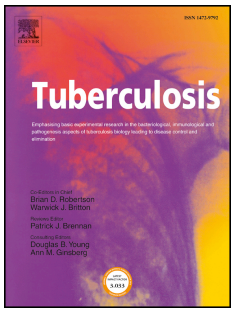
Accepted Manuscript



Identification and evaluation of new *Mycobacterium bovis* antigens in the *in vitro* interferon gamma release assay for bovine tuberculosis diagnosis

María E. Eirin, Analia Macias, Gabriel Magnano, Claudia Morsella, Laura Mendez, Federico C. Blanco, María V. Bianco, Walter Severina, Alicia Alito, Maria de los Angeles Pando, Mahavir Singh, Ralph Spallek, Fernando A. Paolicchi, Fabiana Bigi, Angel A. Cataldi

PII: S1472-9792(15)20768-8

DOI: 10.1016/j.tube.2015.07.009

Reference: YTUBE 1365

To appear in: *Tuberculosis*

Received Date: 5 March 2015

Accepted Date: 29 July 2015

Please cite this article as: Eirin ME, Macias A, Magnano G, Morsella C, Mendez L, Blanco FC, Bianco MV, Severina W, Alito A, Pando MdlA, Singh M, Spallek R, Paolicchi FA, Bigi F, Cataldi AA, Identification and evaluation of new *Mycobacterium bovis* antigens in the *in vitro* interferon gamma release assay for bovine tuberculosis diagnosis, *Tuberculosis* (2015), doi: 10.1016/j.tube.2015.07.009.

Title page

Identification and evaluation of new *Mycobacterium bovis* antigens in the *in vitro* interferon gamma release assay for bovine tuberculosis diagnosis

María E. Eirin[1§]; Analia Macias[2]; Gabriel Magnano[2]; Claudia Morsella[3]; Laura Mendez[3]; Federico C. Blanco[1]; María V. Bianco[1]; Walter Severina[4]; Alicia Alito[1]; Maria de los Angeles Pando[5], Mahavir Singh[6]; Ralph Spallek[6]; Fernando A. Paolicchi[3]; Fabiana Bigi[1]; and Angel A. Cataldi[1].

[1]Laboratorio de Tuberculosis Bovina, Instituto de Biotecnología, CICVyA, INTA-Castelar, Buenos Aires, Argentina.

[2]Departamento de Patología Animal, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina.

[3]Laboratorio de Bacteriología, EEA-Balcarce, INTA, Buenos Aires, Argentina.

[4]Private Activity, 722 Piacenza Street, Post code 5917, Arroyo Cabral, Córdoba, Argentina.

[5]Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), UBA-CONICET, Facultad de Medicina, Buenos Aires, Argentina.

[6]LIONEX GmbH, Braunschweig, Germany.

E-mail addresses:

Maria Emilia Eirin: eirin.maria@inta.gob.ar
Analia Macias: amacias@ayv.unrc.edu.ar
Gabriel Magnano: gmagnano@ayv.unrc.edu.ar
Claudia Morsella: morsella.claudia@inta.gob.ar
Laura Mendez: mendez.laura@inta.gob.ar
Federico Blanco: blanco.federico@inta.gob.ar
Maria Verónica Bianco: bianco.maria@inta.gob.ar
Walter Severina: walterseverina@hotmail.com
Alicia Alito: alicia.alito@gmail.com
Maria de los Ángeles Pando: mpando@fmed.uba.ar
Mahavir Singh: info@lionex.de

Ralph Spallek: rsp@lionex.de

Fernando Paolicchi: paolicchi.fernando@inta.gob.ar

Fabiana Bigi: bigi.fabiana@inta.gob.ar

Angel Cataldi: cataldi.angeladrian@inta.gob.ar

[§]Corresponding author: María Emilia Eirin. de los Reseros y las Cabañas s/n, Instituto de Biotecnología, CICVyA, INTA, Castelar, Buenos Aires, Argentina. Post Code 1686, Telephone +54-11-4621-1447 ext 109; Fax: +54-11-4621-0199; e-mail: eirin.maria@inta.gob.ar

SUMMARY

Bovine tuberculosis (bTB) is a common zoonotic disease, caused by *Mycobacterium bovis* (*M. bovis*), responsible for significant economic losses worldwide. Its diagnosis is based on the detection of cell mediated immunity under the exposure to protein purified derivative tuberculin (PPD), a complex and poorly characterized reagent. The cross-reactivity to non-tuberculous mycobacterium species (false-positive results) has been crucial to develop a more proper antigen. In the present study, we selected six *M. bovis* Open Reading Frames (Mb1992, Mb2031c, Mb2319, Mb2843c, Mb2845c and Mb3212c) by *in-silico* analysis and evaluated them in experimental and natural infection; none of these antigens had been previously assessed as diagnostic antigens for bTB. The reactivity performance was tested in animals with both positive and negative Tuberculin Skin Test (TST) results as well as in cattle infected with *Mycobacterium avium* subesp. p*aratuberculosis* (MAP). The six recombinant antigens individually induced an IFN-γ response, with overall responder frequency ranging from 18.3 to 31%. Mb2845c was the most valuable antigen with the potential to discriminate TST-positive cattle from either TST-negative or MAP infected animals. Mb2845c showed similar performance to that observed with ESAT-6 and PPD-B among TST and MTC specific-PCR positive animals, although this result needs to be proven in further studies with a higher sample size. Our data confirm the feacibility to implement bioinformatic screening tools and suggest Mb2845c as a potential diagnostic antigen to be tested in protein cocktails to evaluate their contribution to bTB diagnosis.

KEYWORDS

Bovine; tuberculosis; diagnosis; new antigens; IGRA.

TEXT

1. INTRODUCTION

Mycobacterium bovis (*M. bovis*) is the etiological agent of bovine tuberculosis (bTB) and a member of the *Mycobacterium tuberculosis* complex (MTC), which comprises the causative agents of tuberculosis (TB) in different animal species [1]. Although cattle are the primary host for *M. bovis* infection, wild animals can act as a reservoir. Bovine TB is spread worldwide and, despite the control efforts and extensive eradication programs based on the test-and-slaughter strategies, remains an animal health concern in several countries [2,3,4]. Moreover, bTB persists as a zoonotic disease in certain areas, especially in diary regions [5,6].

The diagnostic test for the control and surveillance of bTB that is used worldwide is the Tuberculin Skin Test (TST), which is based on the *in vivo* intradermal inoculation of purified protein derivative from *M. bovis* (PPD-B). An available ancillary test quantifies the *in vitro* IFN-γ release in a whole blood culture under PPD-B stimulation (IGRA) [7]. PPD-B has been largely used for bTB diagnosis as primary diagnostic reagent. However, false-positive reactions can occur because it has components common to other mycobacterium species such as non tuberculous mycobacteria (NTM) [8,9,10].

