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Field Evaluation of a Novel Differential Diagnostic Reagent for the Detection of *Mycobacterium bovis* Infected Cattle

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Short title – *M. bovis* diagnostic reagent for cattle

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ABSTRACT

In the search for improved tools with which to control bovine tuberculosis, the development of enhanced immunodiagnostic reagents is a high priority. Such reagents are required to improve the performance of tuberculin based reagents and to allow the discrimination of vaccinated cattle from those infected with *Mycobacterium bovis*. In this study we identified the immunodominant, frequently recognized peptides from Rv3873, Rv3879c, Rv0288, and Rv3019c, which, together with peptides comprising the current lead diagnostic antigens, ESAT-6 and CFP-10, were formulated into a peptide cocktail. Testing naturally infected cattle, this cocktail was significantly better in identifying skin-test negative animals with confirmed bovine tuberculosis, than tuberculin. In addition, the specificity of this cocktail was not compromised by BCG vaccination. In summary, our results prioritize this peptide-based, fully synthetic, reagent for assessment in larger trials.

INTRODUCTION

For the last two decades cases of bovine tuberculosis (BTB) in Great Britain have been increasing at an annual rate of 16% (1). At present the BTB control program involves regular skin-testing with the single intradermal comparative tuberculin test (SICTT) followed by compulsory slaughter of cattle with a positive result. In addition, the bovine gamma interferon (IFN-γ) ELISA (BOVIGAM™), also using tuberculin, has been used recently in the UK as an ancillary test to supplement the SICTT in specific situations. Vaccination has also been considered as a viable method of BTB control, however the only potentially available vaccine at present, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), sensitizes animals to the SICTT (2, 4, 29). Nevertheless, it is likely that the next generation of BTB vaccines will focus on the augmentation of BCG or its inclusion in prime-boost vaccination regimes (10). Therefore, the use of such vaccination strategies will require the development of differential diagnostic reagents that can distinguish vaccination from infection (7).

Recently, we described the identification of 13 candidate diagnostic antigens that were deleted from the genome of BCG Pasteur during its attenuation, but are still present in the *M. bovis* genome (7). Pools of overlapping peptides, representing the amino acid sequences of the deleted genes, were screened for their ability to stimulate IFN-γ in whole blood from cattle experimentally infected with *M. bovis*, from cattle vaccinated with BCG or from naïve cattle. This systematic screen resulted in a hierarchical list of immunogenic antigens, some of which were suitable candidates for the development of diagnostic reagents to either differentiate between *M. bovis* infected and BCG vaccinated animals or to improve the specificity of tuberculin *per se* (7, 11) Interestingly, the

immunogenicity of the most strongly recognized antigens was frequently concentrated in a single peptide pool rather than distributed amongst all peptide pools needed for complete coverage of the test protein. Among the potential diagnostic proteins prioritized for further evaluation were the RD1 region products Rv3873 and Rv3879c (7). However, individual peptides carrying immunodominant epitopes were not identified during these experiments.

In addition to the antigens identified in our previous study, a number of groups including ourselves have demonstrated the immunogenicity of the ESAT-6 family of proteins. In particular, ESAT-6 and CFP-10 have been shown to be outstanding diagnostic target proteins for cattle and humans (6, 12, 23, 27). Moreover, other ESAT-6-like proteins including Rv0288 and Rv3019c are also targets of human and bovine T cell responses (23, 28), and so were incorporated into this present study.

Although experimentally infected animals are useful for the initial assessment of diagnostic reagents, there are limitations of using such animals that can only be addressed by using naturally infected animals. For example, due to differences in responses between experimentally infected cattle and field reactors, it is important that assay cut-offs for positivity are defined by testing naturally infected cattle. Once such cut-offs have been set, the sensitivity of the reagents can be evaluated in populations of cattle with different infection and disease status.

The objective of this current study was to identify and formulate a sensitive and specific diagnostic cocktail of peptides. We describe the definition of immunodominant peptides from the *M. tuberculosis* genes Rv3873, Rv3879c, Rv3019c, Rv0288, ESAT-6 and CFP-10, and demonstrate the diagnostic potential of a cocktail composed of these

dominant peptides using naturally infected field animals.

