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ORIGINAL ARTICLE

Evaluation of the use of recombinant proteins of *Mycobacterium bovis* as antigens in intradermal tests for diagnosis of bovine tuberculosis#

Evaluación de uso de las proteínas recombinantes de *Mycobacterium bovis* como antígenos en las pruebas intradérmicas para el diagnóstico de la tuberculosis bovina

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RESUMEN

La prueba itradérmica de la piel con derivados de proteína (PPD) purificada a partir de *Mycobacterium bovis* se ha utilizado para el diagnóstico de la tuberculosis bovina. Sin embargo, debido a la especificidad subóptima de esta mezcla de proteínas, la mejora de las pruebas basadas en antígenos específicos definidos son necesarias. En el presente estudio, las proteínas recombinantes de *M. bovis* se evaluaron como antígenos en la prueba cutánea. Entre estas, EsxI, Mb0143, PE5 y PE13 son antígenos probados por primera vez en el ganado. Bovinos sensibilizados y no sensibilizados con la cepa AN5 inactivada de *M. bovis* fueron inyectados simultáneamente con cada proteína recombinante, un cóctel con todas las proteínas recombinantes, PPD de *M. bovis*, PPD de *M. avium* y salina. Solo las proteínas EsxI, Mb0143 y PE5 fueron capaces de diferenciar ganado sensibilizado de lo no sensibilizado cuando se usó 320 µg de proteína y la lectura realizada 24 horas después de la inyección. EsxI y PE5 exhibieron un nivel de sensibilidad de 83,33% y una especificidad de 100% y 80%, respectivamente. Los resultados del presente estudio sugieren que las proteínas recombinantes tienen el potencial de ser evaluadas como antígenos en las pruebas cutáneas en el ganado.

Palabras clave: tuberculosis bovina, prueba intradérmica, EsxI, PE5.

SUMMARY

The skin test with purified protein derivatives (PPD) from *Mycobacterium bovis* has been used for the diagnosis of bovine tuberculosis. However, due to the suboptimal specificity of this protein mixture, improved tests based on defined specific antigens are needed. In the present study, recombinant proteins from *M. bovis* were evaluated as antigens in the skin test. Among these proteins, EsxI, Mb0143, PE5, and PE13 are antigens tested for the first time in skin test on cattle. Sensitised and non-sensitised cattle to the inactivated AN5 strain of *M. bovis* were simultaneously injected with each recombinant protein, a cocktail with all recombinant proteins, *M. bovis* PPD, *M. avium* PPD and saline. Only the proteins EsxI, Mb0143 and PE5 were able to differentiate sensitised and non-sensitised cattle when 320 µg of protein was used and the reading done 24 hours post-injection. EsxI and PE5 exhibited a sensitivity level of 83.33% and specificity of 100% and 80%, respectively. The results of the present study suggest that the recombinant proteins have potential to be assessed as antigens in skin tests in cattle.

Key words: bovine tuberculosis, recombinant proteins, skin test, EsxI, PE5.

INTRODUCTION

Bovine tuberculosis is a disease of both economic and zoonotic importance caused by members of the *Mycobacterium tuberculosis* complex, particularly *Mycobacterium bovis* (Wobeser 2009, Michel *et al* 2010). Bovine tuberculosis eradication programs involve the detection and elimination of infected animals (Ministério da Agricultura, Pecuária e Abastecimento 2004, Álvarez *et al* 2012). The detection of infected cattle is based mainly on the cell-mediated immune response (McNair *et al* 2007, Good and Duignan 2011) assessed through an intradermal tuberculin test (skin test) and interferon-gamma (IFN-γ) assay (Rothel *et al* 1992).

The skin test involves the measurement of increased skin fold thickness 72 h after the intradermal injection of mycobacterial extracts, termed purified protein derivatives (PPD). However, due the complexity of these reagents and the sharing of antigenic components between pathogenic and nonpathogenic mycobacteria, PPD do not always allow the discrimination between cattle infected with virulent *M. bovis* and non-infected cattle sensitised by environmental mycobacteria and *Mycobacterium avium* subsp. *avium* or *M. avium* subsp. *paratuberculosis* (Waters *et al* 2004, Pollock *et al* 2005, Schiller *et al* 2010,). Thus, the comparison with responses to *M. avium* PPD is often

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used to facilitate the discrimination between cattle infected with *M. bovis* and those exposed to environmental strains (Pollock *et al* 2005).