Two immunodominant and specific proteins, ESAT-6 and CFP-10, have been extensively studied. Both proteins are absent from many NTM and from the *M. bovis* Bacille Calmette Guerin (BCG) strain. More recently, Sidders et al. evaluated the use of Rv3615c in combination with ESAT-6 and CFP-10 and demonstrated that Rv3615c significantly increased diagnostic sensitivity without reducing specificity in BCG-vaccinated populations [11]. Other antigens have been assayed alone or in conjunction with ESAT-6 and CFP-10, in order to study possible optimization of bTB diagnosis [12,13,14]. Furthermore, several studies have been focused in the deciphering of the PPD molecular composition for the formulation of a more proper antigen cocktail. Thus, proteomic approaches have led to the identification of approximately 300 protein components [15,16], which represent about 9% of the total mycobacterium proteome [17].

In the present study we conducted an *in silico* approach to screen the *M. bovis* orpheome and thus search for new and potential candidates, by comparing their performance to a single recombinant antigen ESAT-6 and the cocktail PPD-B in the *in vitro* IFN-γ diagnostic test. Six *M. bovis* uncharacterized proteins were selected, including Mb1992, Mb2031c, Mb2319, Mb2843c, Mb2845c and Mb3212c; none of them have been previously identified in PPD-B [15,16]. Our data showed Mb2845c as the most valuable antigen with the potential to discriminate TST-positive cattle from either TST negative or MAP infected animals.

2. MATERIAL AND METHODS

2.1. Ethics Statement

Animal assays were performed inside the biosafety facilities of the National Institute of Agricultural Technology (INTA), Argentina. This study was carried out under the regulations of the Institutional Animal Care and Use Committee (CICUAE) of CICVyA-INTA in agreement with the European Union Laws for protection of experimental animals. Also, these experiments were authorized by SENASA and the National Consultant Commission of Agricultural Biotechnology (CONABIA). The ethical approval for this study was obtained from CICUAE (nu 18/2011).

2.2. Cattle population

2.2.1. Experimentally infected animals

Five Holando Argentino calves (three to four months old, males) were experimentally infected with a wild type *M. bovis* strain. Details of infection, inoculation and necropsy procedures were previously described [18].

2.2.2. Naturally infected animals

Selection of the population was based on the reference test for diagnosis of bTB: the Tuberculin Skin Test (TST). A total of 121 animals of Holando Argentino breed, which were classified in three groups, were studied. TST-positive animals (n=77) belonged to three bTB infected herds with a previous history of bTB cases confirmed by microbiological culture and PCR [19]. In the present study bTB infection was evidenced in TST-positive animals by a MTC-specific PCR (IS6110) in nasal swabs (n=6) and confirmed by necropsy and microbiological culture in two animals.

The TST-negative group (n=28) included bovines from dairy herds that had been free of bTB for at least the last five years. A third group of animals with MAP infection, which has been confirmed by ELISA, cultures (from feces) and PCR [20], was included in this study (n=16).

2.3 Diagnosis

2.3.1. Tuberculin Skin Testing

The caudal fold tuberculin skin test was performed to evaluate *M. bovis* infection. Animals were intradermally injected with 0.1 mg of PPD-B (CEVA Santé Animale; Argentina) and the thickness of the caudal fold was then measured by using calipers before and 72 hours after injection. An increase ≥ 5mm in the skin thickness was considered positive, as described by SENASA (Resolution 128/12) [21]. In addition, serology for MAP antibodies was assessed in positive and TST-negative animals by

an in-house ELISA [22]. Negative results for MAP serology were considered to include TST-positive and negative samples in the study.

2.3.2. IFN-γ Release Assay (IGRA)

Animals were also screened by IGRA based PPD-B by using a commercial bovine IFN-γ -microplate enzyme-linked immunosorbent assay (Prionics, Shlieren, Zurich, Switzerland). Whole blood (200µl aliquots) was dispensed into individual wells in a 96-well plate. IFN-γ release was measured after stimulation with tested and control antigens. Thus, 4 µg/ml of all tested antigen, sterile PBS 1X, 4µg/ml ESAT-6 (Lionex GmbH, Braunschweig, Germany), 20µg/ml of *M. bovis* PPD-B, or *M. avium* PPD-A (Prionics, Shlieren, Zurich, Switzerland) and 1µg/ml of pokeweed mitogen (Sigma-Aldrich, USA) were used. Blood from six animals with a positive MTC-specific PCR (IS6110) result were stimulated with PPD-B, PPD-A, ESAT-6, sterile PBS 1X, Mb2845c alone (4, 10 or 20µg/ml), or in a cocktail with PPD-B (20µg/ml: 1/3 Mb2845c and 2/3 commercial PPD-B). The plasma were harvested and stored at -80ºC. A result was considered positive if the Optical Density at 450nm (OD_{450}) with antigens minus the OD_{450} without antigens was ≥ 0.1. For comparative analysis, a result was considered positive if the PPD-B OD_{450} minus the PPD-A OD_{450} was ≥ 0.1 and the PPD-B OD_{450} minus the unstimulated control OD_{450} was ≥ 0.1.

2.3.3. Molecular diagnosis

DNA extraction from nasal swabs was performed as previously described [13] and MTC-specific PCR (IS6110) was carried out [19].

2.4. Selection of antigens

A computational search was performed by using an integrated bioinformatic comparative tool (GenoList; www.genolist.pasteur.fr) [23] to identify specific genes from MTC. A reference list was generated, including *M. bovis* strains AF2122/97, BCG and Pasteur 1173P2 and *M. tuberculosis* strains CDC1521, H37Ra and H37Rv. An additional query list included *Mycobacterium* genomes outside the MTC: *M. abscessus* strain ATCC 19977, *M. avium* strain 104, *M. gilvum* strain PYR-GCK, *M. leprae* strain TN, *M. marinum* strain M, *M. paratuberculosis* strain K-10, *M. smegmatis* strain MC2 155, *M. ulcerans* strain agy99 and *M. vanbaalenii* strain PYR-1. A cut-off value of 90% selection and 15% of exclusion were used. A blast search was performed with the selected ORFs (www.ncbi.nlm.nih.gov/BLAST) to exclude those present in other organisms. The selected candidates were then analyzed by using sets of overlapping peptides with the NetMHCII 2.2 Server (www.cbs.dtu.dk/services/NetMHCII) in the context of the BoLA class II molecule. The median affinity constant value for each ORF was obtained thus establishing an order of merit in terms of

159 immunogenicity. The molecular weight of the selected ORFs was predicted by using Uniprot
160 (http://www.uniprot.org/).

161 **2.5. Bacterial growth and DNA isolation**

162 The *M. bovis* AN5 strain was grown in Dorset-Henley medium for 28 days at 37ºC and harvested by
163 centrifugation. The DNA was extracted [24] and used as template to amplify and then clone the
164 selected ORFs.

