 1.0135 76/100 | aPPD | bPPD | aPPD |
| 31  | 489997334, 497663425 | Phosphoglycerate kinase | 0.1 ± 0.0 0.2 ± 0.0 - 1.0415 1.0428 82/88 | aPPD | bPPD | aPPD |
| 32  | 489497373, 489970531 | Hypothetical protein | 0.1 ± 0.0 1.2 ± 0.2 - 1.0209 1.0136 76/100 | aPPD | bPPD | aPPD |

NCBI accession codes and protein names are reported in normal text for bPPD and in italics for aPPD.

AAP average antigenic propensity.

**Table 4 continued**

**Fig. 2** Indirect ELISA for serum reactivity from animals immunised with a aPPD or b bPPD against bPPD, aPPD and P22. As a negative control, serum was obtained from non-immunised mice (yellow line). The inset shows results obtained at serum dilutions of 1:128. OD optical density.
bovine tuberculosis outbreaks in high-prevalence settings. Moreover, some antibody detection techniques have been shown to give the best performance in cattle with gross tuberculosis lesions [29].

We identified 32 highly abundant and immunogenic proteins that contribute to cross-reactivity between aPPD and bPPD (Table 4). These results are consistent with previous proteomics work highlighting the importance of the chaperones DnaK, GroEL, and GroES as well as elongation factor Tu and acyl carrier protein as sources of cross-reactivity [21, 22, 30]. Chaperones, which are conserved among most mycobacteria and other bacteria that cause respiratory disease [31], play important roles in humoral and cellular innate and adaptive immune responses [32, 33]. Our results, then, are consistent with work suggesting that false-positive serodiagnosis of tuberculosis tends to reflect immune responses due to homologous vaccination antigens or environmental mycobacteria [34, 35].

Our results here, based purely on antibody-based immune responses, suggest that P22 may be a superior alternative to bPPD. It would be important to verify this by experimentally assessing T cell-mediated immune responses, which cooperate with antibody-based responses to mount a collaborative defense [36]. We expect that T cell-mediated responses would be similar to the antibody-based responses described here because several of the most abundant proteins in bPPD are powerful T immunogens, including MPB70, MPB83, ESAT-6 and CFP-10 [37–42].

**Conclusion**

On the basis of these results, we propose that a subpreparation of bPPD called P22 may be an alternative to bPPD for tuberculosis diagnosis, offering greater specificity as well as similar or even greater sensitivity for ELISA-type detection of antibodies against *M. tuberculosis* complex. P22 should be tested in field studies of tuberculosis serodiagnosis, and its ability to elicit T cell-mediated responses should be analysed, since it contains several antigens recognised by T cells, including MPB70, MPB83, ESAT-6 and CFP-10. In addition, we have analysed the protein composition of bPPD and aPPD, assessing the relative abundance and immunogenicity of major components. We identified several highly antigenic proteins specific to *M. bovis*, such as MPB70, MPB83, MPB63 and MPB64, which may therefore be the most useful in serological diagnosis of tuberculosis. We also identified several proteins common to bPPD and aPPD that may help explain the cross-reactivity between them in standard tuberculosis tests; these shared proteins include the chaperones GroES and DnaK, meromycolate extension acyl carrier protein, secretion protein and 50S ribosomal protein L7/L12.

**Additional files**

- **Additional file 1.** Peptides and proteins identified in bPPD.
- **Additional file 2.** Peptides and proteins identified in aPPD.
- **Additional file 3.** Peptides and proteins identified in P22.
- **Additional file 4.** Proteins shared between bPPD and aPPD.

**Abbreviations**

AAP: average antigenic propensity; aPPD: avian purified protein derivative; bPPD: bovine purified protein derivative; CFP-10: ESAT-6-like protein EsxB; IFN: interferon; sIT: single intradermal test.

**Authors’ contributions**

JL conceived and designed the study, performed most of the experiments, analysed and interpreted the data and wrote the first draft of the manuscript. IM performed part of the experiments and was involved in the drafting of the manuscript. MAR analysed and interpreted the data and critically revised the manuscript. AR, MV, BR, NI, JF, EP, LJ and CG were involved in the drafting of the manuscript. MD conceived and designed the study, analysed and interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

Datasets supporting the conclusions of this article are included within the article and in Additional files. Mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository under dataset identifiers PXD007766 and https://doi.org/10.6019/PXD007766.

**Consent for publication**

Not applicable.

