

Display of Antigens on Polyester Inclusions Lowers the Antigen Concentration Required for a Bovine Tuberculosis Skin Test

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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 the 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 showed that the use of three or four recombinant mycobacterial proteins, including 6-kDa early secretory antigenic target (ESAT6), 10-kDa culture filtrate protein (CFP10), Rv3615c, and Rv3020c, or a peptide cocktail derived from those proteins, in the skin test greatly enhanced test specificity, with minimal loss of test sensitivity. The proteins are present in members of the pathogenic *Mycobacterium tuberculosis* complex but are absent in or not expressed by the majority of environmental mycobacteria and the BCG vaccine strain. To produce a low-cost skin test reagent, the proteins were displayed at high density on polyester beads through translational fusion to a polyhydroxyalkanoate synthase that mediates the 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 (0.1 to 3 µg of mycobacterial protein/inoculum) in the skin test. 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 high sensitivity for detection of *M. bovis*-infected cattle and induced minimal responses in animals exposed to environmental mycobacteria or vaccinated with BCG.

Control of bovine tuberculosis (TB), which is caused by infection with *Mycobacterium bovis*, is critical, as this disease is of great economic and zoonotic importance. Programs for eradication in cattle are primarily based on use of the tuberculin skin test for diagnosis of the disease, with slaughter of reactor animals. This approach has been instrumental in eradication of this disease from a number of countries (1). The tuberculin skin test is a delayed-type hypersensitivity (DTH) test that was developed more than 100 years ago for the diagnosis of TB, and it has proved to be a simple, inexpensive, robust, and widely accepted test. However, the test lacks specificity when animals have been sensitized to environmental mycobacteria or have been vaccinated with the human TB vaccine, *M. bovis* bacille Calmette-Guérin (BCG) (2). Estimates of sensitivity for a single intradermal test in cattle using purified protein derivative (PPD) prepared from *M. bovis* (bovine PPD) have ranged from 63.2% to 100% (median, 83.9%), with specificity between 75.5% and 99.0% (median, 96.8%) (reviewed in reference 2). Development of a more specific skin test reagent would be highly desirable. The 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). BCG cannot be used currently, as it compromises 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 that are present in nonpathogenic environmental mycobacteria. The two major formats for the tuberculin skin test are the caudal fold test, in which bovine PPD is injected intradermally in the caudal fold of the tail, and the single intradermal comparative cervical test (SICCT), in which 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, namely, 6-kDa early secretory antigenic 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 nonpathogenic environmental mycobacteria and the BCG vaccine strain (4–7). Those proteins, or peptides derived from those proteins, have been shown to enhance skin test specificity in cattle, while maintaining relatively high sensitivity for the diagnosis of bovine TB (8, 9). More recently, the addition of peptides from a fourth specific mycobacterial protein, Rv3020c, further enhanced test sensitivity (10). The tuberculin skin test is used as the primary screening test

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for bovine TB, and three major criteria must be satisfied before bovine PPD can be replaced with specific antigens. Use of specific antigens must provide improved test specificity with minimal loss of test sensitivity, and the cost of the reagents must be similar to that of 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 reagents.

The cost of reagents 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 displaying ESAT-6, CFP10, and Rv3615c on polyester inclusions (biobeads) produced by *Escherichia coli*, and preliminary results have indicated their utility in skin testing for the diagnosis of bovine TB (11). Polyester inclusions are naturally produced by various bacteria during imbalanced nutrient availability in which excess carbon is available and is deposited as spherical water-insoluble cytoplasmic inclusions (12, 13). The polyesters are composed of (*R*)-3-hydroxy-fatty acids with different carbon chain lengths (14). Foreign proteins have been displayed on the polyester beads by translationally fusing them to a polyester synthase (PhaC), which has mediated formation of protein-displaying beads in recombinant *E. coli* (15, 16). The beads are 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 the beads showed adjuvant properties by enhancing the immune response to the displayed antigen, compared to its soluble counterpart (18).

This 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 noninfected animals. In addition, biobeads were designed and produced to display four mycobacterial proteins, i.e., ESAT-6, CFP10, Rv3615c, and Rv3020c (4-protein biobeads) simultaneously, potentially increasing test sensitivity (10). Such biobeads were successfully produced and analyzed, and skin test performance experiments showed that they were effective in identifying *M. bovis*-infected animals in skin tests, using very low concentrations of the mycobacterial proteins.

