Display of antigens on polyester inclusions lowers the antigen concentration required for a bovine tuberculosis skin test

Natalie A. Parlanea†, Shuxiong Chenb†, Gareth J. Jonesc, H. Martin Vordermeierc, D. Neil Wedlocka, Bernd H.A. Rehmdb, Bryce M. Buddlea#

aAgResearch, Hopkirk Research Institute, Palmerston North, New Zealand.
bInstitute of Fundamental Sciences, Massey University, Private Bag 11222, Palmerston North, New Zealand
cTB Immunology and Vaccinology, Animal and Plant Health Agency, New Haw, Addlestone, Surrey, United Kingdom
dMacDiarmid Institute for Advanced Materials and Nanotechnology, New Zealand
ePolybatics Ltd, Palmerston North, New Zealand

† These two co-authors equally contributed to this paper
#Corresponding author, Bryce M. Buddle, bryce.buddle@agresearch.co.nz

Short title: Low dose immobilised antigen for TB skin test
The tuberculin skin test is the primary screening test for the diagnosis of bovine tuberculosis (TB) and use of this test has been very valuable in control of this disease in many countries. However, the test lacks specificity when cattle have been exposed to environmental mycobacteria or vaccinated with *Mycobacterium bovis* bacille Calmette-Guérin (BCG). Recent studies have shown that use of three or four recombinant mycobacterial proteins, including ESAT6, CFP10, Rv3615c and Rv3020c or a peptide cocktail derived from these proteins, in the skin test greatly enhanced test specificity with minimal loss in test sensitivity. These proteins are present in members of the pathogenic *Mycobacterium tuberculosis* complex, but absent or not expressed by the majority of environmental mycobacteria or by the BCG vaccine strain. To produce a low cost skin test reagent these proteins were displayed at high density on polyester beads by translational fusion to a polyhydroxyalkanoate (PHA) synthase which mediated formation of antigen displaying inclusions in recombinant *Escherichia coli*. Display of the proteins on the polyester beads greatly increased their immunogenicity, allowing for the use of very low concentrations of proteins in the skin test, between 0.1 to 3 µg of mycobacterial protein/inoculum. Polyester beads simultaneously displaying all four proteins were produced in a single fermentation process. The polyester beads displaying three or four mycobacterial proteins were shown to have a high sensitivity for detection of *M. bovis*-infected cattle and induced minimal response in animals exposed to environmental mycobacteria or vaccinated with BCG.
INTRODUCTION

Control of bovine tuberculosis (TB), caused by infection with *Mycobacterium bovis*, is critical as this disease is of great economic and zoonotic importance. Eradication programs in cattle are primarily based on the use of the tuberculin skin test for diagnosis of the disease and slaughter of reactor animals. This regime has been instrumental in the eradication of this disease from a number of countries (1). The tuberculin skin test is a delayed-type hypersensitivity (DTH) test developed more than 100 years ago for the diagnosis of TB and has proved to be a simple, inexpensive, robust and widely accepted test. However, the test lacks specificity when animals are sensitized to environmental mycobacteria or if animals are vaccinated with the human TB vaccine, *M. bovis* bacille Calmette-Guérin (BCG) (2). Estimates of sensitivity for the single intradermal test in cattle using purified protein derivative (PPD) prepared from *M. bovis* (bovine PPD) have ranged from 63.2% to 100%, median of 83.9% and specificity between 75.5% and 99.0%, median of 96.8% (reviewed in (2)). Development of a more specific skin test reagent would be highly desirable. Use of BCG vaccine alone or as part of a heterologous prime-boost combination is currently being considered by a number of countries for control of bovine TB (3). Currently BCG cannot be used as it compromises the interpretation of the tuberculin skin test and development of a test to differentiate infected from vaccinated animals (DIVA) would be essential.

The tuberculin skin test utilizes bovine PPD which is a poorly defined mixture of proteins, lipids and carbohydrates, including components present in non-pathogenic environmental mycobacteria. The two major formats for the tuberculin skin test are the caudal fold test where bovine PPD is injected intradermally in the caudal fold of the tail
and the single intradermal comparative cervical test (SICCT) where PPDs are injected in the neck. The SICCT compares reactions induced following intradermal injection of bovine PPD and *Mycobacterium avium*-derived PPD (avian PPD) to control for environmental sensitization.