165 **2.6. Cloning, expression and purification of the recombinant antigens**

166 The gene sequences were amplified with *Pfx* DNA polymerase (Invitrogen), cloned into a
167 pENTR™/D-TOPO® vector and sub-cloned in the pDEST17 vector (Gateway System, Invitrogen).
168 The recombinant proteins tagged with histidine (Hisx6) were expressed in chemically competent
169 BL21-AI *Escherichia coli* cells (Invitrogen) and recovered from inclusion bodies. The induced
170 bacterial culture (200ml) was harvested by centrifugation and resuspended in 4 ml of lysis buffer (50
171 mM Tris-HCL pH8.0, 100 mM NaCl, 0.5% Triton X-100) by sonication (4ºC, 3 min, 1 second
172 interval, output 5). DNAse (0.01 mg/ml) and Lysozyme (0.1 mg/ml) were added at room temperature
173 (20 minutes) and subsequently centrifugation was performed (4500g, 15 minutes, 4ºC). The pellet
174 was resuspended in lysis buffer and sonication and centrifugation was performed as above. Inclusion
175 bodies were resuspended in 4ml of wash buffer (50m M Tris-HCL pH8.0, 100 mM NaCl),
176 centrifuged (4500g, 15 minutes, 4ºC) and resuspended in 500 µl of wash buffer with sonication (10
177 seconds). Suspension was dissolved drop wise into urea 8M and the supernatant was recovered at
178 maximum speed centrifugation. Proteins were purified by affinity chromatography on an Agarose-
179 Nickel column (Qiagen) under denaturing conditions following the manufacturer's instructions.
180 Dialysis was performed (molecularporous membrane Spectra/Por) in stirring urea 4M, 2M, 1M,
181 0.5M and 0.25M solution at 4ºC. A final dialysis step was carried out at 4ºC overnight in stirring
182 fresh PBS 1X buffer. Proteins were quantified by Bradford protein assay (BioRad).

183 **2.7. SDS-PAGE Coomassie blue staining and Western Blot analysis**

184 Protein expression was confirmed by Coommassie blue stained polyacrylamide gel and Western blot
185 assay. Blots were probed with a mouse anti-his monoclonal antibody (GE) at a 1:3000 dilution. As a
186 secondary antibody, alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma) was
187 used at a dilution of 1:30,000. For detection, BCIP/NBT colour development substrates (Promega,
188 Madison, WI) were used.

189

2.8. Statistical analysis

Responder frequencies for every antigen; including PPD-B and PPD-A, were calculated as the percentage of animals in which the OD450 antigen minus the OD450 nil was ≥ 0.1. Statistical comparison and frequencies were performed with EpiDat 3.0 version software (Xunta de Galicia, OPS-OMS). Statistical difference in the magnitude response (ΔOD_{450}) between those induced by control and tested antigens was determined by using ANOVA. P values computed ≤ 0.05 were reported as measures of statistical significance. Graphics were constructed by using GraphPad prism 5.03 software (GraphPad Software, San Diego California USA).

3. RESULTS

3.1. Selection of antigens

Fifteen completely sequenced *Mycobacterium* genomes were analyzed comparatively. A total of 44 ORFs specific to MTC members were identified. Nine of these ORFs were excluded for sharing similarity to *M. kansasii* proteins, thus resulting in a final selected group of 35 ORFs.

The median affinity constant in terms of immunogenicity was calculated based on the identification of peptide binding core motifs in the full linear aminoacid sequence. Seven ORFs (Mb1753c, Mb1979c, Mb1990, Mb2749, Mb2851c, Mb3135 and Mb3458c) did not show any antigenic determinant. Therefore, the final candidate group comprised 28 ORFs: Mb0061, Mb0613c, Mb0627c, Mb0629, Mb1015, Mb1024, Mb1077c, Mb1401, Mb1700c, Mb1764c, Mb1992, Mb2031c, Mb2039, Mb2049c, Mb2166c, Mb2298, Mb2317, Mb2319, Mb2627, Mb2842c, Mb2843c, Mb2844c, Mb2845c, Mb2847c, Mb2848c, Mb3212c, Mb3412c and Mb3558c. Their sequences exhibited an amino acid homology ranging from 99.2 to 100% with their orthologous proteins in *M. tuberculosis*.

Six candidates were selected for further analysis (Mb1992, Mb2031c, Mb2319, Mb2843c, Mb2845c and Mb3212c) because they exhibited the highest predicted immune score. All proteins differed in the number of predicted immunological epitopes in decreasing order as follows: Mb3212c, Mb2031c, Mb1992, Mb2319, Mb2843c and Mb2845c (Table 1).

3.2. Reactivity of the recombinant candidates in experimentally infected cattle

The selected candidates were first studied by analyzing the blood from five experimentally infected calves with confirmed *M. bovis* infection by necropsy and microbiological culture for *M. bovis*. Mb1992 and Mb2845c antigens induced IFN-γ release in the five animals. Mb2319 exhibited a positive response in three out of five infected animals, whereas Mb3212c stimulated IFN-γ release only in one animal. Mb2031c and Mb2843c did not induce IFN-γ release in any of the blood samples. In all cases, PPD-B elicited a higher response than the recombinant proteins followed by ESAT-6. We also analyzed the reactivity under stimulation with PPD-A (Figure 1).

3.3. Reactivity of the recombinant candidates in naturally infected cattle

IGRA based PPD-B was carried out in order to classify the studied animals as bTB positive or negative by an ancillary diagnostic test (Bovigam, Prionics). A total of 77 TST-positive animals were evaluated, of which 66% (51/77) were positive and 34% (26/77) resulted negative. To compare the antigenic performance between the PPD-B, PPD-A, ESAT-6 and the six proteins codified by the selected *M. bovis* ORFs, we tested their individual ability to stimulate IFN-γ release in 71 TST-positive animals. The responder frequencies were similar in the recombinant tested proteins ($p >$

0.05) and statistically lower than the responder frequency obtained with PPD-B ($p < 0.0001$): Mb2319 (31%), Mb1992 (29.5%), Mb3212c (25%), Mb2031c and Mb2843c (24%), and Mb2845c (18.3%) in the TST-positive group (Figure 2). The magnitude of the IFN-γ response for each antigen is shown in supplementary material (Figure 1, Supplementary Data).

The *M. bovis* infection was confirmed in two animals (by necropsy and microbiological culture): one of them with reactivity to ESAT-6, and the other animal exhibited reactivity to ESAT-6, Mb2319 and Mb3212 (tested individually).

Then, the analysis was focused on the IGRA based PPD-B nonreactive samples (n=24), which were named as "discordant" because they showed a positive TST and a negative IGRA based PPD-B result. Twelve discordant samples were positive by IGRA under the stimulation of the individual recombinant candidates', six of them in conjunction to ESAT-6 and the remaining six samples only under stimulation with the uncharacterized antigens. A variable reactivity pattern was observed, thus suggesting differences in the abilities of the animals' immune systems to recognize antigens (Table 2).