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 noninfected animals were from TB-free herds located in TB-free regions of New Zealand, some of which had been naturally exposed to environmental mycobacteria, as indicated by strong skin test or gamma interferon (IFN- γ) responses to avian PPD. The BCG-vaccinated cattle were cattle that had been vaccinated subcutaneously with BCG (2×10^5 to 8×10^5 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). The 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 described previously (20). The naturally *M. bovis*-infected cattle were from infected herds from the west coast of the South Island and Waikato, New Zealand, and the three groups of animals were identified as infected with *M. bovis* on the basis of positive results for initial caudal fold skin tests with bovine PPD. All of the experimentally *M. bovis*-infected cattle and all except one of the naturally *M. bovis*-infected cattle were confirmed as infected through culture of *M. bovis* from tissues obtained at slaughter,

TABLE 1 Cattle tested with comparative cervical skin tests with biobead skin test reagents

Group	No. of animals	Age when tested (mo)	Biobeads tested	Time after vaccination, challenge, or skin test
Control	12	27	3-protein ^a	NA ^b
	24	9	4-protein ^c	NA
BCG-vaccinated	12	27	3-protein	11 wk after vaccination
Experimentally <i>M. bovis</i> -infected	10	12	3-protein	27 wk after challenge
	12	33	3-protein	11 wk after challenge
	10	12	4-protein	10 wk after challenge
Naturally <i>M. bovis</i> -infected	11	Mixed ages	3-protein	11 wk after initial skin test
	9	Mixed ages ^d	3-protein and 4-protein	15 wk after initial skin test
	7	Mixed ages ^d	4-protein	10 wk after initial skin test

^a The 3-protein biobeads displayed three mycobacterial proteins (ESAT-6, CFP10, and Rv3615c) on their surfaces.

^b NA, not applicable.

^c The 4-protein biobeads displayed four mycobacterial proteins (ESAT-6, CFP10, Rv3615c, and Rv3020c) on their surfaces.

^d Cattle were also tested in the caudal fold test with the 4-protein biobeads.

with confirmation for the remaining animal on the basis of typical gross and histopathological lesions. All animal manipulations were approved by the Grasslands Animal Ethics Committee, New Zealand.

Antigens. Bovine and avian PPDs were supplied by AsureQuality (Upper Hutt, New Zealand) and Prionics (Lelystad, The Netherlands). Purified recombinant proteins, i.e., 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, i.e., 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 that simultaneously displayed four mycobacterial antigens, i.e., 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 by Genscript (USA), with codon optimization for expression in *E. coli*. 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. The plasmid pMCS69 contains the genes *phaA* and *phaB* from *Ralstonia eutropha*, which both mediate synthesis of the polyester precursor (*R*)-3-hydroxybutyryl-CoA.

To confirm that the bead preparations were sterile, samples of the preparations were spread on LB agar. In the later experiments 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 the biobead preparations to keep the beads in suspension.