Despite the broad usage of this assay, skin test sensitivity reports range from 68 to 95%, whereas specificity (for the comparative test) ranges from 96 to 99% (Monaghan *et al* 1994, Whelan *et al* 2004, De La Rua-Domenech *et al* 2006). Furthermore, there is evidence that PPD inoculation may sensitise the animals, thereby affecting the result of a subsequent skin test in the same animal (Thom *et al* 2004). It is possible that repeated tuberculin testing may affect the results of other blood-based immune assays used to diagnose tuberculosis (Thom *et al* 2004, Coad *et al* 2010). The complexity of tuberculin, the presence of cross-reactive components, and the low specificity have fueled the search for new antigens and diagnostic assays specific to *M. bovis* (Pollock *et al* 2005, Aagaard *et al* 2010).

In recent years, a large number of proteins from *M. bovis* have been purified and characterised (Harboe *et al* 1996, Wiker *et al* 1998, Rhodes *et al* 2000, Mustafa *et al* 2006, Sidders *et al* 2008, Jones *et al* 2010). However, only some of them have been evaluated as antigens in the skin test on cattle as an alternative to traditional PPD, such as ESAT-6 (Pollock *et al* 2003, Whelan *et al* 2010, Flores-Villalva *et al* 2012), CFP-10, MPB70 and MPB83 (Whelan *et al* 2010). Although promising results have been observed with the use of ESAT-6 and CFP-10 either individually (Pollock *et al* 2003) or in cocktail combinations (Whelan *et al* 2010, Casal *et al* 2012), there is no sufficient evidence to date to support the replacement of traditional PPD with these recombinant proteins, mainly due to problems regarding sensitivity (Pollock *et al* 2003, Whelan *et al* 2010). Jones *et al* (2010) reported a sensitivity level of 87.5% and specificity of 97.6% using a protein cocktail of ESAT-6/CFP-10/Rv-3615c with the addition of Rv3020c. Whelan *et al* (2010) reported sensitivity and specificity of 73.6% and 100% respectively using a cocktail of recombinant proteins composed of ESAT-6/CFP-10/MPB70/MPB83. Thus, there is a need to evaluate other recombinant proteins from *M. bovis* in skin tests on cattle.

Based on previous reports of diagnosis of tuberculosis in cattle using IFN-γ assays (EsxI, Mb0143, PE5, PE13 and TB10.4) (Aagaard *et al* 2006, Jones *et al* 2010, Meikle *et al* 2009), the aim of the present study was to assess these recombinant proteins with respect to the ability to differentiate *M. bovis* sensitised and non-sensitised cattle by skin test, for further evaluation of the best antigens in infected animals.

MATERIAL AND METHODS

ETHICAL ASPECTS

The use of cattle was approved by the Ethics Committee on Animal Use of the Universidade Federal de Mato Grosso do Sul, Brazil (protocol 321/11). All experiments were carried out in accordance with international norms and regulations.

CATTLE SENSITISATION

Cattle from the Caracu breed (n = 29), approximately 24 months of age and with a mean weight of 408 kg, raised at Embrapa Beef Cattle, Campo Grande, MS, Brazil, were used. The animals came from a herd with no clinical or pathological history of tuberculosis during the last 3 years, as evidenced by inspection at slaughter. In addition, all the animals used were negative in ELISA based on MPB70 and P27 recombinant antigens, according to Farias *et al* (2012). Twenty-four animals were sensitised by subcutaneous injection of 10 mg of inactivated heat (96-105 °C for 30 min) *M. bovis* AN5 strain, which was supplied by the LANAGRO/Brazilian Ministry of Agriculture (Pedro Leopoldo, Brazil) to simulate the immunological response observed in *M. bovis* infected animals. In addition, five animals were only inoculated with sterile saline solution to simulate non-infected cattle. After sensitisation, all animals were kept in the isolation area of Embrapa Beef Cattle, Brazil. In order to avoid any possibility of prior sensitization of experimental animals, they have not been previously submitted to comparative intradermal tuberculin test (CITT).