MATERIAL AND METHODS

Antigens and Peptides

Bovine tuberculin (PPD B) and avian tuberculin (PPD A) were supplied by the Tuberculin Production Unit at the Veterinary Laboratories Agency, Weybridge, Surrey, UK and were used to stimulate whole blood at 10μg/ml. Staphylococcal enterotoxin B (SEB) was used as a positive control at 5μg/ml. Peptides between 16-20 amino acids in length, were synthesized, quality assessed and formulated into a peptide cocktail (10μg/ml/peptide), as previously described (7).

Cattle used in this study

All animal experiments were conducted within the limits of a UK Home Office licence under the Animal (Scientific Procedures) Act 1986, which was approved by the local Ethical Reviews Committee.

Uninfected controls

Heparinised blood was obtained from tuberculin skin-test negative calves in herds free of bovine tuberculosis and located in non-TB endemic regions of England (n=55). These animals produced IFN-γ *in vitro* after stimulation with tuberculin from *M. avium,* indicating that they had been exposed to environmental mycobacterial species.

Experimental infection of cattle with *M. bovis*

Calves (n=6) (6 months old, Friesian) were infected with an *M. bovis* field strain from Great Britain (GB) (AF 2122/97) (9), by intratracheal instillation of between 1 x 10^2 colony forming unit (CFU) and 1 x 10^3 CFU as previously described (8). Bovine tuberculosis was confirmed in these animals by the presence of visible lesions in lymph

nodes and/or lungs found at *post-mortem* examinations, by the histo-pathological examination of lesioned tissues and the culture of *M. bovis* from tissue samples collected from lymph nodes and lungs.

Cattle naturally infected with *M. bovis*

Heparinised blood samples were obtained from herds with a history of BTB infection by the State Veterinary Service and contained naturally infected, SICTT positive reactors and SICTT negative cattle (n=58). The status of the animals were confirmed at *post-mortem* by the examination of the animal for lesions and culture of *M. bovis* from tissues.

BCG vaccination

Calves (n=10) (6 months old, Friesian) were vaccinated with BCG Pasteur by subcutaneous injection of $1x10^6$ CFU into the side of the neck as described previously (4, 26). The inoculum was prepared by the dilution of a frozen stock of BCG Pasteur of known CFU in phosphate buffered saline (PBS). The vaccinating dose was confirmed by plating the inoculum on 7H10 agar supplemented with OADC and incubated for 4 weeks at 37^{o}C. Blood was taken between 4-6 weeks post-vaccination.

IFN-γ enzyme-linked immunosorbent assay

0.1ml of heparinised blood was mixed with 0.1ml of antigen solution, SEB or medium (RPMI containing 5% complete processed serum replacement-type 3 [CPSR-3], Sigma, Poole, Dorset) in 96-well plates and incubated for 48hrs at 37°C and 5% CO_2. IFN-γ concentration in culture supernatants was measured using the BOVIGAM ELISA kit (Prionics, Zurich, Switzerland) following the manufacturers instructions. Optical

density was determined at 450nm (OD_{450}) and response to medium control was subtracted from the response to antigen (ΔOD450), positive response: ΔOD450>0.1.

ROC curve analysis

Analysis was preformed using Analyse-it™ software (Leeds, UK).

RESULTS

Identification of individual immunodominant peptides for a diagnostic cocktail

To identify the most frequently and strongly recognized peptides within the immunodominant pools of Rv3873 (representing amino acid residues 89-188), Rv3879c (representing amino acid residues 1-92), Rv0288, and of Rv3019c, individual peptides were assayed in experimentally *M. bovis* infected cattle. As shown in figure 1, dominant peptides could be readily identified. For example, peptide 9.3 and 9.9 from Rv3879c were predominantly recognized by 80 and 50% of cattle tested, respectively, which responded to the total pool of 11 peptides, whilst the other 7 peptides were recognized by <20% of cattle, or not at all (Fig. 1). Peptides were also assayed in uninfected cattle where no responses were observed (data not shown).

Using this data, peptides were selected for further study if they generated strong responder frequencies individually, or if they had a synergistic effect when assayed in combination with other peptides, on the basis that peptides fulfilling these criteria would increase the sensitivity of a peptide cocktail. In a further attempt to increased signal strength, several peptides were selected that stimulated strong IFN-γ responses despite the fact that they provided poor responder frequencies. Peptides identified by this screen were then formulated into a single peptide cocktail (Cocktail 1), that also included peptides from ESAT-6 and CFP-10 that we had previously demonstrated to be highly immunogenic (27, 28) (Table 1).