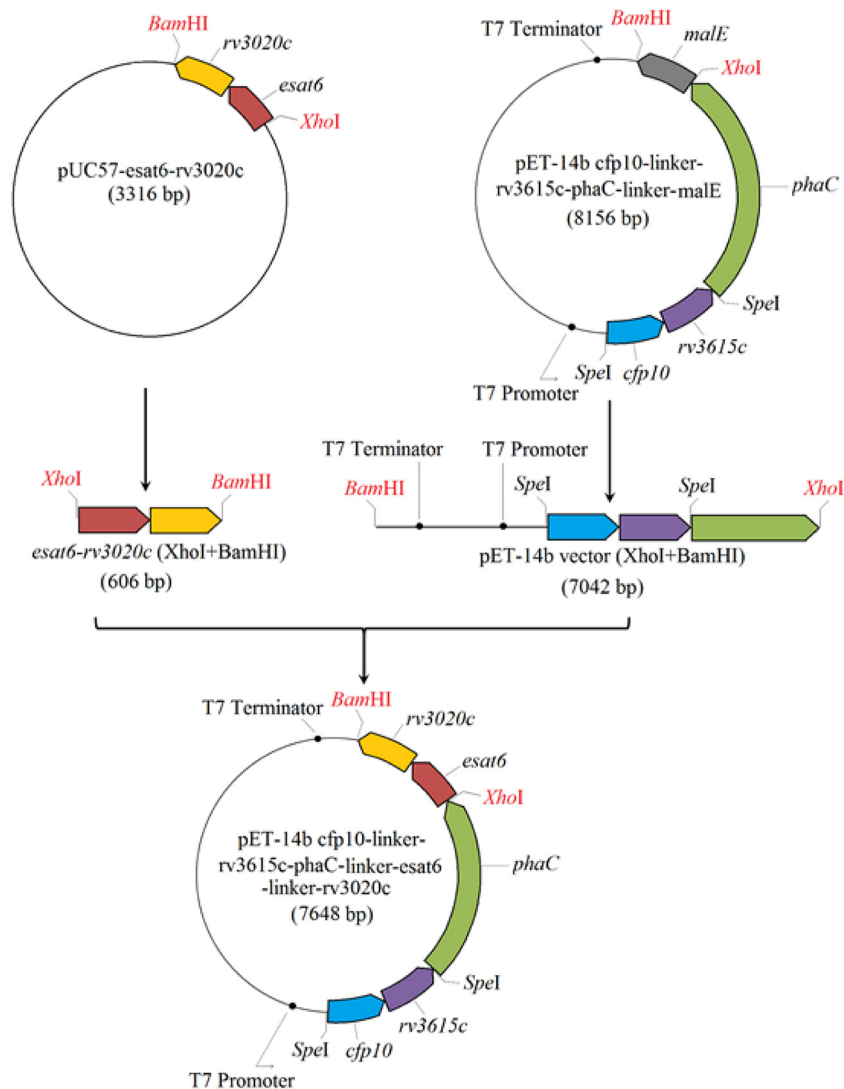


FIG 1 Cloning strategy for production of the CFP10-Rv3615c-PhaC-ESAT6-Rv3020c fusion protein.

Analysis of proteins attached to 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 on a 8% polyacrylamide gel and was stained with Coomassie blue. Proteins of interest were excised from the gels and subjected to tryptic peptide fingerprinting using matrix-assisted laser desorption ionization–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. Then the proportion of protein attributed to the fusion protein was determined by densitometry, and the amounts of mycobacterial proteins contained in the fusion protein were calculated from the molecular masses of the mycobacterial proteins in comparison with that of the PhaC protein.

Testing for sensitizing effects. The method used to determine whether the biobeads had sensitizing effects 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 were injected intradermally on the abdominal flank with 0.1 ml of the 4-protein biobead skin test reagent, containing 0.3 μ g of mycobacterial proteins, on three occasions, with intervals of 5 days. The concentration of biobeads was one-tenth of the highest cattle dose

and was equivalent to the one-tenth of the cattle dose of bovine tuberculin (500 IU) used to assess 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 biobead reagent. The guinea pigs were examined 24 to 28 h 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 spaces between the sites. The hair at each site was clipped, and the skin thickness at the injection site was measured with calipers immediately prior to injection and 72 h later, with results being expressed as the change in skin thickness (in millimeters). Changes in skin thickness of <1 mm could not be measured accurately, and the positive cutoff value was set at a ≥ 1 -mm increase in skin thickness between 0 and 72 h postinoculation. Different concentrations of the biobeads displaying mycobacterial proteins were prepared by dilution in phosphate-buffered saline (PBS). Bovine PPD (5,000 IU/0.1 ml for PPD fromASUREQuality [Upper Hutt, New Zealand] or 3,000 IU/0.1 ml for PPD from Prionics [Lelystad, The Netherlands]) was included in each test. Manufacture and

supply of the AsureQuality PPDs were discontinued midway through the study. The comparative caudal fold skin test was performed by intradermally injecting a 0.1-ml volume containing the 4-protein biobead reagent into the caudal fold on one side of the tail and a 0.1-ml volume containing bovine PPD (3,000 IU; Prionics) into the caudal fold on the other side. The caudal folds were palpated 72 h after injection, and any detectable lumps were measured with calipers. 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 being 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 treatment serving as the fixed effect and animal as the random effect. For analysis of data for experimentally 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 treatment serving as the fixed effect and animal plus treatment nested within experiment as the random effects. A mixed-effects model was used for comparison of injection sites, reagents, and times of test reading (hours), with site, reagent, and time as the fixed effects and animal as the random effect. The results from these models provided multiple comparisons of the predicted means, with *P* values adjusted by the Benjamini-Hochberg method (23). The analyses were performed with the R packages nlme, lme4', and predictmeans in R 3.2.0 (24, 25). Statistical significance was denoted by *P* values of <0.05.