GENE AMPLIFICATION

M. bovis DNA (AN5 strain) was purified from cultures in Stonebrink's medium using a commercial kit (DNeasy Blood & Tissue kit, Qiagen).

Primer sets were used to amplify the genes *mb0143* (Rv0138), *pe5* (Rv0285), *pe13* (Rv1195) *and tb10.4* (Rv0288) as described by Souza *et al* (2012). The primer set for the *esxI* (Rv1037c) gene (forward: 5' TATCAATTCGGGGACGTCGACGCTCACG 3' and reverse: 5' GGCGCTGTCGGTTTGTGCCATGTTGTTG 3') were designed using the *PrimerSelect* program (DNAStar). The primers were designed to amplify the full length of the genes.

Polymerase chain reactions were performed in a volume of 25 µl containing 20 mM of Tris (pH 8.4), 50 mM of KCl, 1.5 mM of $MgCl_2$, 250 µM of each dNTP, 100 ηg of each primer, 0.2 U of *Taq* DNA polymerase (Invitrogen) and 50 ηg of *M. bovis* DNA. The amplification protocol was as follows: 95 °C for 4 min followed by 30 cycles of 95 °C for 1 min (denaturation), annealing for 30 sec at 56 °C (*pe5*), 58°C (*pe13, tb10.4*), 64 °C (*mb0143*) and 65 °C (*esxI*), extension for 30 sec at 72 °C. A final step of extension was performed at 72 °C for 4 min.

GENE CLONING, PRODUCTION AND EVALUATION OF RECOMBINANT PROTEINS

Following amplification, the genes were initially cloned into the *pGEM-T Easy* (Promega) plasmid, following the manufacturer's instructions, and subcloned into *pET47-b* (Novagen), except *tb10.4*, which was subcloned into *pET28-a* (Novagen) at the *Eco*RI site. The plasmid was thus extracted, purified and sequenced in order to evaluate the integrity of the cloned gene sequence.

The *Escherichia coli* Rosetta strain was used as the host cells for all DNA constructs. The induction of gene expression was performed using 1 mM of isopropyl-β-D-galactopyranoside (IPTG) in 500 ml of Luria-Bertani broth supplemented with 50 µg/ml of chloramphenicol and 30 µg/ml of kanamycin at 30 °C for 4 h at 200 rpm. Gene expression was confirmed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with the anti-6x-histidine monoclonal antibody (Sigma).

Recombinant proteins were solubilized with 6 M of HCl-guanidine and purified using agarose-nickel resin His-Trap HP (GE Healthcare), following the manufacturer's instructions. Recombinant proteins were dialyzed with PBS at 4 ºC for 48 h and concentrations were determined by comparisons with known concentrations of bovine serum albumin in SDS-PAGE using the LabImage v.3.3.2 image analysis software (Loccus, Brazil).

Molecular masses of the recombinant proteins were analysed by MALDI-TOF mass spectrometry using an Autoflex III spectrometer (Bruker Daltonics). Proteins were mixed with 2.5-dihydroxybenzoic acid (Sigma-Aldrich) by dried droplet method and spectra were acquired after 1000 laser shots in linear positive mode under external calibration with ProteoMass calibrants (Sigma-Aldrich).

All recombinant proteins used in this study were previously evaluated in ELISA as described by Souza *et al* (2012) and were recognised by antibodies (IgG) of cattle naturally infected with *M. bovis*.