Receiver Operator Characteristic (ROC) Curve Analysis

Test cut-off values for the peptide cocktail were determined by Receiver Operator Characteristic (ROC) curve analysis using a group of field animals with known *M. bovis* disease status (confirmed positives), and a group of animals free of *M. bovis* infection (confirmed negatives) (22). All *M. bovis* infected cattle were classified by the presence of visible lesions (VL) at slaughter and by the culture of *M. bovis* from tissue samples.

The performance of the peptide cocktail (Cocktail 1) was compared with the previously described lead diagnostic reagent consisting of a complete set of overlapping peptides representing the antigens ESAT-6 and CFP-10, and the comparison of responses against bovine and avian tuberculin (PPD-B – PPD-A). The area under the ROC curve represents the accuracy of a test using a given antigen and was used to determine the ability of each diagnostic reagent to discriminate correctly between cattle with or without disease (15). Table 2A shows the results of such an analysis: Cocktail 1 was found to be more accurate at diagnosing *M. bovis* infection in cattle than the ESAT-6/CFP-10 cocktail. Furthermore, both these peptide cocktails were more accurate than the bovine tuberculin biased response, as indicated by their superior area under the ROC curve (Table 2A), although the differences in the diagnostic accuracy were not statistically different.

Using the data from the graph it was also possible to establish, at predetermined specificities, the relative sensitivity of each peptide cocktail, or tuberculin, in relation to different OD450 cut-off values (Table 2B). The corresponding sensitivity levels were determined at specificities set at 91, 93 and 98%. From this analysis, the following

conclusions could be drawn. ESAT-6/CFP-10 and Cocktail 1 have an increased sensitivity at all three specificity levels chosen compared with that of tuberculin. With specificities set at 91% and 93%, respectively, Cocktail 1 detected 5.2% more infected cattle than ESAT-6/CFP-10 and 12.1% more than using avian and bovine tuberculin. ESAT-6/CFP-10 peptides and Cocktail 1 demonstrated equal sensitivity when the specificity was set at 98% (Table 2B).

Sensitivity of diagnostic reagents in relation to SICTT status

Figure 2 demonstrates the ability of ESAT-6/CFP-10 peptides, Cocktail 1 and the tuberculins, to diagnose *M. bovis* infection in animals with *M. bovis* culture confirmed bovine tuberculosis, that were either SICTT positive, SICTT negative or SICTT-suspects (inconclusive reactors, IR). We assessed their capabilities by performing ROC curve analysis using results from the different SICTT groups of *M. bovis* infected animals in conjunction with the cohort of uninfected cattle. Results are expressed as areas under the curve.

When the SICTT +ve animals were assessed, all three reagents performed at comparable levels. This was also the case for animals presenting with suspect tuberculin skin-test results (SICTT-IR, Fig. 2). In contrast, test performance, in detecting SICTT –ve animals, as measured by the areas under the ROC curve was better after the application of Cocktail 1 compared to ESAT-6/CFP-10 peptides and tuberculin, although the difference reached only statistical significance when compared to tuberculin ($p=0.0372$, Fig.2). In addition, the performance of ESAT-6/CFP-10 was not significantly better than that of tuberculin in this SICTT category (Fig.2).

Diagnosis of early infection

In order to ascertain whether the enhanced sensitivity of Cocktail 1, in *M. bovis* infected, visible lesioned, SICTT –ve cattle, was due to its earlier recognition after infection, ESAT-6/CFP-10 peptides, Cocktail 1, and avian and bovine PPD were assayed in cattle experimentally infected with *M. bovis*, at set time-points post-infection. A set of six experimentally infected calves were used in this time-course experiment. The initial responses to Cocktail 1 was detectable up to 1 week earlier than those against the ESAT-6/CFP-10 peptide cocktail in two animals, and 2 weeks earlier in a third calf (data not shown). However, the responses in the other 3 animals developed at the same time as those towards ESAT-6/CFP-10 peptides (data not shown). Although not statistically conclusive, this data suggests that the improved performance of Cocktail 1 by skin-test negative cattle could be due to its earlier recognition post-infection.