Three highly immunogenic, specific TB antigens, 6-kDa early secretory antigen target (ESAT6), 10-kDa culture filtrate protein (CFP10) and Rv3615c are expressed by members of the pathogenic *M. tuberculosis* complex which includes *M. bovis*, but are not expressed by the majority of non-pathogenic environmental mycobacteria and the BCG vaccine strain (4, 5, 6, 7). These proteins or peptides derived from these proteins have been shown to enhance skin test specificity in cattle, yet maintaining a relative high sensitivity for the diagnosis of bovine TB (8, 9). More recently, the addition of peptides from a fourth specific mycobacterial protein, Rv3020c has further enhanced test sensitivity (10). The tuberculin skin test is used as the primary screening test for bovine TB and three major criteria must be satisfied before bovine PPD could be replaced with specific antigens. Use of specific antigens must provide improved test specificity, with minimal loss in test sensitivity and the cost of the reagents needs to be similar to that for PPD. Recent research has indicated that the first two criteria could be met, while use of recombinant proteins or a peptide cocktail would increase the cost of the reagent.

The cost of the reagent could be reduced by using low concentrations of the proteins by displaying them on nanoparticles, potentially increasing their immunogenicity and producing them as a recombinant fusion protein. This has been achieved by display of ESAT-6, CFP10 and Rv3615c on polyester inclusions (biobeads).
produced by *Escherichia coli* and preliminary results indicated their utility in the skin test for diagnosis of bovine TB (11). Polyester inclusions are naturally produced by various bacteria during imbalanced nutrient availability in which excess carbon is available and deposited as spherical water-insoluble cytoplasmic inclusions (12, 13). These polyesters are composed of R-3-hydroxy fatty acids of varying carbon chain length (14). Foreign proteins have been displayed on the polyester beads by translationally fusing them to a polyester synthase (PhaC) which mediated formation of protein displaying beads in recombinant *E. coli* (15, 16). The size of the beads is between 100 to 500 nm in diameter and beads contain an amorphous hydrophobic polyester core surrounded by proteins, including the fusion protein composed of PhaC and foreign proteins (12, 17). Interestingly, immunological studies using antigen displaying beads revealed that respective beads showed adjuvant properties by enhancing the immune response to the displayed antigen when compared to its soluble counterpart (18).

The current paper extends findings from the earlier study (11) by demonstrating that biobeads displaying ESAT-6, CFP10 and Rv3615c (3-Protein biobeads) were effective in identifying experimentally and naturally *M. bovis*-infected cattle as well as distinguishing them from BCG-vaccinated and non-infected animals. In addition, biobeads were designed and produced to simultaneously display the four mycobacterial proteins, ESAT-6, CFP10, Rv3615c and Rv3020c (4-Protein biobeads), potentially increasing test sensitivity (10). These biobeads were successfully produced, analysed and skin test performance experiments showed that they were effective in identifying *M. bovis*-infected animals in the skin test using very low concentrations of the
MATERIALS AND METHODS

Animals. The groups of cattle used to assess the skin test performance of the biobead reagents are shown in Table 1. The non-infected animals were sourced from TB-free herds, located in TB-free regions of New Zealand; some of which had been naturally exposed to environmental mycobacteria as indicated from a strong skin test or interferon-γ (IFN-γ) response to avian PPD. The BCG-vaccinated cattle consisted of cattle vaccinated subcutaneously with BCG (2-8 X 10^5 CFU; Statens Serum Institute, Denmark) at 2 to 4 weeks of age and revaccinated with the same dose of vaccine at 2 years of age (19). These animals were skin tested 11 weeks after revaccination. The three groups of cattle had been experimentally-infected endobronchially with approximately 6,000 CFU of *M. bovis* as previously described (20). The cattle naturally-infected with *M. bovis* were sourced from infected herds from the West Coast of the South Island and Waikato, New Zealand and these three groups of animals were identified as infected with *M. bovis* from an initial positive caudal fold skin test with bovine PPD. All of the experimentally *M. bovis*-infected and all except one, naturally *M. bovis*-infected cattle were confirmed as infected by culture of *M. bovis* from their tissues at slaughter, with the remaining animal confirmed from typical gross and histopathological lesions. All animal manipulations were approved by an independent Animal Ethics committee.

Antigens. Bovine and avian PPDs were supplied by AsureQuality, (Upper Hutt, New Zealand) and Prionics Lelystad BV (Lelystad, The Netherlands). Purified mycobacterial proteins.
recombinant proteins, ESAT-6, CFP10 and Rv3615c were supplied by Lionex Diagnostics and Therapeutics GmbH (Germany).