RESULTS

Engineering of *E. coli* for production of 4-protein biobeads. A plasmid encoding PhaC and the mycobacterial genes *esat6*, *cfp10*, *Rv3615c*, and *Rv3020c* was 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 *E. coli* cells. Formation of intracellular polyester beads in the *E. coli* cells was indicated by isolation of a white suspension from disrupted cells (data not shown). Gas chromatography-mass spectrometry (GC-MS) analysis confirmed that the cells were accumulating the polyester polyhydroxybutyrate, which constitutes the core of the polyester beads, contributing about 34% of the cellular dry weight. SDS-PAGE analysis demonstrated that antigen-displaying beads showed a prominent protein with an apparent molecular mass of 107 kDa for the PhaC-4 mycobacterial fusion protein, compared to a molecular mass of 98 kDa for the PhaC-3 mycobacterial fusion protein (data not shown). The molecular mass of the PhaC protein alone was 64 kDa. The components of the fusion protein were identified by tryptic peptide fingerprinting using MALDI-TOF MS (see Table S1 in the supplemental material). Densitometric analysis of the SDS-PAGE results 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 fractions (data not shown).

Sedimentation of the beads in the injection syringe was overcome by the addition of dextran to a final concentration of 15% (wt/vol). All animal studies with the 4-protein biobeads, and with the 3-protein biobeads used for comparison with the 4-protein biobeads, utilized preparations that contained 15% dextran and had been 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 and γ -irradiation did not affect the magnitude of the skin test responses (data not shown).

Testing for sensitizing effects in guinea pigs. The 4-protein biobeads were tested for induction of sensitization at a dose of 0.3 μ g mycobacterial protein (one-tenth of the cattle dose). There was no difference in the reactions at the skin test sites of the guinea pigs that had received multiple doses of the skin test reagent at 5-day intervals versus those that had received a single dose of the reagent, when examined 25 h postinoculation. Each of the three vaccinated guinea pigs had a small zone of erythema at the site of inoculation (3, 4, and 5 mm in diameter), with identical readings for the three nonvaccinated animals. In addition, two of the three vaccinated guinea pigs had a small area of induration (2 mm in diameter; 0 mm for the other guinea pig), while all three nonvaccinated 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 (≥ 1 -mm increase in skin thickness) 72 h following injection of the 3-protein biobeads (3 μ g of mycobacterial protein), three recombinant proteins (30 μ g of total mycobacterial protein; 10 μ g of each protein), and bovine PPD (5,000 IU; AsureQuality). Twenty-one of the 22 cattle tested positive with the 3-protein biobeads (1 μ g of mycobacterial protein/dose) (Fig. 2A). The only significant difference between the reagents was that the mean response with bovine PPD was greater than that with the 3-protein biobeads (1 μ g/dose; *P* < 0.05). In naturally infected cattle, the two concentrations of the 3-protein biobeads (1 and 3 μ g/dose) produced responses in 10 of the 11 infected animals, while all tested positive with bovine PPD (5,000 IU; AsureQuality) (Fig. 2B). The mean response for the 3-protein biobeads (3 μ g/dose) was significantly greater than that for bovine PPD (*P* < 0.01). One animal tested negative with the two concentrations of the 3-protein biobeads (Fig. 2B) and also with the three recombinant proteins used at a dose of 10 μ g of total mycobacterial protein (data not shown). There was insufficient recombinant protein for testing at the recommended dose of 30 μ g of total recombinant protein. Twelve cattle that had been vaccinated with BCG vaccine at 2 to 4 weeks of age and revaccinated 2 years later were skin tested 11 weeks after revaccination. Eleven of the 12 cattle tested positive with bovine PPD (5,000 IU; AsureQuality), 2 of 12 tested positive with the 3-protein biobeads (3 μ g/dose), and none tested positive with the 3-protein biobeads (1 μ g/dose) or the recombinant proteins (30 μ g/dose) (Fig. 2C). The noninfected cattle included 12 animals, some of which had been naturally exposed to environmental mycobacteria; six of those animals responded positively to avian PPD (2,500 IU; AsureQuality) in the skin test (data not shown). All tested negative with the 3-protein biobeads (1 and 3 μ g/dose) and the recombinant proteins (30 μ g/dose), while two animals showed reactivity with 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 μ g of mycobacterial protein were tested in the comparative cervical skin test in five cattle that had been 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, or 0.11 μ g mycobacterial protein. In contrast, the mean responses for the 4-protein biobeads containing 0.04 or 0.01 μ g mycobacterial protein were significantly lower than those