Differential diagnosis of BCG vaccination

Both ESAT-6/CFP-10 and Cocktail 1 were also assayed alongside avian and bovine tuberculin in 10 cattle vaccinated with BCG Pasteur. As expected, the specificity of avian and bovine PPD was compromised, as three of the ten animals responded positively. However, none of the BCG vaccinated animals gave positive IFN-γ responses after stimulation with ESAT-6/CFP-10 or Cocktail 1 (data not shown).

DISCUSSION

By using a peptide based approach, a diagnostic cocktail of immunodominant peptides was formulated from the sequences of six antigens, four of whose genes are deleted from the genome of BCG Pasteur. Peptides that contained immunogenic specific

epitopes were selected on the basis of their ability to stimulate elevated levels of IFN-γ in animals experimentally infected with *M. bovis*. Interestingly, the majority of peptides from Rv0288 and Rv3019c demonstrated immunogenicity, with no clear dominant peptide. This is similar to responses seen in other ESAT-6 family members (23, 24) and is most likely a result of the high epitope density within these antigens. By contrast, the antigenicity of the Rv3879c peptide pool was mainly a consequence of the recognition of two peptides, 9.3 and 9.9, with all others peptides generating responder frequencies of below 20%. We have demonstrated that the peptides described are recognized in animals from different herds, which implies that they are recognized promiscuously in the context of multiple BoLA-class II alleles - a prerequisite for diagnostic reagents. Such promiscuous peptides have been discussed frequently for antigens from *M. tuberculosis* and *M. bovis* (16, 18, 27).

The peptides were identified using blood samples from cattle experimentally infected with *M. bovis* taken at a set time-point post-infection. Whilst the use of experimentally infected cattle is convenient for first-line screening, the responses to antigens of naturally infected cattle can be different for a number of reasons. For example, the sample time-points in relation to the time of infection, and consequently their disease status, is not uniform in animals that were naturally infected. Also, repeat exposure to *M. bovis* may have an influence on the immune response not observed in experimentally infected cattle. Finally, the same strain of *M. bovis* (AF2122/97) was administered in our experimental model, while many different strains are isolated from the British herd that may differ in their ability to stimulate IFN-γ-mediated immune

responses (25). For these reasons, therefore, it is important to validate these defined reagents in field experiments (5, 27).

In this study we used results obtained from such naturally infected cattle with confirmed BTB, together with results from animals free of BTB, to perform ROC analysis. The area under the curve generated by peptide Cocktail 1 was greater than that of both ESAT6/CFP10 and tuberculin, thus highlighting its advantage in correctly identifying infected animals. The explanation for this increase in sensitivity is the addition of peptides from Rv3873, Rv3879c, Rv0288 and Rv3019c, which are not present in the ESAT6/CFP10 peptide cocktail (data not shown).

M. bovis infected cattle that have been skin-tested prior to subsequent slaughter and post-mortem, can be sub-divided into several groups depending on their tuberculin skin-test results and pathological status. In regard to the SICTT they can be designated either skin-test positive, negative or inconclusive. Herd removal operations were carried out on farms with persistent and severe BTB herd breakdowns. In these cases the whole herd is depopulated and all cattle are slaughtered irrespective of their SICTT response. This therefore, provided the opportunity to sample animals that present a spectrum of SICTT responses and pathological descriptions.

Our data demonstrated that the main advantage of Cocktail 1 as a diagnostic reagent will be the capability to detected infected animals escaping skin-testing (false negatives), at a level exceeding that of tuberculin and ESAT-6/CFP-10. It is likely that a proportion of skin-test negative tuberculous cattle are at an earlier time-point post-infection than skin-test positive animals because, cellular immune responses dominate early after infection (21), and it has been speculated that skin-test responses develop

subsequently to *in vitro* IFN-γ responses (3, 13, 17). It is therefore, interesting to speculate the reason for the increase in sensitivity of Cocktail 1 over that of ESAT-6/CFP-10, in detecting skin-test negative tuberculous cattle. During early infection stages it is likely that only a limited number of antigens will be recognized. Our data is suggestive that Cocktail 1 detected animals at early stages of infection, i.e. the additional antigens present in Cocktail 1 may be expressed early during *M. bovis* infection, or during acute *M. bovis* infection.