**Production of polyester beads displaying mycobacterial proteins.** Polyester beads displaying three mycobacterial proteins ESAT-6, CFP10 and Rv3615c (3-Protein biobeads) were produced in *E. coli* BL21(DE3) as described previously (11).

In order to produce polyester beads which simultaneously display all four mycobacterial antigens ESAT-6, CFP10, Rv3615c and Rv3020c (4-Protein biobeads), we designed and constructed a hybrid gene encoding all four antigens fused to a polyester synthase as a single polypeptide. Briefly, the DNA fragment encoding the antigens ESAT-6 and Rv3020c was synthesized as codon optimized for expression in *E. coli* by Genscript (USA). This DNA fragment was subcloned directly into the 3’ end of the polyester synthase gene from the plasmid construct pET-14b cfp10-linker-rv3615c-phaC-linker-malE resulting in a hybrid gene encoding the single fusion protein CFP10-Rv3615c-PhaC-ESAT6-Rv3020c. The cloning strategy is outlined in Fig. 1. The resulting plasmid pET-14b cfp10-linker-rv3615c-phaC-linker-esat6-linker-rv3020c, was transferred into *E. coli* BL21 (DE3) (pMCS69) to assess production of polyester beads. Plasmid pMCS69 contains the genes *phaA* and *phaB* from *Ralstonia eutropha* which both mediate synthesis of the polyester precursor R-3-hydroxybutyryl-CoA.

To determine that the bead preparations were sterile, samples of the preparations were spread on LB agar. In the later experiment that compared the use of the 4-Protein and 3-Protein biobead preparations, the beads were γ-irradiated (12.5 kGy, MSD Ltd., Upper Hutt, New Zealand) as an additional step to ensure sterility. Dextran at a final
concentration of 15% (USP grade, Pharmacosmos A/S, Holbaek, Denmark) was added to these biobead preparations to keep the beads in suspension.

**Analysis of proteins attached to the polyester beads.** For analysis of the proteins, the fusion protein consisting of mycobacterial proteins and PhaC protein, was separated from the polyester beads by SDS-PAGE using 8% polyacrylamide gel and stained with Coomassie blue. Proteins of interest were excised from the gels and subjected to tryptic peptide finger-printing using matrix-assisted laser desorptionization-time of flight mass spectrometry (MALDI-TOF MS) (21). The mycobacterial protein concentration contained in the beads was estimated by first measuring the total amount of protein contained in the beads. The proportion of protein attributed to the fusion protein was determined by densitometry and the amount of mycobacterial proteins contained in the fusion protein calculated from the molecular weights of the mycobacterial proteins in comparison to that for the PhaC protein.

**Testing for a sensitizing effect.** The method used to determine whether the biobeads induced a sensitizing effect was based on the method used to test batches of bovine tuberculin (22). Briefly, a group of three guinea pigs that had not been treated previously with any material that could interfere with the test was injected intradermally on the abdominal flank with 0.1 ml volume of the 4-Protein biobead skin test reagent containing 0.3 µg of mycobacterial proteins on three occasions at intervals of 5 days. The concentration of biobeads was 1/10 of the highest cattle dose and was equivalent to the 1/10 cattle dose of bovine tuberculin (500 IU) used for assessing sensitization in guinea pigs. Each guinea pig, together with each of three control guinea pigs that had not been injected previously, was injected intradermally in the abdominal flank 15 days
after the third injection with the same dose of the bBiobead reagent. The guinea pigs were examined 24-28 hours later, to determine whether there was any difference in the reactions between the two groups of animals.