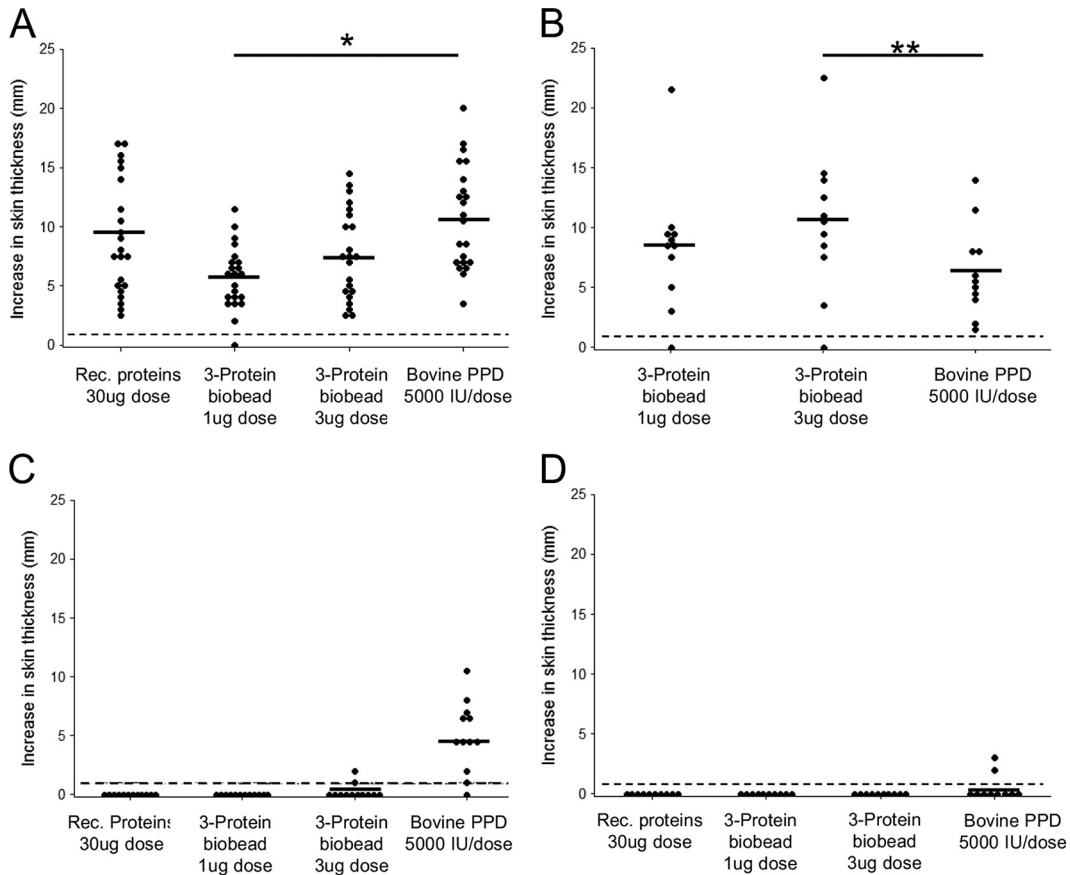


FIG 2 Skin test responses to the 3-protein biobeads in cattle. Comparative cervical skin test responses for recombinant (Rec.) ESAT-6, CFP10, and Rv3615c proteins (total protein dose of 30 μ g), 3-protein biobeads (ESAT-6, CFP10, and Rv3615c) at 1 μ g mycobacterial protein and 3 μ g mycobacterial protein, and bovine PPD (5,000 IU/dose; AsureQuality) are shown. Results are presented as increases in skin thickness between 0 and 72 h postinoculation. (A) Cattle experimentally infected with *M. bovis* ($n = 22$). (B) Cattle naturally infected with *M. bovis* ($n = 11$); the recombinant proteins (30 μ g dose) were not available for testing for this group. (C) BCG-vaccinated cattle ($n = 12$). (D) Naive (noninfected and nonvaccinated) cattle ($n = 12$). Dashed horizontal lines, 1-mm positive cutoff value. *, $P < 0.05$; **, $P < 0.01$. In panel A, there were no significant differences between the recombinant proteins and the two doses of the 3-protein biobeads ($P > 0.05$). Horizontal bars, means.