SKIN TESTING

Sixty days after sensitization, the animals were separated into three groups. Two groups were each composed of twelve sensitised cattle and the third group was formed by five non-sensitised animals. All animals received an intradermal inoculation of 100 µg of *M. bovis* PPD (2606 IU) and 50 µg of *M. avium* PPD (2397 IU) at a volume of 0.1 ml (following the technical regulations of the National Brucellosis and Tuberculosis Control and Eradication Program of the Brazilian Ministry of Agriculture [PNCBT]) (Ministério da Agricultura, Pecuária e Abastecimento 2004). All PPD were supplied by the *Instituto de Tecnologia do Paraná* – TECPAR (Curitiba, Brazil). The first group of sensitised cattle received 160 µg of each recombinant protein (EsxI, Mb0143, PE5, PE13 and TB10.4), a cocktail containing 32 µg of each protein and sterile saline at final volume of 0.16 ml. The second sensitised group received 320 µg of each recombinant protein, a cocktail containing 64 µg of each protein and saline at final volume of 0.32 ml. Three non-sensitised animals received all proteins injections at final volume of 0.16 ml and two non-sensitised animals received injections at final volume of 0.32 ml. The protein concentrations used in this experiment were established based on previous evaluations in experiments with guinea pigs (*Cavia porcellus*) (data not shown).

Nine intradermal injection sites were established on each animal, four on one side and five on the other side of the neck, with a distance of approximately 15 cm between inoculation sites. The inoculation sites were alternated systematically in each animal to minimize any bias related to the inoculation site.

The results were expressed as the difference in skin thickness in millimeters (mm) between the pre- and post-skin test measurements at 24, 48 and 72 h following intradermal inoculation (Ministério da Agricultura, Pecuária e Abastecimento 2006). The technicians were blinded to both identification of inoculum and the sites of the inoculations during measurements. The comparative intradermal tuberculin test (CITT) was interpreted based on the technical regulations of the PNCBT/Brazil (positive reactions = Δ skin thickness for *M. bovis* PPD – *M. avium* PPD ≥ 4mm).

STATISTICAL ANALYSIS

The increase in skin fold thickness (in mm) with each combination of protein, concentration and post-injection reading time was compared with respective measurements in non-sensitised animals using the nonparametric Mann-Whitney test. Differences in skin fold thickness between non-sensitised animals inoculated with 0.16 ml or 0.32 ml of inoculum were also analysed using the Mann-Whitney test. The sensitivity and specificity of the best combinations (protein, concentration and reading time), resulting in a significant difference between sensitised and non-sensitised cattle, were interpreted using a cut-off point ≥ 1mm, as used by Whelan (2010), also in experiments with recombinant proteins in cattle. *P* values below 0.05 were considered statistically significant. The statistical analyses were performed using BioEstat version 5.0 (*Sociedade Mamirauá*, Belém, Brazil).

RESULTS

Gene expression was determined by SDS-PAGE and Western blot. In Western blot, all recombinant proteins were recognized by the anti-6x-histidine antibody, thereby confirming gene expression. The molecular masses of the recombinant proteins was confirmed by MALDI-TOF mass spectrometry revealed the proteins EsxI, Mb0143, PE5, PE13 and TB10.4 to have 18.4, 20.4, 7.9, 17.3 and 13.9 KDa, respectively.

The *M. bovis* sensitised animals used in this experiment exhibited a significant response to *M. bovis* PPD (median = 17.75 mm; interquartile range = 16.8 to 21.0). All were considered positive to the cervical comparative tuberculin skin test interpreted based on Brazilian legislation. The non-sensitised cattle exhibited a weak response to *M. bovis* PPD (median = - 0.6 mm; interquartile range = - 1.2 to 1.0) and all were negative based on the same interpretation criteria (Δ skin thickness ≥ 4 mm, 72 h post-injection). The responses to *M. avium* PPD showed a median of increase in skin fold thickness of 0.4 mm (interquartile range = - 0.85 to 0.95) in non-sensitised cattle, and 3.5 mm (interquartile range = 2.5 to 4.2) in sensitised animals.

No significant differences (P > 0.05) were observed between non-sensitised animals inoculated with 0.16 or 0.32 mL of inoculum. Thus, these animals were analysed as a single group.