**Skin testing of cattle.** The comparative cervical skin test in cattle was undertaken by injecting 0.1 ml volumes of the reagents intradermally in the mid-neck region. Up to six inoculation sites were used on each side of the neck, with at least 50 mm space between each site. The hair at each site was clipped and skin thickness at the site of injection was measured with callipers immediately prior to injection and 72 h later, with results expressed as the change in skin thickness (mm). Changes in skin thickness of < 1 mm could not be measured accurately and the positive cut-off was set at ≥ 1 mm increase in skin thickness between 0 and 72 h post-inoculation. Different concentrations of the biobeads displaying mycobacterial proteins were prepared by diluting in PBS. Bovine PPD (5,000 IU/0.1 ml; AsureQuality, Upper Hutt, New Zealand or 3,000 IU/0.1 ml; Prionics, Lelystad, The Netherlands) was included in each test. Manufacture and supply of the AsureQuality PPDs was discontinued mid-way through the study. The comparative caudal fold skin test was undertaken by intradermally injecting a 0.1 ml volume containing 4-Protein biobead reagent into the caudal fold on one side of the tail and 0.1 ml injection of bovine PPD (3,000 IU; Prionics) in the caudal fold on the other side. The caudal folds were palpated at 72 hours after injection and any detectable lumps were measured with callipers. For one group of seven cows, two different doses of the 4-Protein biobead reagent were injected intradermally approximately 50 mm apart in one caudal fold, with bovine PPD injected in the other caudal fold.
Statistical analyses. A mixed effects model was used for comparisons of different test reagents or concentrations of reagents (Treatment) with the Treatment serving as the fixed effect and the Animal as the random effect. For the analysis of experimental-infected cattle tested with the 3-Protein biobeads, the results from two studies were combined, requiring a meta-analysis. For this analysis, a mixed effects model was used with the Treatment serving as the fixed effect and Animal plus Treatment nested within Experiment as the random effects. A mixed effects model was used for comparing injection sites, reagents and time of reading the test (hours) and in this model the Site, Reagents and Hours were fixed and Animals as the random effect. The results from these models provided a multiple comparison of the predicted means with p-values adjusted by the ‘BH’ method (23). The analyses were done by the R packages ‘nlme’, ‘lme4’ and ‘predictmeans’ in R 3.2.0. (24, 25). Statistical significance was denoted when \( P < 0.05 \).

RESULTS

Engineering of \textit{E. coli} for the production of 4-Protein biobeads. A plasmid encoding PhaC and the four mycobacterial genes, \textit{esat-6}, \textit{cfp-10}, \textit{Rv3615c} and \textit{Rv3020c} were constructed as described in Materials and Methods. Briefly, antigen Rv3615c was inserted between CFP10 and the N terminus of PhaC and antigens ESAT6 and Rv3020c were fused to the C terminus of PhaC resulting in a single fusion protein containing the four mycobacterial proteins. Recombinant production of this fusion protein facilitated the formation of intracellular polyester beads in the \textit{E. coli} cells. Formation of intracellular polyester beads in the \textit{E. coli} cells was indicated by isolation of a white
suspension from disrupted cells (data not shown). GC-MS analysis confirmed that cells were accumulating the polyester, polyhydroxybutyrate, which constitutes the core of the polyester beads contributing to about 34% of the cellular dry weight. SDS-PAGE analysis demonstrated that antigen displaying beads showed a prominent protein with an apparent molecular weight of 107 kDa for PhaC-4 mycobacterial fusion protein, compared to a molecular weight of 98 kDa for the PhaC-3 mycobacterial protein fusion (data not shown). The molecular weight of the PhaC protein alone was 64 kDa. The components of the fusion protein were identified by tryptic peptide fingerprinting using MALDI-TOF/MS (Table S1). Densitometry analysis of the SDS-PAGE showed that the fusion proteins, CFP10-Rv3615c-PhaC-ESAT6-Rv3020c and CFP10-Rv3615c-PhaC-ESAT6 accounted for approximately 70.5% of the total protein in their corresponding bead fraction (data not shown).

Sedimentation of the beads in the injecting syringe was overcome by addition of dextran to a final concentration of 15% (w/v). All animal studies with the 4-Protein, and the 3-Protein biobead preparations used for comparing with the 4-Protein biobeads, utilized preparations containing 15% dextran and were γ-irradiated as an added safeguard to ensure sterility. Testing of the 4-Protein biobeads in experimentally-infected cattle demonstrated that the addition of 15% dextran or γ-irradiation did not affect the magnitude of the skin test responses (data not shown).

Assessment for sensitizing effect in guinea pigs. The 4-Protein biobeads were tested for induction of a sensitizing effect at a dose of 0.3 µg mycobacterial protein (1/10 cattle dose) There was no difference in the reaction at the skin test site of the guinea pigs which received multiple doses of skin test reagent at 5 day intervals.
compared to those that received a single dose of the reagent when examined at 25 h post-inoculation. Each of the three vaccinated guinea pigs had a small zone of the erythema at the site of inoculation (3, 4 and 5 mm in diameter), with identical readings for the three non-vaccinated animals. In addition, two of the three vaccinated guinea pigs had a small area of induration, 2 mm in diameter (zero for the other guinea pig), while all three non-vaccinated guinea pigs had a 2 mm diameter area of induration at the site of inoculation.