Studies in the early 1990s, indicated that a clear specificity overlap existed between the repertoire of human and bovine $CD4^+$ T cells during tuberculous infections (e.g. ESAT-6 and CFP-10) (12, 19, 20, 26, 27). Recently, we employed cattle models of *M. bovis* infection and BCG vaccination to identify highly immunogenic antigens from genomic regions absent in BCG Pasteur and 7/28 tested antigens were recognized at high frequencies by T cells from infected cattle (7). Liu and co-workers tested 4/7 of these antigens in humans and found them to be equally immunogenic (14), with the ranking of responder frequencies identical between infected cattle and humans (7, 14). These results therefore enforce the notion that the cattle model is useful for predicting antigens and immune responses in humans, thereby facilitating the prioritization of candidate diagnostic and vaccine antigens. In addition, we could also show that overlaps in epitope repertoires exist between mycobacterial epitopes recognized by human and bovine CD4 T cells (28). Therefore our study may also be of relevance for the diagnosis of human tuberculosis.

In conclusion, our results demonstrate that a cocktail of defined immunodominant peptides can result in a novel diagnostic reagent, which when used in the field can detect

M. bovis infected animals that escaped skin-testing. Consequently, the findings reported in this paper could have consequences for the potential improvement of the IFN-γ test already used in the field to supplement the skin-test. Taken together, these results therefore prioritize this peptide cocktail for further detailed field evaluation.

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REFERENCES

1. **Anonymous.** 2006. Animal Health 2005 - The report of the Chief Veterinary Officer. *In* DEFRA (ed.).

2. **Berggren, S. A.** 1981. Field experiment with BCG vaccine in Malawi. Br. Vet. J. **137:**88 - 94.

3. **Buddle, B. M., G. W. de Lisle, A. Pfeffer, and F. E. Aldwell.** 1995. Immunological responses and protection against Mycobacterium bovis in calves vaccinated with a low dose of BCG. Vaccine **13:**1123-1130.

4. **Buddle, B. M., D. Keen, A. Thomson, G. Jowett, A. R. McCarthy, J. Heslop, G. W. De Lisle, J. L. Stanford, and F. E. Aldwell.** 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed Mycobacterium vaccae. Res. Vet. Sci. **59:**10-16.

5. **Buddle, B. M., A. R. McCarthy, T. J. Ryan, J. M. Pollock, H. M. Vordermeier, R. G. Hewinson, P. Andersen, and G. W. de Lisle.** 2003. Use of mycobacterial peptides and recombinant proteins for the diagnosis of bovine tuberculosis in skin test-positive cattle. Vet Rec **153:**615-620.

6. **Buddle, B. M., T. J. Ryan, J. M. Pollock, P. Andersen, and G. W. de Lisle.** 2001. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol **80:**37-46.

7. **Cockle, P. J., S. V. Gordon, A. Lalvani, B. M. Buddle, R. G. Hewinson, and H. M. Vordermeier.** 2002. Identification of novel Mycobacterium tuberculosis antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect Immun **70:**6996-7003.

8. **Dean, G. S., S. G. Rhodes, M. Coad, A. O. Whelan, P. J. Cockle, D. J. Clifford, R. G. Hewinson, and H. M. Vordermeier.** 2005. Minimum infective dose of Mycobacterium bovis in cattle. Infect Immun **73:**6467-6471.

9. **Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P. R. Wheeler, J. Parkhill, B. G. Barrell, S. T. Cole, S. V. Gordon, and R. G. Hewinson.** 2003. The complete genome sequence of Mycobacterium bovis. Proc Natl Acad Sci U S A **100:**7877-7882.

10. **Hewinson, R. G.** 2005. TB vaccines for the world. Tuberculosis (Edinb) **85:**1-6.

11. **Hewinson, R. G., H. M. Vordermeier, N. H. Smith, and S. V. Gordon.** 2006. Recent advances in our knowledge of Mycobacterium bovis: a feeling for the organism. Vet Microbiol **112:**127-139.

12. **Lalvani, A., P. Nagvenkar, Z. Udwadia, A. A. Pathan, K. A. Wilkinson, J. S. Shastri, K. Ewer, A. V. Hill, A. Mehta, and C. Rodrigues.** 2001. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J Infect Dis **183:**469-477.