Significant differences in the increase in skin fold between *M. bovis* sensitised (n = 12) and non-sensitised cattle (n = 5) were observed only with the proteins EsxI (P = 0.039; 95% CI -0.136 to 2.036), Mb0143 (P = 0.039; 95% CI 0.054-1.409) and PE5 (P = 0.030; 95% CI 0.727-2.781), when 320 µg of protein was used and the reading performed 24 hours post-injection (figure 1).

Table 1 displays the sensitivity and specificity of EsxI, Mb0143 and PE5 (320 µg/24 h) in the skin test to differentiate between sensitised and non-sensitised animals, based on a criteria of interpretation ≥ 1 mm.

Figure 2 displays the comparison of reaction sizes to *M. bovis* PPDs (read at 72 h post-injection) and EsxI, Mb0143 and PE5 (read at 24 h post-injection). Significant differences (P = 0.001) were found in skin fold produced by the PPD in relation to the recombinant proteins.

All animals used in this experiment were slaughtered (at the end of experiment) and no lesions suggestive of BTB were found during meat inspection at an official slaughterhouse.

DISCUSSION

ESAT-6 and CFP-10, are two important proteins for the diagnosis of *M. bovis* in cattle by the skin test and IFN-γ assays (Vordermeier *et al* 2001, Aagaard *et al* 2003, Waters *et al* 2004, Aagaard *et al* 2006, Whelan *et al* 2010, Casal *et al* 2012). Despite the promising results obtained by the use of ESAT-6 and cocktails combinations of ESAT-6 and CFP-10 in previous studies (Pollock *et al* 2003, Whelan *et al* 2003, Whelan *et al* 2010, Casal *et al* 2012), there is no sufficient evidence to date to support the replacement of traditional PPD by these recombinant proteins, showing that it is necessary to evaluate other proteins as antigens.

In the present study only the proteins EsxI, Mb0143 and PE5 (320 µg/ 24 h) induced significant differences between sensitised and non-sensitised cattle. Readings at 48 and 72 hours did not allow the differentiation between *M. bovis* sensitised and non-sensitised cattle. However, the animals used in this experiment were experimentally sensitised with the inactivated AN5 strain of *M. bovis* and the delayed-type hypersensitivity (DTH) response may be significantly different from the response observed in naturally infected cattle, especially in the chronic phase of infection (Cockle *et al* 2006).

In the present study, proteins, such as EsxI (Jones *et al* 2010), Mb0143, PE5, PE13 and TB10.4 previously assessed in IFN-γ assays (Aagaard *et al* 2006, Meikle *et al* 2009), were evaluated in skin test in cattle. Excluding TB10.4, which was recently evaluated in skin test in cattle by Xin *et al* (2013), all the other proteins were assessed for the first time in skin test in bovines.

In previous assessments of recombinant proteins as antigens in skin test in cattle, only experimentally infected (Pollock *et al* 2003, Xin *et al* 2013) or naturally infected (Whelan *et al* 2010, Casal *et al* 2012) animals had been used. In the present study, for the first time, sensitised animals were used. Although performing the skin test in cattle experimentally infected with virulent *M. bovis* might have been preferable, the model using sensitisation with a subcutaneous inoculation of an AN5 strain appears to be suitable, since an intense *M. bovis* PPD response was observed in all of the sensitised animals. This method represents an interesting alternative for the screening of antigens for a skin test in cattle.

In the present study, EsxI and PE5 exhibited a sensitivity level of 83.3% (10/12), and specificity of 100% (5/5) and

Table 1. Sensitivity and specificity at the specific cut-off point of recombinant EsxI, Mb0143, PE5 and *M. bovis* PPD as antigens in a tuberculin skin test in cattle.

La sensibilidad y la especificidad en el punto de corte específico de EsxI, Mb0143, PE5 recombinantes y PPD de *M. bovis* como antígenos en la prueba cutánea de la tuberculina en el ganado.