**Reactivity of 3-Protein biobeads in cattle.** All of the 22 experimentally-infected cattle produced positive responses in the comparative cervical skin test at 72 hours post-injection (≥ 1 mm increase in skin thickness) following injection of the 3-Protein biobeads (3 µg of mycobacterial protein), three recombinant proteins (30 µg of total mycobacterial protein; 10 µg of each protein) and bovine PPD (5,000 IU; AsureQuality).

Twenty-one of the 22 were positive for the 3-Protein biobeads (1 µg of mycobacterial protein /dose) test reagent (Fig. 2A). The only significant difference between the reagents was that the mean response for bovine PPD was greater than that for the 3-Protein biobeads, 1 µg/dose ($P < 0.05$). In naturally-infected cattle, the two concentrations of the 3-Protein biobeads (1 and 3 µg/doses) produced responses in 10 of the 11 infected animals, while all were positive for bovine PPD (5,000 IU, AsureQuality; Fig. 2B). The mean response for the 3-Protein biobeads (3 µg/dose) was significantly greater than that for bovine PPD ($P < 0.01$). One animal was negative for the two concentrations of the 3-Protein biobeads (Fig. 2B) and also for the three recombinant proteins used at a dose of 10 µg total mycobacterial protein (data not shown). There was insufficient recombinant protein for testing at the recommended dose of 30 µg of total
recombinant protein. Twelve cattle which were vaccinated with BCG vaccine at 2-4
weeks of age and revaccinated 2 years later, were skin tested 11 weeks after
revaccination. Eleven of the 12 cattle responded positively for bovine PPD (5,000 IU,
AsureQuality), two of 12 were positive for the 3-Protein biobeads (3 µg/dose) and none
for the 3-Protein biobeads (1 µg/dose) and the recombinant proteins (30 µg/dose) (Fig.
2C). The non-infected animals consisted of 12 cattle, some of which were naturally
exposed to environmental mycobacteria; six of these animals responded positively in
the skin test to avian PPD (2,500 IU; AsureQuality) (data not shown). All were negative
for the 3-Protein biobeads (1 and 3 µg/dose) and recombinant proteins (30 µg/dose),
while two animals showed reactivity for bovine PPD (5,000 IU) (Fig. 2D).

Reactivity of 4-Protein biobeads in cattle. Concentrations of the 4-Protein
biobeads, ranging from 3 to 0.01 µg of mycobacterial protein were tested in the
comparative cervical skin test in five cattle experimentally-challenged with M. bovis, 10
weeks previously. There were no significant differences between the mean responses for
the 4-Protein biobead preparations containing 3, 1, 0.33 and 0.11 µg mycobacterial
protein. In contrast, the mean response for the 4-Protein biobeads containing 0.04 or
0.01 µg mycobacterial protein were significantly lower than those for the four higher
doses of the biobeads ($P < 0.05$; Fig. 3). A total of 24, 9-month old, non-infected
animals were tested with 4-Protein biobeads (3 µg/dose) and bovine PPD (3,000 IU;
Prionics) in the comparative cervical skin test and no detectable increases in skin
thickness ≥ 1 mm were detected in any of these animals (data not shown).

In a comparative cervical skin test, all nine naturally-infected animals produced
positive responses to the 3- and 4-Protein biobead preparations (3 µg mycobacterial
protein) and bovine PPD (3,000 IU, Prionics) (Table 2). The mean response for the bovine PPD was significantly greater than those for the 3- and 4-Protein biobeads ($P < 0.01$), while responses for the two biobead preparations were very similar. In a comparative caudal fold test which was undertaken on the same day as the cervical skin test, there were no significant differences between the mean responses for the 4-Protein biobeads (3 µg mycobacterial protein) and bovine PPD (3,000 IU, Prionics) (Table 2). Similar results were observed in a second group of seven naturally-infected animals where two doses of the 4-Protein biobeads were compared with bovine PPD in the cervical and caudal fold formats of the skin tests conducted on the same day (Fig. 4). The skin responses were significant greater for bovine PPD compared to the two doses of the 4-Protein biobeads in the comparative cervical test ($P < 0.001$), but not in the caudal fold test. For this latter group of animals, the overall responses in the cervical test were significantly greater than those in the caudal fold test ($P < 0.001$). No significant differences were detected between reading the tests at 72 and 96 hours post-injection. A total of 24 non-infected cattle, 9 months old were tested in the comparative cervical skin test with 4-Protein biobeads (3 µg/dose) and bovine PPD (3,000 IU; Prionics) and there were no responses of ≥1 mm increase in skin thickness.