13. **Lilenbaum, W., J. C. Schettini, G. N. Souza, E. R. Ribeiro, E. C. Moreira, and L. S. Fonseca.** 1999. Comparison between a gamma-IFN assay and intradermal tuberculin test for the diagnosis of bovine tuberculosis in field trials in Brazil. Zentralbl Veterinarmed B **46:**353-358.

14. **Liu, X. Q., D. Dosanjh, H. Varia, K. Ewer, P. Cockle, G. Pasvol, and A. Lalvani.** 2004. Evaluation of T-cell responses to novel RD1- and RD2-encoded Mycobacterium tuberculosis gene products for specific detection of human tuberculosis infection. Infect Immun **72:**2574-2581.

15. **Metz, C. E.** 1978. Basic principles of ROC analysis. Semin Nucl Med **8:**283-298.

16. **Mustafa, A. S., and F. A. Shaban.** 2005. ProPred analysis and experimental evaluation of promiscuous T-cell epitopes of three major secreted antigens of Mycobacterium tuberculosis. Tuberculosis (Edinb).

17. **Neill, S. D., J. Cassidy, J. Hanna, D. P. Mackie, J. M. Pollock, A. Clements, E. Walton, and D. G. Bryson.** 1994. Detection of Mycobacterium bovis infection in skin test-negative cattle with an assay for bovine interferon-gamma. Vet Rec **135:**134-135.

18. **Panigada, M., T. Sturniolo, G. Besozzi, M. G. Boccieri, F. Sinigaglia, G. G. Grassi, and F. Grassi.** 2002. Identification of a promiscuous T-cell epitope in Mycobacterium tuberculosis Mce proteins. Infect Immun **70:**79-85.

19. **Pathan, A. A., K. A. Wilkinson, P. Klenerman, H. McShane, R. N. Davidson, G. Pasvol, A. V. Hill, and A. Lalvani.** 2001. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in Mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. J Immunol **167:**5217-5225.

20. **Pollock, J. M., and P. Andersen.** 1997. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J Infect Dis **175:**1251-1254.

21. **Ritacco, V., B. Lopez, I. N. De Kantor, L. Barrera, F. Errico, and A. Nader.** 1991. Reciprocal cellular and humoral immune responses in bovine tuberculosis. Res Vet Sci **50:**365-367.

22. **Ryan, T. J., B. M. Buddle, and G. W. De Lisle.** 2000. An evaluation of the gamma interferon test for detecting bovine tuberculosis in cattle 8 to 28 days after tuberculin skin testing. Res Vet Sci **69:**57-61.

23. **Skjot, R. L., I. Brock, S. M. Arend, M. E. Munk, M. Theisen, T. H. Ottenhoff, and P. Andersen.** 2002. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. Infect Immun **70:**5446-5453.

24. **Skjot, R. L., T. Oettinger, I. Rosenkrands, P. Ravn, I. Brock, S. Jacobsen, and P. Andersen.** 2000. Comparative evaluation of low-molecular-mass proteins

from Mycobacterium tuberculosis identifies members of the ESAT-6 family as immunodominant T-cell antigens. Infect Immun **68:**214-220.

25. **Smith, N. H., J. Dale, J. Inwald, S. Palmer, S. V. Gordon, R. G. Hewinson, and J. M. Smith.** 2003. The population structure of Mycobacterium bovis in Great Britain: clonal expansion. Proc Natl Acad Sci U S A **100:**15271-15275.

26. **Vordermeier, H. M., P. C. Cockle, A. Whelan, S. Rhodes, N. Palmer, D. Bakker, and R. G. Hewinson.** 1999. Development of diagnostic reagents to differentiate between Mycobacterium bovis BCG vaccination and M. bovis infection in cattle. Clin Diagn Lab Immunol **6:**675-682.

27. **Vordermeier, H. M., A. Whelan, P. J. Cockle, L. Farrant, N. Palmer, and R. G. Hewinson.** 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol **8:**571-578.

28. **Vordermeier, M., A. O. Whelan, and R. G. Hewinson.** 2003. Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. Infect Immun **71:**1980-1987.