Antigen	Sensitivity	Specificity	Cut-off (mm)
EsxI	83.33%	100%	≥ 1
Mb0143	33.33%	80%	≥ 1
PE5	83.33%	80%	≥ 1
M. bovis PPD*	100%	100%	≥ 4.0**

* *M. bovis* PPD used in Comparative Intradermal Tuberculin Test.
** Standard PNCBT-Brazil.
* PPD de *M. bovis* usado en la Prueba de Tuberculina Intradérmica Comparativa.
** Estándar PNCBT-Brasil.

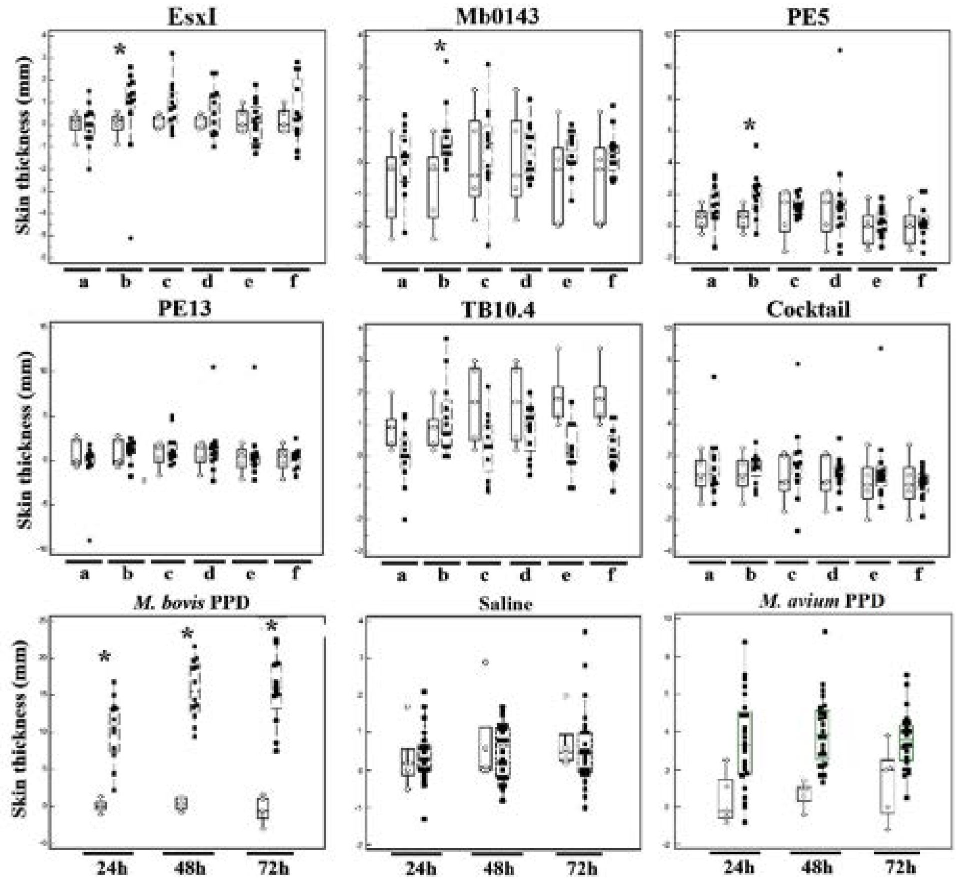

Figure 1. Skin test response to individual proteins of *M. bovis*, a cocktail of all proteins, *M. bovis* and *M. avium* PPD; response measured in sensitised (■) and non-sensitised (○) cattle; letters represent amount of injected protein and post-injection reading time: a) 160 µg/ 24 h; b) 320 µg/ 24 h; c) 160 µg/ 48 h; d) 320 µg/ 48 h; e) 160 µg/ 72 h; f) 320 µg/ 72 h; closed circles (●) represent outliers; asterisk (*) indicates significant difference between sensitised and non-sensitised cattle.