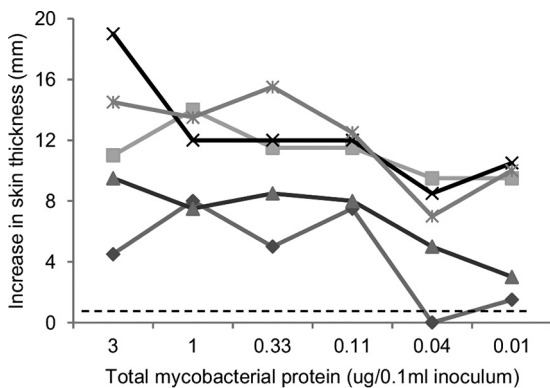


FIG 3 Skin test results for 4-protein biobead dose titration. Comparative cervical skin test responses in experimentally infected cattle ($n = 5$) for dilutions of the 4-protein biobeads (ESAT-6, CFP10, Rv3615c, and Rv3020c) containing 3, 1, 0.33, 0.11, 0.04, or 0.01 μ g of total mycobacterial protein/dose are shown. Responses for different animals are shown with different symbols. Results are presented as increases in skin thickness between 0 and 72 h postinoculation. Dashed horizontal lines, 1-mm positive cutoff value. Mean responses for dilutions of 4-protein biobeads containing 3, 1, 0.33, and 0.11 μ g mycobacterial protein were significantly greater than those for dilutions containing 0.04 and 0.01 μ g protein ($P < 0.05$).

for the four higher doses of the biobeads ($P < 0.05$) (Fig. 3). A total of 24 noninfected animals (9 months of age) were tested with the 4-protein biobeads (3 μ g/dose) and bovine PPD (3,000 IU; Prionics) in the comparative cervical skin test, and no detectable increases in skin thickness of ≥ 1 mm were detected in any of those 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 μ g mycobacterial protein) and bovine PPD (3,000 IU; Prionics) (Table 2). The mean response for bovine PPD was significantly greater than those for the 3-protein and 4-protein biobeads ($P < 0.01$), while responses for the two biobead preparations were very similar. In a comparative caudal fold test that was performed on the same day as the cervical skin test, there were no significant differences between the mean responses for the 4-protein biobeads (3 μ g mycobacterial protein) and bovine PPD (3,000 IU; Prionics) (Table 2). Similar results were observed in a second group of 7 naturally infected animals in which two doses of the 4-protein biobeads were compared with bovine PPD in the cervical and caudal fold skin tests conducted on the same day (Fig. 4). The skin responses were significantly greater for bovine PPD than for the two doses of the 4-protein biobeads in

TABLE 2 Skin test responses for 3- and 4-protein biobeads in naturally *M. bovis*-infected cattle in comparative cervical and caudal fold tests performed on the same day ($n = 9$)^a

Animal no.	Increase in skin fold thickness (mm) ^b				
	Comparative cervical skin test			Comparative caudal fold skin test	
	3-protein biobeads	4-protein biobeads	Bovine PPD	4-protein biobeads	Bovine PPD
1	1.5	2	5.5	2	1.5
2	2.5	4.5	9	5	4
3	3	4	8	2	6
4	4.5	4.5	9.5	1.5	3
5	10.5	10	25	5	6
6	11	11.5	14.5	7	6
7	12.5	12	12	9	3
8	12.5	14.5	11	9	4
9	13.5	15.5	18	9	5
Mean ± SEM	7.9 ± 1.6	8.7 ± 1.7	12.5 ± 2.0 ^c	5.5 ± 1.0	4.3 ± 0.5

^a Comparative cervical skin test responses to 3-protein biobeads (ESAT-6, CFP10, and Rv3615c) (3 µg total mycobacterial protein), 4-protein biobeads (ESAT-6, CFP10, Rv3615c, and Rv3020c) (3 µg total mycobacterial protein), and bovine PPD (3,000 IU/dose; Prionics) and comparative caudal fold skin test responses to 4-protein biobeads (ESAT-6, CFP10, Rv3615c, and Rv3020c) (3 µg total mycobacterial protein) and bovine PPD (3,000 IU/dose; Prionics) are shown. Animals are listed from lowest to highest for responses to 3-protein biobeads in the comparative cervical skin test. SEM, standard error of the mean.