Of the total TST-positive samples (n=77), 44 (57%) showed a positive IFN-γ release under ESAT-6 stimulation, whereas 33 (43%) remained negative. The proportion of reactors under ESAT-6 stimulation was significantly higher than the one obtained with the uncharacterized antigens ($p < 0.0005$). However, among the ESAT-6 negative samples, all the recombinant antigens induced IFN-γ release in different, but not significant ($p > 0.05$), proportions (Data not shown). Most of these ESAT-6 non reactive samples were negative under PPD-B stimulation by IGRA.

M. bovis diagnosis investigation underlies the discovery of new diagnostic reagents in order to discriminate between a real infection (caused *by M. bovis*) and other infections (caused by MNT species). For this purpose, the reactivity of recombinant candidates was further studied in MAP infected cattle. The individual recombinant candidates, with the exception of Mb2845c, showed a positive response by IGRA under single antigen stimulation as follows: Mb1992 (1/16), Mb2031c (1/16), Mb2319 (5/16), Mb2843 (1/16) and Mb3212c (1/16).

With the samples from TST-negative cattle, Mb2845c did not induce a positive response by IGRA as well as Mb2031c and Mb3212c, whereas the remaining antigens induced IFN-γ release in a few animals (Figure 2). Our data suggest Mb2845c as the most relevant protein with the potentiality to avoid a possible false-positive IGRA result.

A group of six animals with TST and MTC-specific PCR positive results was studied in order to evaluate the antigen Mb2845c. A responder frequency of 50% (3/6) was observed for Mb2845c either alone (at 10μg/ml and 20μg/ml, with no positive reactors at 4μg/ml) or in a protein cocktail with PPD-B. This responder frequency was similar to that for the single recombinant ESAT-6 protein

266 (83.3%, p = 0.54) and for the PPD-B cocktail (66.7%, p=1). It is interesting to note that one of the
267 Mb2845c positive animal; also positive with ESAT-6, exhibited a discordant result for PPD-B (TST
268 positive/IGRA negative).
269

4. CONCLUSIONS

In this study we identified six specific *Mycobacterium bovis* proteins by bioinformatics comparisons of the genomes of different mycobacteria and by prediction of binding to the BoLA class II molecule. None of the analyzed proteins has been previously tested for the diagnosis of bTB in cattle.

The bioinformatics screening tool implemented in this study led to the selection of uncharacterized *M. bovis* ORFs whose proteins exhibited immunogenic reactivity in experimental and natural bTB conditions.

The criteria established in the *in silico* search to select ORFs exclusive to members of the *Mycobacterium tuberculosis* Complex was confirmed with previously published data about on orthologous genes in *M. tuberculosi*s.

In spite of the search criteria used here, Mb1992, Mb2031c, Mb2319, Mb2843c and Mb3212c exhibited reactivity in TST-negative and/or MAP-infected cattle; which suggests cross-reactivity between these uncharacterized antigens and those in NTM species.

A highest performance for PPD-B and the single recombinant ESAT-6 antigen in comparison to the tested candidates was confirmed.

The results obtained in experimental and natural infected animals suggest to Mb2845c as a valuable protein for diagnostic testing that need to be further explored in order to characterize sensitivity/specificity as well as the optimal cut-off associated to this recombinant antigen, either alone or as part of antigenic cocktails.

5. DISCUSSION

In the present study, six uncharacterized *M. bovis* proteins were assayed by the IGRA in their ability to induce IFN-γ release in experimental and natural *M. bovis* infection. Although the six candidates were not previously tested as diagnostic antigens, published data on their orthologous genes in *M. tuberculosis* support the bioinformatics selection. For instance, Rv2819c (Mb2843c ortholog) is specific to the *M. tuberculosis complex* species [25], whereas Mb2843c and Mb2845c exhibit 100% homology to CRISPR-associated proteins Csm5 (Rv2819c) and Cmr4 (Rv2821c), only described in *M. tuberculosis* and *M. bovis* [26].

PPD-B has been the most widely used reagent for bTB diagnosis [27]. However, the description of specific reagents provided evidences about the usefulness and advantages to use a more proper antigenic cocktail to avoid non-specific reactors [8,9,10]. MAP and other non-pathogenic mycobacterium infections have been pointed out as a cause of cross-reactivity reactions. In a previous study, the authors reported higher IFN-γ responses for PPD-A than for PPD-B in TST-negative animals from Northern Ireland, Mexico and Argentina, and concluded that this result suggest an exposure to environmental mycobacterials [10]. Similarly, our data showed a median OD_{450} for PPD-A higher than for PPD-B in the TST-negative group, although these values were not significantly different ($p > 0.05$).

Mb1992, Mb2319 and Mb2843c induced reactivity in some TST negative cattle. Considering the negativity for MAP serology of these animals, the results could be explained by a possible non-pathogenic environmental mycobacterium infection that would induce an immune cross-reactivity with the recombinant antigens. Some samples from the MAP group reacted to Mb1992, Mb2031c, Mb2319, Mb2843 and Mb3212c stimulation (positive response by IGRA). Accordingly, these data suggest that five out of six tested proteins may share epitopes with NTM species in spite of the specificity of the bioinformatics selection criteria. Nevertheless, it offers a useful tool for short-term results at a low cost, especially when the input to be analyzed is as vast as the *M. bovis* orpheome [17].

Previous studies showed ESAT-6 responder frequency ranging from 66% to 78% in European cattle herds. In our country, a 78% of reactors were described in high prevalence bTB herds [10]. Although in the present study a lower responder frequency out of the reported interval was obtained for ESAT-6 (57%), the frequency was similar to those previously described ($p=0.056$). Thus, the different proportions observed in the studied regions could be attributed to factors such as variation in prevalence rates at the sampling site and the host genetic background, among others.

Rv3615c has been shown as an antigen that did not overlap ESAT-6/CFP-10 immune reactivity in the IGRA and thus a potential candidate to increase ESAT-6/CFP-10 cocktail sensitivity in naturally infected cattle [11,28,29]. In this study, among all tested proteins, Mb2845c seemed to be the most promising antigen, with the potential to avoid cross reactivity with NTM species. However, in contrast to Rv3615, Mb2845c response overlapped the ESAT-6 reactivity. with the exception of a discordant sample that only reacted upon Mb2845c stimulation.

In the case of Mb1992 the immune reactivity observed in the bovine host, either in the experimental or natural *M. bovis* infection, confirmed its cell mediated inducer nature, which has been previously described for its ortholog in *M. tuberculosis* Rv1957 (which shares 100% homology with Mb1992), in humans. However, in contrast to that for the current study where the Mb1992 responder frequency was significantly lower than that for ESAT-6, the study in humans showed that the response for Rv1957 was similar to that for ESAT-6 [30].

A thirth of the TST-positive samples showed a PPD-B based discordant result (TST positive/ IGRA negative). This pattern was also detected in two out of the six positive animals for MTC-specific IS6110 PCR. Although PCR is not considered a “gold standard” technique, it represents an evidence of infection and thus this result supports the idea that these two discordant animals would be infected but no reactors to the *in vitro* diagnostic test. Furthermore, it was reported that the sensitivity of the *in vitro* IFN-γ test may be reduced with extended periods from blood collection to culture [31]. In the present study, the TST-positive samples were collected from two different geographical areas and samples differed in the time period from collection to culture. However, OD_{450} PKW mitogen was similar in these two groups ($p > 0.05$) (Figure 2, Supplementary data). This finding suggests that PBMC viability would not be the reason for the discordance observed.