Respuesta de la prueba cutánea a las proteínas individuales de *M. bovis*, un cóctel de todas las proteínas, PPD de *M. bovis* y *M. avium*; la respuesta se mide en el ganado sensibilizado (■) y no sensibilizado (○); las letras representan cantidad de proteína inyectada y tiempo de lectura después de la inyección: a) 160 µg/ 24 h; b) 320 µg/ 24 h; c) 160 µg/ 48 h; d) 320 µg/ 48 h; e) 160 µg/ 72 h; f) 320 µg/ 72 h; círculos cerrados (●) representan los valores extremos; asterisco (*) indica diferencias significativas entre el ganado sensibilizado y no sensibilizado.

80% (4/5) respectively, using a cut-off point ≥ 1 mm. Based on the same criteria of interpretation, Whelan *et al* (2010) reported a sensitivity level of 73.6% and specificity of 100% using a protein cocktail (ESAT-6, CFP-10, MPB70 and MPB83) in cattle naturally exposed to *M. bovis,* whereas Jones *et al* (2010) reported 75% and 87.5%, respectively, using a peptide combination of ESAT-6/CFP-10/Rv3615c or ESAT-6/CFP-10/Rv3615c/Rv3020c. Recently, Xin *et al* (2013) reported sensitivity and specificity of 94.02% and 95.2% respectively, using the same criteria of interpretation (cut-off point ≥ 1 mm) and a protein cocktail composed by CFP10/ESAT-6/TB10.4.

Although artificially sensitised animals were used in the present study, and as mentioned above, the immune response may be significantly different from naturally infected cattle, the parameters of sensitivity and specificity observed for EsxI and PE5 were very close to those observed with other proteins in skin tests (Jones *et al* 2010, Whelan *et al* 2010). These results are very interesting because EsxI is a secreted protein member of the ESAT-6 family (Mtb 9.9 subfamily) (Jones *et al* 2010), and perhaps its inclusion in a protein cocktail with ESAT-6 and CFP-10 could improve the sensitivity and specificity of the skin test. On the other hand, PE5 is a member of the PE family that is present in the mycobacterial cell wall, and is considered, along with TB10.4 and PE13, to be an antigen that is sensitive and highly specific in the IFN-γ assay (Aagaard *et al* 2006). Although Mb0143 was also considered a satisfactory antigen in IFN-γ assay to identify experimentally infected cattle (Meikle *et al* 2009), its sensitivity levels were not satisfactory in the skin test.

In the present study, the combination of different antigens in a cocktail did not favour the differentiation of sensitised and non-sensitised cattle, although some proteins allowed such a differentiation individually. A definitive explanation for these results is not known.

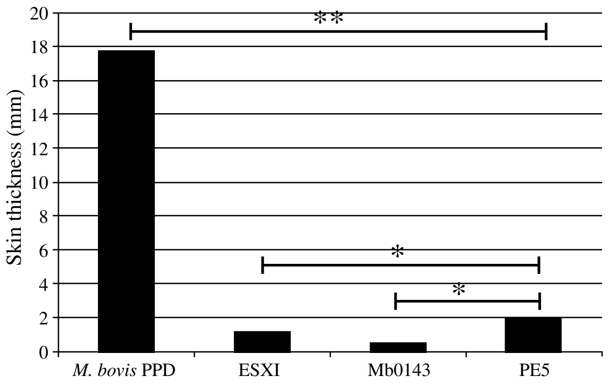

Figure 2. Comparison of median of skin test responses induced by *M. bovis* PPD, protein EsxI, Mb0143 and PE5 in twelve cattle sensitised with inactivated *M. bovis* AN5 strain. The statistical difference between the antigens was determined by the Mann-Whitney test (*P < 0.05; **P < 0.001).

Comparación de la mediana de las respuestas de las pruebas cutáneas inducidas por PPD de *M. bovis*, proteínas EsxI, Mb0143 y PE5 en doce bovinos sensibilizados con la cepa *M. bovis* AN5 inactivada. La diferencia estadística entre los antígenos se determinó mediante la prueba de Mann-Whitney (*P < 0,05; **P < 0,001).

However, the amount of each protein injected in a cocktail format (32 or 64 µg/ protein), in contrast to the amount used individually (160 or 320 µg/ protein) may have influenced these findings. In addition, Kalra *et al* (2010) proposed that protein-protein interaction and their immunocompatibility, in terms of recognition of different leukocyte antigen alleles and competition for antigen processing and presentation, should be considered before combining two or more antigens.