^b Increase in skin fold thickness between 0 and 72 h after inoculation.

^c The mean for bovine PPD was significantly greater than the means for the 3-protein and 4-protein biobeads in the comparative cervical skin test ($P < 0.01$).

the comparative cervical test ($P < 0.001$) but not in the caudal fold test. For the 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 when the tests were read at 72 and 96 h postinjection.

DISCUSSION

Recent studies have demonstrated that skin testing with three or four specific mycobacterial proteins, or peptides derived from those proteins, could be used to diagnose bovine TB in cattle (8, 10). The 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 animals vaccinated with BCG vaccine. As the skin test is the primary screening test for TB diagnosis in cattle, the cost of the skin test reagents is an important consideration. It was reported recently that display of three 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 is required with the proteins displayed as a fusion protein on single biobeads.

In the current study, no significant differences in mean increases in skin thicknesses were observed between the 3-protein biobeads and the three recombinant proteins in the comparative cervical skin test with experimentally infected cattle, despite concentrations of the mycobacterial proteins in the biobeads being 10- and 30-fold lower than those of 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 with the low dose (1 µg of mycobacterial protein) than with bovine PPD in experimentally infected animals, while significantly greater mean skin thicknesses were observed with the high dose (3 µg of mycobacterial protein) than with bovine PPD in a group of naturally infected animals.

The comparative sizes of responses to recombinant proteins versus bovine PPD also varied in studies undertaken in Spain (9) and the United Kingdom (8) with naturally infected cattle; no