Frequently, evaluations of diagnostic antigens are accompanied by statistical tests such as receiving operating curves (ROC) to estimate their performance. Indeed, samples should be assessed by the accepted ‘gold standard’ to confirm infection/disease (culture of *M. bovis* from tissues or visible lesions). Considering that only two animals included in the current study were slaughtered, ROC was not used to statistically evaluate the performance of recombinant candidates.

Mb2845c was the most valuable antigen because did not induce a positive IFN-γ release neither in TST-negative nor MAP groups. Results obtained in experimental and natural infected animals suggest that this antigen has properties that need to be further explored in order to characterize sensitivity/specificity as well as the optimal cut-off associated to this recombinant antigen, either alone or as part of antigenic cocktails.

ACKNOWLEDGEMENTS

The authors thank to veterinarians Alejandro Abdala for gently providing samples and Sergio Garbaccio for assisting with the experimental infection. We gratefully acknowledge the continuous technical support provided by Valeria Rocha. The authors also thank Julia Sabio y García for the English revision of the manuscript and to Luis Fernandez for providing bibliography. MEE, FB, MP, FB and AC are fellows of the National Research Council of Argentina (CONICET).

The present study was supported by the following fundings sources: Instituto Nacional de Tecnología Agropecuaria AEBIO243512, PNBIO1131034 and UNMdP AGR 422/13.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

1. van Ingen J, Rahim Z, Mulder A, Boeree MJ, Simeone R, Brosch R, van Soolingen D. Characterization of Mycobacterium orygis as M. tuberculosis complex subspecies. Emerg Infect Dis 2012; 18:653-5.
2. Abernethy DA, Upton P, Higgins IM, McGrath G, Goodchild AV, Rolfe SJ, Broughan JM, Downs SH, Clifton-Hadley R, Menzies FD, et al. Bovine tuberculosis trends in the UK and the Republic of Ireland, 1995-2010. Vet Rec 2013; 172:312.
3. de la Cruz ML, Perez A, Bezos J, Pages E, Casal C, Carpintero J, Romero B, Dominguez L, Barker CM, Diaz R, et al. Spatial dynamics of bovine tuberculosis in the Autonomous Community of Madrid, Spain (2010-2012). PLoS One 2014; 9:e115632.
4. Byrne AW, White PW, McGrath G, J OK, Martin S. Risk of tuberculosis cattle herd breakdowns in Ireland: effects of badger culling effort, density and historic large-scale interventions. Vet Res 2014; 45:109.
5. Torres-Gonzalez P, Soberanis-Ramos O, Martinez-Gamboa A, Chavez-Mazari B, Barrios-Herrera MT, Torres-Rojas M, Cruz-Hervert LP, Garcia-Garcia L, Singh M, Gonzalez-Aguirre A, et al. Prevalence of latent and active tuberculosis among dairy farm workers exposed to cattle infected by Mycobacterium bovis. PLoS Negl Trop Dis 2013; 7:e2177.
6. de Kantor IN, Ritacco V. An update on bovine tuberculosis programmes in Latin American and Caribbean countries. Vet Microbiol 2006; 112:111-8.
7. Rothel JS, Jones SL, Corner LA, Cox JC, Wood PR. A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. Aust Vet J 1990; 67:134-7.
8. Aranaz A, De Juan L, Bezos J, Alvarez J, Romero B, Lozano F, Paramio JL, Lopez-Sanchez J, Mateos A, Dominguez L. Assessment of diagnostic tools for eradication of bovine tuberculosis in cattle co-infected with Mycobacterium bovis and M. avium subsp. paratuberculosis. Vet Res 2006; 37:593-606.
9. Michel AL. Mycobacterium fortuitum infection interference with Mycobacterium bovis diagnostics: natural infection cases and a pilot experimental infection. J Vet Diagn Invest 2008; 20:501-3.
10. Aagaard C, Govaerts M, Meikle V, Gutierrez-Pabello JA, McNair J, Andersen P, Suarez-Guemes F, Pollock J, Espitia C, Cataldi A. Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity. Prev Vet Med 2010; 96:161-9.

11. Sidders B, Pirson C, Hogarth PJ, Hewinson RG, Stoker NG, Vordermeier HM, Ewer K. Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the Mycobacterium tuberculosis complex. Infect Immun 2008; 76:3932-9.

12. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci 2006; 81:190-210.

13. Aagaard C, Govaerts M, Meikle V, Vallecillo AJ, Gutierrez-Pabello JA, Suarez-Guemes F, McNair J, Cataldi A, Espitia C, Andersen P, et al. Optimizing antigen cocktails for detection of Mycobacterium bovis in herds with different prevalences of bovine tuberculosis: ESAT6-CFP10 mixture shows optimal sensitivity and specificity. J Clin Microbiol 2006; 44:4326-35.

14. Vordermeier M, Gordon SV, Hewinson RG. Mycobacterium bovis antigens for the differential diagnosis of vaccinated and infected cattle. Vet Microbiol 2011; 151:8-13.

15. Cho YS, Dobos KM, Prenni J, Yang H, Hess A, Rosenkrands I, Andersen P, Ryoo SW, Bai GH, Brennan MJ, et al. Deciphering the proteome of the in vivo diagnostic reagent "purified protein derivative" from Mycobacterium tuberculosis. Proteomics 2012; 12:979-91.

16. Prasad TS, Verma R, Kumar S, Nirujogi RS, Sathe GJ, Madugundu AK, Sharma J, Puttamallesh VN, Ganjiwale A, Myneedu VP, et al. Proteomic analysis of purified protein derivative of Mycobacterium tuberculosis. Clin Proteomics 2013; 10:8.

17. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C,Monsempe C, et al. The complete genome sequence of Mycobacterium bovis. Proc Natl Acad Sci U S A 2003 ;100(13):7877-82.

18. Blanco FC, Bianco MV, Garbaccio S, Meikle V, Gravisaco MJ, Montenegro V, Alfonseca E, Singh M, Barandiaran S, Canal A, et al. Mycobacterium bovis Deltamce2 double deletion mutant protects cattle against challenge with virulent M. bovis. Tuberculosis (Edinb) 2013; 93:363-72.

19. Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon JL, Gicquel B. Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. J Clin Microbiol 1990; 28:2668-73.

20. Collins DM, Stephens DM, de Lisle GW. Comparison of polymerase chain reaction tests and faecal culture for detecting Mycobacterium paratuberculosis in bovine faeces. Vet Microbiol 1993; 36:289-99.

21. http://www.senasa.gov.ar/

22. Paolicchii FA, Zumarraga MJ, Gioffre A, Zamorano P, Morsella C, Verna A, Cataldi A, Alito A, Romano M. Application of different methods for the diagnosis of paratuberculosis in a dairy cattle herd in Argentina. J Vet Med B Infect Dis Vet Public Health 2003; 50:20-6.