Inconsistent results have been observed in different cattle studies regarding the amount of protein necessary to produce a perceptible DTH. The use of 10 µg (Whelan *et al* 2010) up to 400 µg (Pollock *et al* 2003) of recombinant protein has been reported. In the present study, the amount of protein evaluated (160 and 320 µg) was based on a previous evaluation in an experiment with guinea pigs (data not shown) and significant differences in skin fold between *M. bovis* sensitised and non-sensitised cattle were observed only when 320 µg of EsxI, Mb0143 or PE5 were used.

Despite the significant difference in skin fold thickness produced by the *M. bovis* PPD in relation to the recombinant proteins, it was possible to differentiate sensitised from non-sensitised cattle with EsxI and PE5, with satisfactory levels of sensitivity and specificity. Significant differences in the magnitude of the reactions of *M. bovis* PPD and recombinant proteins have been reported in previous studies (Pollock *et al* 2003, Whelan *et al* 2010), and consequently cut-off points of few millimeters have been utilized to interpret recombinant proteins or synthetic peptide skin tests (Pollock *et al* 2003, Jones *et al* 2010, Whelan *et al* 2010, Casal *et al* 2012). In the present study, the median of increase in skin fold thickness in the reactions to the Mb0143 antigen was only 0.5 mm (interquartile range = 0.25 to 1), whereas median values were 1.2 mm (interquartile range = 0.87 and 1.82) for EsxI and 1.95 mm (interquartile range = 1.15 to 2.52) for PE5. Although the full explanation for this finding is not known, the inherent antigenicity of each protein may be considered.

In experiments with recombinant proteins produced in *E. coli*, endotoxin contamination should be considered as a possible inducer of unspecific skin reactions. Although the endotoxin level in the samples of recombinant proteins was not measured in the present study, the influence of this toxin in the skin reactions is believed to have been non-significant. Skin fold thickness induced by recombinant proteins EsxI, Mb0143 and PE5 (24 h post-injection) in non-sensitised animals exhibited no significant differences in the Mann-Whitney test in relation to the reactions induced by sterile saline.

The results of this comparison suggest that reactions observed in sensitised animals are specific to the recombinant proteins and not due to endotoxin or other *E. coli* protein contamination. Moreover, nonspecific reactions due to sensitization with environmental mycobacteria were unlikely, since skin thickness induced by *M. avium* PPD in the non-sensitised animals was negligible. The reactions among sensitised animals were consistent with the findings of Almeida *et al* (2006) (median of increase in skin fold = 6.15 mm, interquartile range = 3.87 to 7.77) in cattle sensitised by heat inactivated *M. bovis* AN5 and non-reactive for *M. avium* in the IFN-γ assay.

Another important issue in the results observed in the present study is related to the time at which the skin test reactions induced by recombinant proteins (mainly EsxI and PE5) peaked (24 h post-injection). Skin thickness induced by PPD normally peaks after 72 h, and it has been found that recombinant proteins such as ESAT-6 also peak after approximately 72 h (Pollock *et al* 2003, Whelan *et al* 2010). This was not observed for any of the proteins used in the present study. Immunologically, a DTH is a local T-cell mediated inflammatory reaction that evolves over 24-72 h, and cutaneous DTH in cattle have been reported to peak at 24 h (Hernández *et al* 2005). Therefore, it is not possible to exclude the hypothesis that the reactions observed in the present study were DTH reactions. However, an incontestable confirmation would only be possible by performing histological analysis of the inoculation sites. Although interesting results were obtained with the proteins EsxI and PE5 in artificially sensitised animals, an assessment with naturally infected animals is required to assess the real potential of these proteins as antigens in skin tests.

The results reported herein suggest that the proteins EsxI and PE5 have potential to be assessed as antigens in skin tests in cattle. However, more studies are needed, especially in cattle naturally infected by *M. bovis*.

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