**DISCUSSION**

Recent studies have demonstrated that skin testing with three or four specific mycobacterial proteins or peptides derived from these proteins could be used to diagnosis bovine TB in cattle (8,10). These reagents at a recommended dose of 10 µg for each protein or peptide pool were shown to be more specific than bovine PPD.
(tuberculin) and could also be used as a DIVA reagent to differentiate *M. bovis*-infected animals from those vaccinated with BCG vaccine. As the skin test is the primary screening test for TB diagnosis in cattle, the cost of the skin test reagent is an important consideration. It has been reported recently that display of three of these specific mycobacterial proteins (ESAT-6, CFP10 and Rv3615c) on bacteria-produced polyester inclusions (biobeads) could greatly increase their immunogenicity for the skin test (11). This markedly reduces the cost of the reagents as lower concentrations of the proteins can be used and only a single fermentation will be required with the proteins displayed as a fusion protein on a single biobead.

In the current study, no significant differences in the mean increases in skin thicknesses were observed between the 3-Protein biobeads and the three recombinant proteins in the comparative cervical skin test for experimentally-infected cattle. This was despite using concentrations of the mycobacterial protein in the biobeads at 10 and 30-fold lower than that for the recombinant proteins. Comparisons between the 3-Protein biobeads and bovine PPD revealed contrasting results for different groups of infected animals. Significantly smaller mean increases in skin thickness were observed in experimentally-infected animals for the low dose (1 µg of mycobacterial protein) compared to that for bovine PPD, while in a group of naturally-infected animals, significantly greater mean skin thicknesses were observed for the high dose (3 µg of mycobacterial protein) compared to that for bovine PPD.

The comparative size of responses to recombinant proteins versus bovine PPD have also varied in studies undertaken in Spain (9) and the UK (8) of naturally-infected cattle where no differences were noted for the pool the three recombinant proteins
compared to that for bovine PPD in one study (9), while responses were smaller for the proteins in another study (8). Comparisons of test sensitivity in naturally-infected animals need to be interpreted with caution as often the animals are selected for re-testing based on an initial positive response to bovine PPD. The size of the response to specific proteins or to the complex mix of components in PPD could depend on the stage of infection and the infecting strain of \textit{M. bovis}. Test sensitivities for specific proteins would be expected to be lower than those for bovine PPD due to the large number of immunogenic proteins present in PPD. However, a recent study in guinea pigs showed that some protein-protein interactions in PPD may abrogate the DTH response for TB, possibly by induction of an anti-inflammatory T cell type immune response (26). In the current study, the low dose of the 3-Protein biobeads (1 µg mycobacterial protein) and the three recombinant proteins had a high specificity with no positive responses in naïve or BCG-vaccinated animals. It is possible that low concentrations of \textit{E. coli} products in the biobead preparations could produce weak skin test responses, although minimal responses have been observed following testing of control biobeads (11).

Studies in the UK have shown that the addition of peptides derived from the mycobacterial protein, Rv3020c to a peptide cocktail of derived from ESAT-6/CFP10/Rv3615c proteins increased test sensitivity without compromising specificity (10). Based on these findings, a biobead was constructed which contained a fusion protein CFP10/Rv3020c/PhaC/Rv3615c/ESAT6, which included the enzyme PhaC required for the biobead formation. Analyses by tryptic peptide fingerprinting using MALDI-TOF/MS, SDS-PAGE and ELISA confirmed the identity of the fusion protein.
proteins and functionality. A critical requirement for a skin test reagent is that it must not induce a sensitizing effect. Using the OIE protocol for testing batches of tuberculin for a sensitizing effect, a 1/10 cattle dose of the 4-Protein biobeads did not induce a sensitizing effect in guinea pigs.

Dilutions of the 4-Protein biobeads were tested in the comparative cervical skin test in experimentally-infected cattle and dilutions containing 3 to 0.11 µg of mycobacterial protein/0.1 ml dose induced similar size skin test responses. In contrast to these findings, Whelan et al. (8) noted a marked decline in the size of skin test responses for naturally TB-infected animals when the dose of recombinant proteins decreased from 5 or 10 µg to 1 µg for each individual protein. This more rapid decline in dose response may relate to the testing of naturally infected animals where the responses are often weaker and more variable.