**Ethics approval**

All animal experiments in this study were conducted according to Spanish regulations (RD 53/2013) and European regulations (EU Directive 2010/63/EU), and the experimental protocols were approved by the Ethics Committee of the Instituto de Salud Carlos III (permit CBA22_2014-v2) and by the Community of Madrid (permit PROEX 278/14).
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References
1. Schiller I, Waters WR, Vordermeier HM, Jenmi T, Welsh M, Keck N, Whelan A, Gormley E, Boschioli ML, Moyen JI, Vela C, Cagiola M, Buddle BM, Palmer M, Thacker T, Oesch B. Mycobacterium bovis infection and tuberculosis in cattle. Vet J. 2002;163:115–27.
2. Reviriego Gordejo FJ, Vemmersch JP. Towards eradication of bovine tuberculosis in the European Union. Vet Med. 2006;61:101–9.
3. Hart D, Sutherland J. The immunity conferred by BCG vaccination in relation to the degree of induced tuberculosis sensitivity. Tuberkulózova. 1966;18:38–40.
4. Bats JH. Mycobacterium avium disease: progress at last. Am J Respir Crit Care Med. 1996;153:1737–8.
5. Berggren SA. Field experiment with BCG vaccine in Malawi. Br Vet J. 1981;137:86–96.
6. Whelan AG, Coad M, Upadhya BL, Clifford DJ, Hewinson RG, Vordermeier HM. Lack of correlation between BCG-induced tuberculin skin test sensitisation and protective immunity in cattle. Vaccine. 2011;29:5453–8.
7. Alvarez J, de Juan L, Bezos J, Romero B, Saez JL, Reviriego Gordejo FJ, Briones V, Moreno MA, Mateos A, Dominguez L, Arana A. Interference of paratuberculosis with the diagnosis of tuberculosis in a goat flock with a natural mixed infection. Vet Microbiol. 2008;128:72–80.
8. Alvarez J, de Juan L, Bezos J, Romero B, Saez JL, Marques S, Dominguez C, Minguez O, Fernandez-Mardomingo B, Mateos A, Dominguez L, Arana A (2009) Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay. Vet Microbiol. 2009;135:389–93.
9. McNair J, Welsh MD, Pollock JM. The immunology of bovine tuberculosis and progression toward improved disease control strategies. Vaccine. 2007;25:5504–11.
10. Welsh MD, Cunningham RT, Corbett DM, Girvin RM, McNair J, Skuce RA, Bryson DG, Pollock JM. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology. 2005;110:141–7.
11. Green LR, Jones CC, Sherwood AL, Garkavi IV, Cangeli GA, Thacker TC, Palmer MV, Waters WR, Rathe CV. Single-antigen serological testing for bovine tuberculosis. Clin Vaccine Immunol. 2009;16:1309–13.
12. Waters WR, Buddle BM, Vordermeier HM, Gormley E, Palmer MV, Thacker TC, Bannantine JP, Stabel JR, Linscott R, Martel E, Milan F, Foshaug W, Lawrence JC. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. Clin Vaccine Immunol. 2011;18:1896–70.
13. Waters WR, Palmer MV, Stafne MR, Bass KE, Maggioli MF, Thacker TC, Linscott R, Lawrence JC, Nelson JT, Esfandiari J, Greenwald R, Lysachenko KP. Effects of serial skin testing with purified protein derivative on the level and quality of antibodies to complex and defined antigens in mycobacterium bovis-infected cattle. Clin Vaccine Immunol. 2015;22:641–9.
14. Whelan C, Whelan AO, Shuralev E, Kwock HF, Hewinson G, Clarke J, Vordermeier HM. Performance of the Enferplex TB assay with cattle in Great Britain and assessment of its suitability as a test to distinguish infected and vaccinated animals. Clin Vaccine Immunol. 2010;7:183–7.
15. Beltran-Beck B, Romero B, Boadella M, Casal C, Bezos J, Mazanioghos M, Martin M, Galindo RC, Perez de la Peña JM, Villar M, Garrido JM, Sevilla IA, Asensio F, Sicilia J, Lysachenko KP, Dominguez L, Juste RA, de la Fuente J, Gortazar C. Tonsils of the soft palate do not mediate the response of pigs to oral vaccination with heat-inactivated Mycobacterium bovis. Clin Vaccine Immunol. 2014;21:1128–36.
16. Lilenbaum W, Ribeiro ER, Souza GA, Moreira EC, Fonseca LS, Ferreira MA, Schettini J. Evaluation of an ELISA-PPD for the diagnosis of bovine tuberculosis in field trials in Brazil. Res Vet Sci. 1996;66:191–5.
17. Amadori M, Tamerini S, Scaccaglia P, Cavirani S, Archetti I, Gisbenedico RQ. Antibody tests for identification of Mycobacterium bovis-infected bovine herds. J Clin Microbiol. 1998;36:596–8.
18. Boadella M, Lysachenko K, Greenwald R, Esfandiari J, Jarosost J, Garrido JM, Vicente J, de la Fuente J, Gortazar C. Serologic tests for detecting antibodies against Mycobacterium bovis and Mycobacterium avium subspecies paratuberculosis in Eurasian wild boar (Sus scrofa scrofa). J Vet Diagn Invest. 2011;23:77–83.