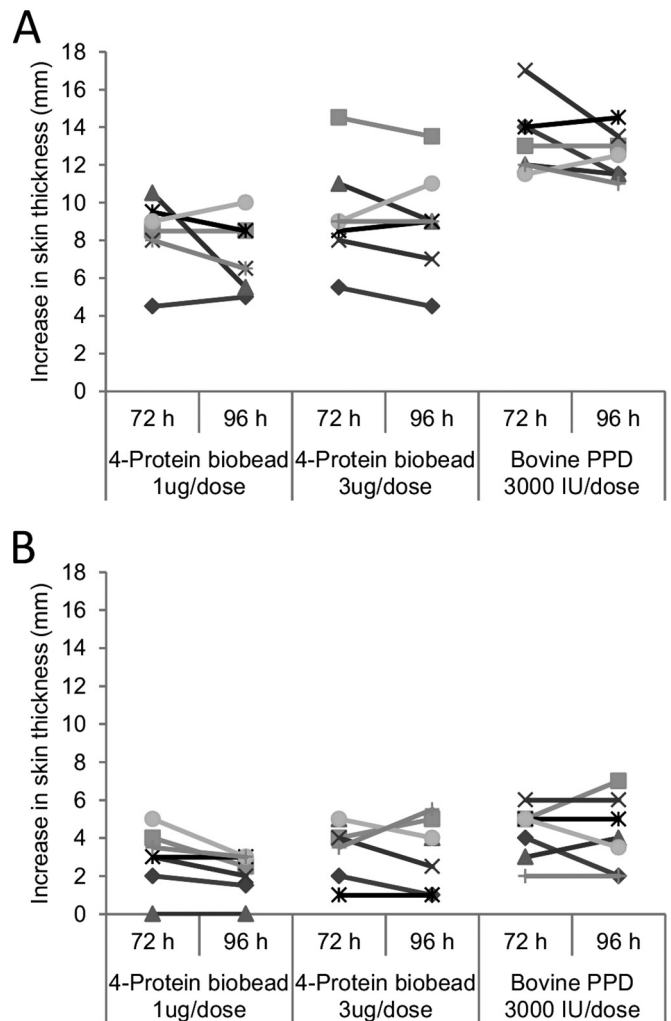


FIG 4 Skin test responses to two doses of the 4-protein biobeads in naturally *M. bovis*-infected cattle ($n = 7$) in comparative cervical and caudal fold tests performed on the same day. Responses for different animals are shown with different symbols. (A) Comparative cervical skin test responses for 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 doses of 1 and 3 µg mycobacterial protein and bovine PPD (3,000 IU/dose, Prionics). The mean response for bovine PPD was significantly greater ($P < 0.05$) than those for the two doses of the 4-protein biobeads in the comparative cervical skin test but not in the comparative caudal fold skin test. Overall responses in the comparative cervical skin test were significantly greater ($P < 0.001$) than those in the caudal fold skin test, while there were no significant differences between readings at 72 and 96 h postinoculation.

differences were noted for the pool of the three recombinant proteins versus bovine PPD in one study (9), while responses were smaller for the proteins in the other study (8). Comparisons of test sensitivity in naturally infected animals need to be interpreted with caution, as the animals often are selected for retesting based on initial positive responses to bovine PPD. The sizes of the responses to specific proteins or to the complex mixture of components in PPD could depend on the stage of infection and the infecting strain of *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 through induction of an anti-inflammatory T cell-type immune response (26). In the current study, the low dose of the 3-protein biobeads (1 μ g mycobacterial protein) and the three recombinant proteins had high specificity, with no positive responses in naive or BCG-vaccinated animals. It is possible that low concentrations of *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 United Kingdom showed that the addition of peptides derived from the mycobacterial protein Rv3020c to a peptide cocktail derived from ESAT-6, CFP10, and Rv3615c proteins increased test sensitivity without compromising specificity (10). Based on these findings, biobeads were constructed that contained the fusion protein CFP10-Rv3020c-PhaC-Rv3615c-ESAT6, which included the enzyme PhaC required for biobead formation. Analyses by tryptic peptide fingerprinting using MALDI-TOF MS and SDS-PAGE confirmed the identity of the fusion proteins and functionality. A critical requirement for a skin test reagent is that it must not induce sensitization. Using the Office International des Epizooties (OIE) World Organisation for Animal Health protocol for testing batches of tuberculin for sensitizing effects (22), one-tenth of the cattle dose of the 4-protein biobeads did not induce sensitization in guinea pigs.

Dilutions of the 4-protein biobeads were tested in the comparative cervical skin test with experimentally infected cattle, and dilutions containing 3 to 0.11 μ g of mycobacterial protein/0.1-ml dose induced skin test responses of similar sizes. In contrast to those findings, Whelan et al. (8) noted marked decreases in the sizes of skin test responses for naturally TB-infected animals when the dose of recombinant proteins was decreased from 5 or 10 μ g to 1 μ g for each individual protein. The more rapid decline in the dose-response relationship may be related to the testing of naturally infected animals, for which responses often are weaker and more variable.

The 4-protein biobead preparation (3 μ g mycobacterial protein/0.1 ml dose) was shown to be effective in both the comparative cervical and caudal fold skin tests for identifying naturally infected cattle. Although the 4-protein biobeads (3 μ g/dose) and bovine PPD positively identified the same numbers of animals, the sizes of the responses were greater for bovine PPD in two groups of naturally infected animals with the cervical test but not the caudal fold test. In addition, the sizes of the responses overall were greater in the cervical test than 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 were no differences in the responses for 1- and 3- μ g/dose 4-protein biobead preparations (Fig. 4). Overall, there were no differences in the responses to the 4-protein biobeads or bovine PPD when the cervical and caudal fold tests were read at 72 versus 96 h postinoculation. Pollock et al. (28) considered that skin test reactions to ESAT-6 were often greatest at 96 h postinoculation, while Whelan et al. (8) considered that there were no differences between the reactions to ESAT-6, CFP10, and Rv3615c proteins at 72 versus 96 h. Measuring responses to both bovine PPD and specific mycobacterial proteins at 72 h postinoculation would be the most practical option.

In recent caudal fold field testing of the 4-protein biobeads in noninfected cattle, a small hard lump, <1 mm in size, was observed at the inoculation site for the 3- μ g protein dose in a small proportion of the animals, while such reactions were less frequent with the 1- μ g protein dose (B. Buddle, unpublished observations). This suggested that the 1- μ g 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 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 with 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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