The 4-Protein biobead preparation (3 µg mycobacterial protein/0.1 ml dose) was shown to be effective in both the comparative cervical and caudal fold skin tests for diagnosing naturally-infected cattle. Although, the 4-Protein biobeads (3 µg/dose) and bovine PPD positively-diagnosed the same number of animals, the size of the responses was greater for bovine PPD in two groups of the naturally-infected animals for the cervical test, but not for the caudal fold test. In addition, the size of the responses overall, in the cervical test was greater than those in the caudal fold test. Francis et al. (27) considered that tuberculin skin testing in the cervical region was more sensitive than that in the caudal fold of the tail. In the comparative cervical test, responses to the 3- and 4-Protein biobead preparations were very similar for individual animals (Table 2) and there was no difference between responses for 1 and 3 µg/dose 4-Protein biobead
preparations (Fig. 4). Overall, there were no differences in the responses to the 4-Protein biobeads and bovine PPD when the cervical and caudal fold tests were read at 72 and 96 hours post-inoculation. Pollock et al. (28) considered that skin test reactions to ESAT-6 were often greatest at 96 hours post-inoculation, while Whelan et al. (8) considered that there was no difference between the reactions to ESAT-6, CFP10 and Rv3615c proteins at 72 and 96 hours. Measuring responses to both bovine PPD and specific mycobacterial proteins at 72 hours post-inoculation would be the most practical option.

From recent caudal fold field testing of the 4-Protein biobeads in non-infected cattle, a small hard lump, < 1 mm in size, has been observed at the inoculation site of the 3 μg protein dose in a small proportion of the animals, while these reactions were less frequent with the 1 μg protein dose (B. Buddle unpublished observations). This suggested that the 1 μg protein dose may be the preferable dose for skin testing.

Overall, this study has demonstrated that bacterial polyester beads displaying three or four TB specific antigens have a high sensitivity and specificity in the skin test when used at very low concentrations. A large field trial involving up to 50,000 cattle is currently in progress in New Zealand to determine test sensitivity and specificity for the 4-Protein biobead reagent in comparison to those for bovine PPD using the comparative caudal fold test. The display of mycobacterial proteins on polyester beads should allow the development of a highly specific, cost-effective skin test reagent for the diagnosis of *M. bovis* infection in cattle.

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REFERENCES


FIGURE LEGENDS

FIG 1. Cloning strategy for the production of the CFP10-Rv3615c-PhaC-ESAT6-Rv3020c fusion protein.

FIG 2. Skin test responses to the 3-Proteins biobeads in cattle. Comparative cervical skin test responses for recombinant ESAT-6, CFP10 and Rv3615c proteins (total protein dose of 30 µg), 3-Protein biobeads (ESAT-6, CFP10 and Rv3615c, 1 µg mycobacterial protein), 3-Protein biobeads (ESAT-6, CFP10 and Rv3615c, 3 µg mycobacterial protein) and bovine PPD (5,000 IU/dose, AsureQuality). Results represented as the increase in skin thickness between 0 and 72 h post-inoculation. Cattle groups: (A) experimentally-infected by *M. bovis* (n=22) (B) naturally-infected with *M. bovis* (n=11), the recombinant protein (30 µg dose) were not available for testing for this group, (C) BCG-vaccinated (n=12), (D) naïve (non-infected, non-vaccinated, n=12). The dashed horizontal lines indicated the 1 mm positive cut-off. Statistical difference between responses in (A) and (B) denoted as *, P <0.05 and **, P <0.01. In Figure 2A, there were no significant differences between the recombinant proteins and the two doses of the 3-Protein biobeads (P >0.05). Horizontal bar indicates mean.

FIG 3. Skin test for the 4-Protein biobead dose titration. Comparative cervical skin test responses for dilutions of the 4-Protein biobeads (ESAT-6, CFP10, Rv3615c and Rv3020c) containing 3, 1, 0.33, 0.11, 0.04 and 0.01 µg of total mycobacterial protein/dose in experimentally-infected cattle (n=5). Responses for each animal were...
shown with similar symbols. Results represented as the increase in skin thickness between 0 and 72 h post-inoculation. The dashed horizontal line indicated the 1 mm positive cut-off. Mean responses for dilutions of 4-Protein biobeads containing 3, 1, 0.33 and 0.11 µg mycobacterial protein were significantly greater than those for 0.04 and 0.01 µg protein dilutions ($P<0.05$).