23. Lechat P, Hummel L, Rousseau S, Moszer I. GenoList: an integrated environment for comparative analysis of microbial genomes. Nucleic Acids Res 2008; 36:D469-74.

24. Ausubel FM, Cannon FC. Molecular genetic analysis of Klebsiella pneumoniae nitrogen-fixation (nif) genes. Cold Spring Harb Symp Quant Biol 1981; 45 Pt 2:487-99.

25. Rindi L, Lari N, Garzelli C. Genes of Mycobacterium tuberculosis H37Rv downregulated in the attenuated strain H37Ra are restricted to M. tuberculosis complex species. New Microbiol 2001; 24:289-94.

26. He L, Fan X, Xie J. Comparative genomic structures of Mycobacterium CRISPR-Cas. J Cell Biochem 2012; 113:2464-73.

27. Monaghan ML, Doherty ML, Collins JD, Kazda JF, Quinn PJ. The tuberculin test. Vet Microbiol 1994; 40:111-24.

28. Whelan AO, Clifford D, Upadhyay B, Breadon EL, McNair J, Hewinson GR, Vordermeier MH. Development of a skin test for bovine tuberculosis for differentiating infected from vaccinated animals. J Clin Microbiol 2010; 48:3176-81.

29. Casal C, Bezos J, Diez-Guerrier A, Alvarez J, Romero B, de Juan L, Rodriguez-Campos S, Vordermeier M, Whelan A, Hewinson RG, et al. Evaluation of two cocktails containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the interferon-gamma assay for diagnosis of bovine tuberculosis. Prev Vet Med 2012; 105:149-54.

30. Gideon HP, Wilkinson KA, Rustad TR, Oni T, Guio H, Sherman DR, Vordermeier HM, Robertson BD, Young DB, Wilkinson RJ. Bioinformatic and empirical analysis of novel hypoxia-inducible targets of the human antituberculosis T cell response. J Immunol 2012; 189:5867-76.

31. Schiller I, Waters WR, Vordermeier HM, Nonnecke B, Welsh M, Keck N, Whelan A, Sigafoose T, Stamm C, Palmer M, et al. Optimization of a whole-blood gamma interferon assay for detection of Mycobacterium bovis-infected cattle. Clin Vaccine Immunol 2009; 16:1196-202.

Table 1. Predicted epitopes in the selected candidates in the context of Bovine Leucocyte Antigen (BoLA) type II.

ORF denomination	RV designation	Size (bp)	Number of Epitopes	Peptide cores	Median Affinity (nM)*	MW** (KDa)
Mb3212c	Rv3190c	1272	14	LRVDDAFML/ IRVDDRNIF	6	47.4
Mb2031c	Rv2008c	1332	7	ITIDEAQRI	6.3	48.4
Mb1992	Rv1957	550	8	YVYDLTGRL/ VDADPATIS	7.3	20.1
Mb2319	Rv2297	457	7	VRCDNPTLM	10	16.5
Mb2843c	Rv2819c	1134	6	VKLDPAKHR	23.2	42.3
Mb2845c	Rv2821c	715	5	VVRDPLSRL/ YGADTETFY	34.8	25.8

MW: Molecular Weight; Bp: base pair; *High binders peptide cores; **predicted *in silico*

Table 2. Reactivity patterns obtained for discordant samples showing that showed a positive IGRA upon stimulation with the recombinant candidates (n=12).

Mb1992	Mb2031c	Mb2319	Mb2843c	Mb2845c	Mb3212c	ESAT-6
						X*
X		X	X	X	X	X
				X	X	X
X			X	X	X	X
X	X	X	X	X	X	X
					X	
				X		
			X			
	X					
		X				
X	X	X			X	

*This pattern was observed in two samples.

Figure 1. *In vitro* evaluation of single recombinant antigens in experimentally infected cattle.

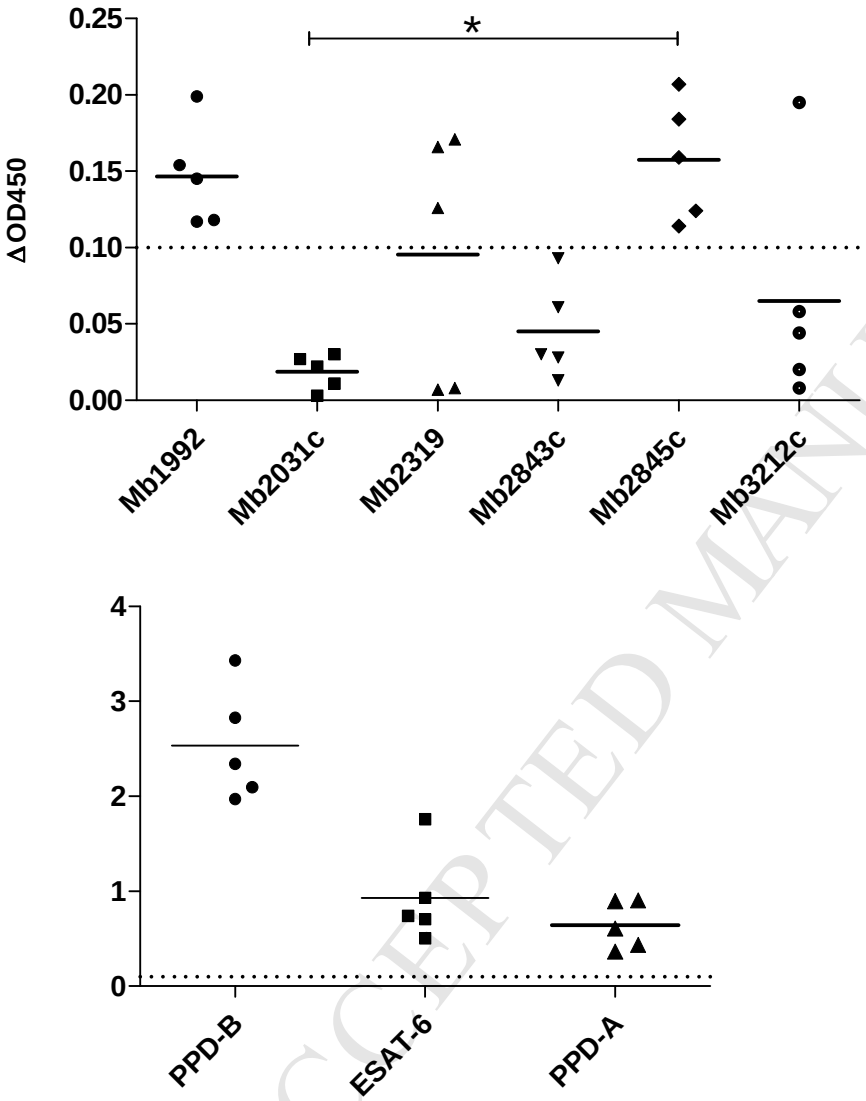
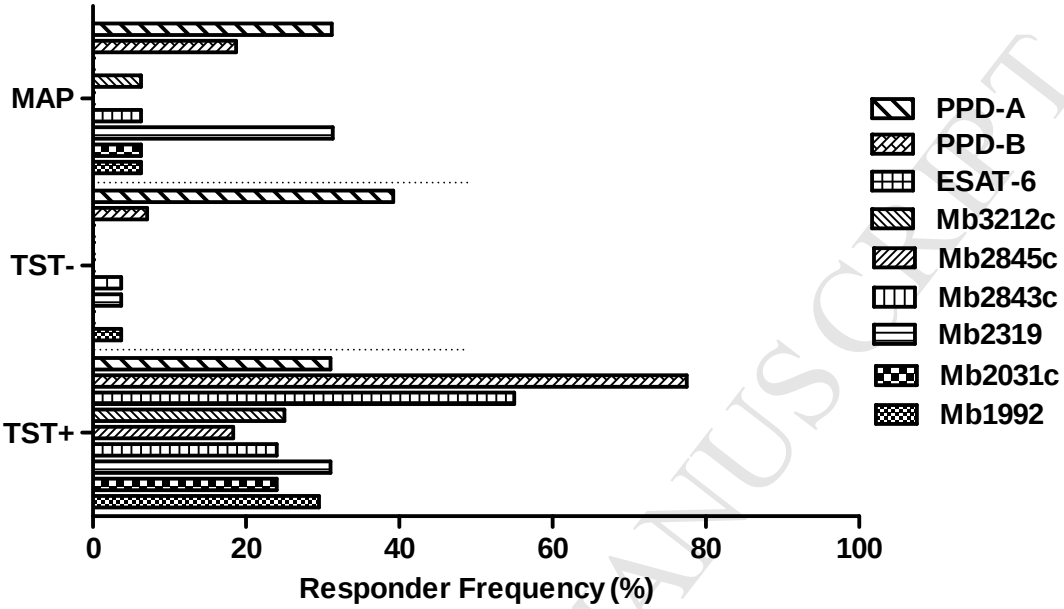