19. Casal C, Infantes JA, Risalde MA, Diez-Guerrer A, Dominguez M, Moreno J, Romero B, de Juan L, Saez JL, Juste R, Gortazar C, Dominguez L, Bezos J. Antibody detection tests improve the sensitivity of tuberculosis diagnosis in cattle. Res Vet Sci. 2017;112:214–21.
20. Cho YS, Jang YB, Lee SE, Cho JY, Ahn JM, Hwang I, Heo E, Nam HM, Cho D, Her M, Jeon YH, Jung SC, Kim JM, Lee HS, Lee K, Belisle JT. Short communication: proteomic characterization of tuberculin purified protein derivative from Mycobacterium bovis. Vet Res. 2015;10:117–9.
21. Borsuk S, Newcombe J, Mendum TA, Dellagostin OA, McFadden J. Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS. Tuberculosis (Edinb). 2009;89:423–30.
22. Rappisilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007;2:1896–906.
23. Liu H, Sadjogy RO, Yates JR III. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem. 2004;76:4193–20.
24. Klein-Nicolas M, Zivy M. Thousand and one ways to quantify and compare protein abundances in label-free bottom-up proteomics. Biochem Biophys Acta. 2016;1864:883–95.
26. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990;276:172–4.
27. Zhu W, Smith JW, Huang CM. Mass spectrometry-based label-free quantitative proteomics. J Biomed Biotechnol. 2010;2010:840518.
28. Harboe M, Viker HG, Duncan JR, Garcia MM, Dukes TW, Brooks BW, Turcotte C, Nagai S. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. J Clin Microbiol. 1990;28:913–21.
29. Pollock JM, Welsh MD, McNair J. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Vet Immunol Immunopathol. 2005;108:37–43.
30. Cho YS, Dobos KM, Prennis J, Yang H, Hess A, Rosenkrands I, Ryoo SW, Bai GH, Brennan MJ, Izzo A, Bielefeldt-Ohmann H, Belisle JT. Deciphering the proteome of the in vivo diagnostic reagent “purified protein derivative” from Mycobacterium tuberculosis. Proteomics. 2012;12:979–91.
31. Prasad TS, Verma R, Kumar S, Niruogi RS, Sathe GJ, Madugundu AK, Sharma J, Puttamallesh VN, Ganjwalve A, Myneedu VP, Chattejee A, Pandey A, Harsha H, Narayana J. Proteomic analysis of purified protein derivative of Mycobacterium tuberculosis. Clin Proteomics. 2013;10:8.
32. Young D, Roman E, Moreno C, O'Brien R, Born W. Molecular chaperones and the immune response. Philos Trans R Soc Lond B Biol Sci. 1993;339:363–7.
33. Colaco CA, Bailey CR, Walker KB, Keeble J. Heat shock proteins: stimulators of innate and acquired immunity. Biomed Res Int. 2013;2013:461230.
34. Harboe M. Antigens of PPD, old tuberculin, and autoclaved Mycobacterium bovis BCG studied by cross-respond immunoelectrophoresis. Am Rev Respir Dis. 1981;124:80–7.
35. Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. Clin Infect Dis. 1993;17:968–75.
36. Claman HN, Chaperon EA, Triplett RF. Thymus-marrow cell combinations. Synergism in antibody production. Proc Soc Exp Biol Med. 1966;122:1167–71.
37. Radford AJ, Wood PR, Billman-Jacobe H, Geysen HM, Mason TJ, Tribbick G. Epitope mapping of the Mycobacterium bovis secretory protein MPB70 using overlapping peptide analysis. J Gen Microbiol. 1990;136:265–72.
38. Tollefsen S, Pollock JM, Lea T, Harboe M, Wiker HG. T- and B-cell epitopes in the secreted Mycobacterium bovis antigen MPB70 in mice. Scand J Immunol. 2003;57:151–61.
39. Vordermeier HM, Cockle PJ, Whelan AO, Rhodes S, Hewinson RG. Toward the development of diagnostic assays to discriminate between Mycobacterium bovis infection and bacille Calmette-Guerin vaccination in cattle. Clin Infect Dis. 2000;30(Suppl 3):S291–8.
40. Vordermeier HM, Brown J, Cockle PJ, Franken WP, Drijfhout JW, Arend SM, Ottenhoff TH, Jahans K, Hewinson RG. Assessment of cross-reactivity between Mycobacterium bovis and M. kansasii ESAT-6 and CFP-10 at the T-cell epitope level. Clin Vaccine Immunol. 2007;14:1203–9.
41. Vordermeier HM, Cockle PC, Whelan A, Rhodes S, Palmer N, Bakker D, Hewinson RG. Development of diagnostic reagents to differentiate between Mycobacterium bovis BCG vaccination and M. bovis infection in cattle. Clin Diag Lab Immunol. 1999;6:675–82.
42. van Pinxteren LA, Ravn P, Agger EM, Pollock J, Andersen P. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. Clin Diag Lab Immunol. 2000;7:155–60.