FIG 4. Skin test responses for two doses of the 4-Protein biobeads in naturally $M. bovis$-infected cattle undertaken in the comparative cervical and caudal fold tests on the same day ($n=7$). (A) Comparative cervical skin test responses for the 4-Protein biobeads (ESAT-6, CFP10, Rv3615c and Rv3020c) at doses of 1 and 3 µg mycobacterial protein and bovine PPD (3,000 IU/dose, Prionics). (B) Comparative caudal fold skin test responses for the 4-Protein biobeads (ESAT-6, CFP10, Rv3615c and Rv3020c) at 1 and 3 µg mycobacterial protein) and bovine PPD (3,000 IU/dose, Prionics). Mean response for bovine PPD in the comparative cervical skin test was significantly greater than those for the two doses of the 4-Protein biobeads ($P<0.05$), but not in the comparative caudal fold skin test. Overall responses in the comparative cervical skin test were significantly greater than those in the caudal fold skin test ($P<0.001$), while there were no significant differences between readings at 72 and 96 h post-inoculation.
Table 1. Cattle tested in the comparative cervical skin test with the biobead skin test reagents

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Age when tested</th>
<th>Biobead type tested</th>
<th>Time after vaccination, challenge or skin test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>27 months</td>
<td>3-Protein</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9 months</td>
<td>4-Protein</td>
<td>N/A</td>
</tr>
<tr>
<td>BCG-vaccinated</td>
<td>12</td>
<td>27 months</td>
<td>3-Protein</td>
<td>11 weeks post-vaccination</td>
</tr>
<tr>
<td>Experimentally <em>M. bovis</em> infected</td>
<td>10</td>
<td>12 months</td>
<td>3-Protein</td>
<td>27 weeks post-challenge</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>33-months</td>
<td>3-Protein</td>
<td>11 weeks post-challenge</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12 months</td>
<td>4-Protein</td>
<td>10 weeks post-challenge</td>
</tr>
<tr>
<td>Naturally <em>M. bovis</em>-infected</td>
<td>11</td>
<td>Mixed age</td>
<td>3-Protein</td>
<td>11 weeks post-initial skin test</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Mixed age</td>
<td>3-, 4-Protein</td>
<td>15 weeks post-initial skin test</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Mixed age</td>
<td>4-Protein</td>
<td>10 weeks post-initial skin test</td>
</tr>
</tbody>
</table>
N/A Not applicable

- 3-Protein biobeads, displayed three mycobacterial proteins (ESAT-6, CFP10 and Rv3615c) on their surface
- 4-Protein biobeads, displayed four mycobacterial proteins (ESAT-6, CFP10, Rv3615c and Rv3020c) on their surface
- Cattle were also tested in the caudal fold test with the 4-Protein biobeads
Table 2. Skin test responses for the 3- and 4-Protein biobeads in naturally *M. bovis*-infected cattle undertaken in the comparative cervical and caudal fold tests on the same day (n=9). Comparative cervical skin test responses of the 3-Protein biobeads (ESAT-6, CFP10 and Rv3615c, 3 µg total mycobacterial protein), 4-Protein biobeads (ESAT-6, CFP10, Rv3615c and Rv3020c, 3 µg mycobacterial protein) and bovine PPD (3,000 IU/dose, Prionics). Comparative caudal fold skin test responses of the 4-Protein biobeads (ESAT-6, CFP10, Rv3615c and Rv3020c, 3 µg total mycobacterial protein) and bovine PPD (3,000 IU/dose, Prionics). Animals were listed, lowest to highest for responses to 3-Protein biobeads in the comparative cervical skin test.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Comparative cervical skin test responses (mm)</th>
<th>Comparative caudal fold skin test responses (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-Protein biobeadsb</td>
<td>4-Protein biobeadsb</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>4.5</td>
</tr>
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<td>10</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>11.5</td>
</tr>
<tr>
<td>7</td>
<td>12.5</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>12.5</td>
<td>14.5</td>
</tr>
<tr>
<td>9</td>
<td>13.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Mean (±SEM)</td>
<td>7.9 (±1.6)</td>
<td>8.7 (±1.7)</td>
</tr>
</tbody>
</table>

- a Increase in skin fold thickness between 0 and 72 h post-inoculation (mm).
- b Dose of the 3- and 4-Protein biobeads was 3 µg/0.1ml dose.
- c Dose of bovine PPD 3,000 IU/0.1ml dose (Prionics).

Mean for bovine PPD was significantly greater than means for the 3-Protein and 4-Protein biobeads in the comparative cervical skin test ($P < 0.01$).