Figure 2. Responder frequencies observed with tested and control (PPD-B, PPD-A and ESAT-6) antigens by the IGRA in the TST-positive, TST-negative and MAP-infected animals.



CAPTIONS TO ILLUSTRATIONS

Title Table 1. Predicted epitopes in the selected candidates in the context of the Bovine Leucocyte Antigen (BoLA) type II.

Legend Table 1. The selected ORFs were screened to analyze the presence of epitopes and predict the immunological affinity to the BoLA class II molecule. Number of predicted peptide cores and the median affinity constant value (nM) for each ORF are shown. Molecular weight of each predicted protein was calculated by using Uniprot database.

Title Figure 1. *In vitro* evaluation of single recombinant antigens in experimentally infected cattle.

Legend Figure 1. Five calves were experimentally infected with the *M. bovis* 04-M303 strain and further screened (100 days post infection) to evaluate the IFN-γ release under stimulation with tested and control antigens by ELISA (Bovigam, Prionics). Data are shown as ΔOD450 (OD450 antigen minus OD450 nil). The horizontal line provides the mean (±SEM). Cut-off for positivity is indicated by dashed lines (ΔOD450 ≥ 0.1). Note that scale for Y axis differs for tested (Mb1992, Mb2031c, Mb2319, Mb2843c, Mb2845c and Mb3212c) and control antigens (PPD-B, PPD-A and ESAT-6). Statistical difference between responses induced by the tested antigens was determined by using ANOVA (*, $p < 0.05$).

Title Figure 2. Responder frequencies observed with tested and control (PPD-B, PPD-A and ESAT-6) antigens by the IGRA in the TST-positive, TST-negative and MAP-infected animals.

Legend Figure 2.

Six *in silico* selected antigens were screened in their ability to induce IFN-γ release in blood samples from TST-positive (TST+; n=71), TST-negative (TST-; n=28) and MAP-infected animals (MAP; n=16) by ELISA (Bovigam, Prionics). Responder frequencies for every antigen; including PPD-B, PPD-A and ESAT-6, were calculated as the percentage of animals in which the subtraction of OD450 antigen and OD450 nil was ≥ 0.1 for each studied group. PPD-B, PPD-A and ESAT-6 were included as reference antigens.

Title Table 2. Reactivity patterns obtained among discordant samples that showed a positive IGRA under the stimulation of the recombinant candidates.

Legend table 2.

Reactivity of 12 discordant samples (TST-positive/IGRA-PPDB negative) by IGRA after individual stimulation with the recombinant candidates. Interestingly, a group of six samples exhibited

reactivity against the recombinant antigens but not under stimulation with ESAT-6. X denotes a positive response with the specified antigen.

Title Supplementary Figure 1. Magnitud of the IFN-γ response for each tested and control antigens in the TST-positive, TST-negative and MAP infected animals.

Legend Supplementary Figure 1.

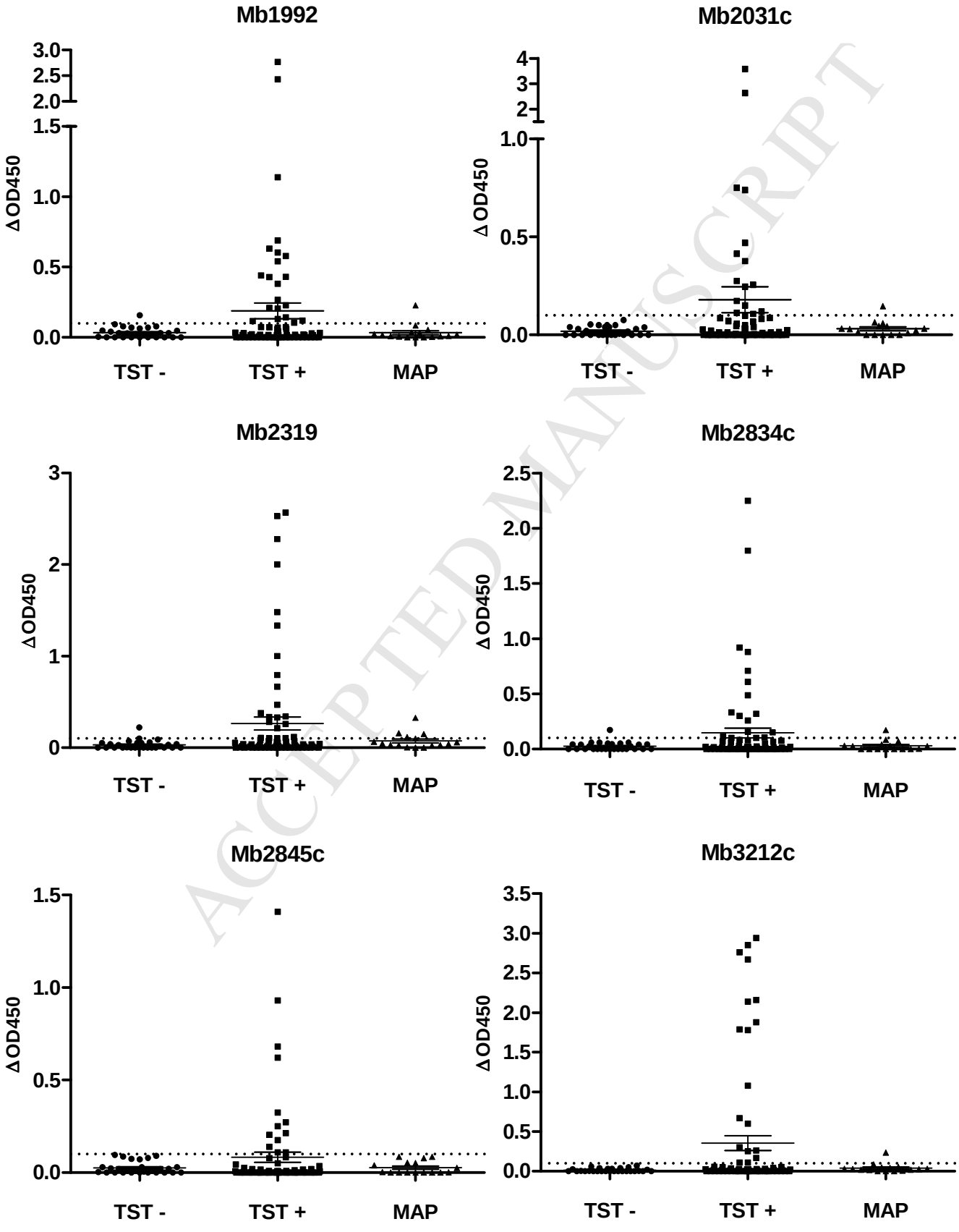
The *in vitro* capacity of the tested antigens (4 μg/ml) to induce IFN-γ was determined in blood from the three groups analyzed: TST-positive (TST+; n=71), TST-negative (TST-; n=28) and MAP infected animals (MAP; n=16). The responses induced by ESAT-6 (4 μg/ml), PPD-B or PPD-A (20 μg/ml) were also studied. The ΔOD450 (OD450 antigen minus OD450 nil) is represented by black circles for each animal. The horizontal line provides the mean and the standard error of the mean (SEM). A thereshold equal to ΔOD450 = 0.1 is shown for every antigen as an horizontal dashed lines.

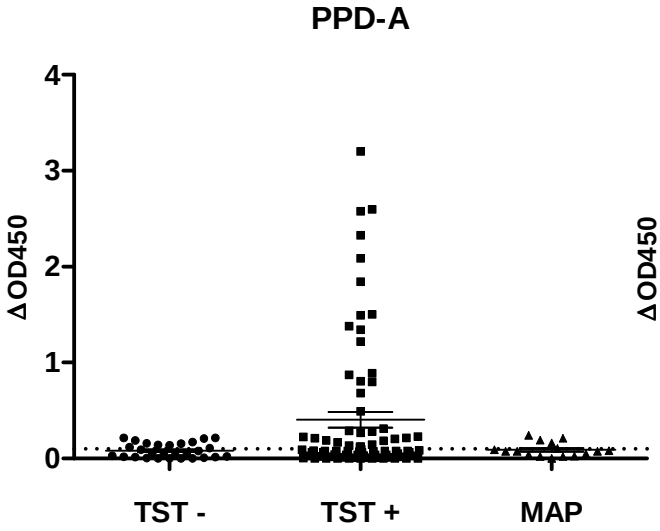
Title Supplementary Figure 2. IFN-γ release upon Pokewed mitogen stimulation in all the samples analyzed.

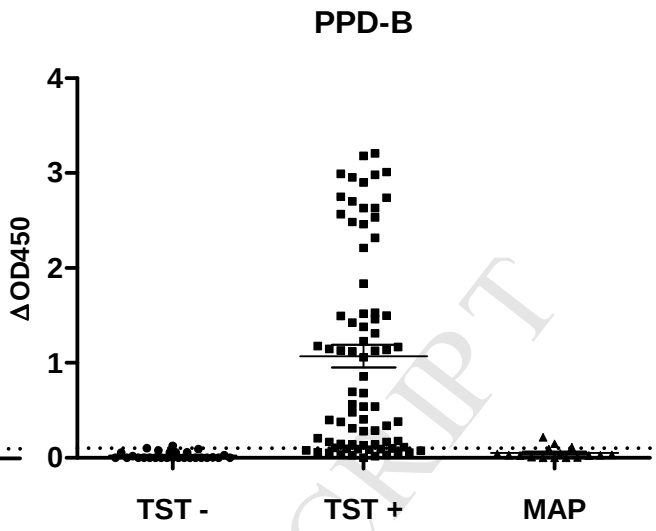
Legend Supplementary Figure 2.

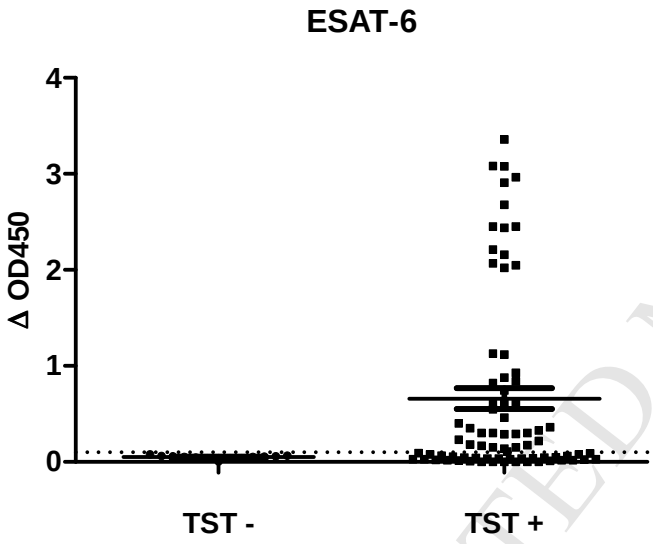
To examine if cell viability could be influenced by different periods from blood collection to culture, all samples were tested by IFN-γ realease upon a mitogen stimulation (1 μg/ml). TST-positive samples differed in the period from blood collection to culture: two fields were located to 8 hours (TST+ “far”, n=65) from the lab and the other to 3 hours (TST+ “near”, n=12) from the lab. TST-negative animals (TST-; n=28), MAP infected cattle (MAP; n=16) and experimentally infected cattle (EIC; n=5) were also included. The median ΔOD450 (OD450 PKW minus OD450 nil) is represented by bars. One way anova was used to statistically compare all groups, showing no differences among them ($p > 0.05$).

Supplementary Figure 1. Magnitud of the IFN-γ response for each tested and control antigens in the TST-positive, TST-negative and MAP infected animals.









Supplementary Figure 2. IFN-γ release upon Pokewed mitogen stimulation in all the samples analyzed.