29. **Waddington, F. G., and D. C. Ellwood.** 1972. An experiment to challenge the resistance to tuberculosis in BCG vaccinated cattle in Malawi. Br. Vet. J. **128:**541 - 552.

Table 1. Amino acid sequence of peptides selected for peptide Cocktail 1.

CDS	Peptide	Amino Acid Sequence
	3.2	TQAMATTPSLPEIAANHITQ
	3.3	SLPEIAANHITQAVLTATNF
Rv3873	3.4	HITQAVLTATNFFGINTIPI
	3.5	ATNFFGINTIPIALTEMDYF
	3.6	TIPIALTEMDYFIRMWNQAA
	9.3	PGGWVEADEDTFYDRAQEYS
	9.6	RVTDVLDTCRQQKGHVFEGG
Rv3879c	9.7	CRQQKGHVFEGGLWSGGAAN
	9.8	FEGGLWSGGAANAANGALGA
	9.9	GAANAANGALGANINQLMTL
	9.10	ALGANINQLMTLQDYLATVI
	0288.5	LQSLGAEIAVEQAALQ
Rv0288	0288.6	EIAVEQAALQSAWQGD
	0288.14	MAMMARDTAEAAKWGG
	3019.1	MSQIMYNYPAMMAHAG
	3019.2	NYPAMMAHAGDMAGYA
Rv3019c	3019.3	AHAGDMAGYAGTLQSL
	3019.4	AGYAGTLQSLGADIAS
	3019.13	SGTHESNTMAMLARDG
	3019.14	MAMLARDGAEAAKWGG
	ESAT6-1	MTEQQWNFAGIEAAAS
	ESAT6-2	AGIEAAASAIQGNVTS
	ESAT6-5	KQSLTKLAAAWGGSGS
ESAT-6	ESAT6-7	EAYQGVQQKWDATATE
	ESAT6-8	KWDATATELNNALQNL
	ESAT6-9	LNNALQNLARTISEAG
	ESAT6-10	ARTISEAGQAMASTEG
	CFP10-1	MAEMKTDAATLAQEAGNF
	CFP10-2	QEAGNFERISGDLKTQ
CFP-10	CFP10-7	VVRFQEAANKQKQELDEI
	CFP10-9	NIRQAGVQYSRADEEQQQ
	CFP10-10	RADEEQQQALSSQMGF

Table 2. ROC curve analysis

A

Curve	Area	SE	p*	95% CI of Area
PPD-B – PPD-A	0.893	0.0358	<0.0001	0.823 to 0.963
ESAT6/CFP10	0.960	0.0203	<0.0001	0.920 to 1.000
Cocktail 1	0.980	0.0114	<0.0001	0.958 to 1.000

B

	Specificity %	91	93	98
Antigens	**PPD-B – PPD-A**	84.5 (0.11)	84.5 (0.13)	81.0 (0.32)
	ESAT6/CFP10	91.4 (0.04)	91.4 (0.04)	91.4 (0.06)
	Cocktail 1	96.6 (0.11)	96.6 (0.11)	91.4 (0.17)

A/ ROC curve statistics

Area under a ROC curve and its statistical analysis, using data from *M. bovis* infected (n=58) and negative/environmentally sensitized cattle (n=55).

*Area values significantly greater than that under the bisecting line.

B/ Sensitivity of antigens at given specificities and the required cut-off.

Sensitivity of antigens expressed as a percent, with the required cut-offs (ΔOD@450nm) in brackets, using data from *M. bovis* infected (n=58) and negative/environmentally sensitized cattle (n=55).

Figure Legends

Figure 1.

Identification of frequently recognized peptides.

Positive response: ΔOD450 (OD450 with peptide minus OD450 media control) > 0.1. Results are expressed as responder frequencies (proportion of animals tested responding to a particular peptide). Calves infected with field strain of *M. bovis*, bloods sampled 16-21 weeks post-infection.

A) IFN-γ responses induced by individual peptides from the second pool of Rv3873 (representing amino acid residues 89-188), tested with Rv3873 responsive *M. bovis* infected cattle (n=14).

B) IFN-γ responses induced by individual peptides from the first pool of Rv3879c (representing amino acid residues 1-92), tested with Rv3879c responsive *M. bovis* infected cattle (n=16).

C) IFN-γ responses induced by individual peptides from Rv0288, tested with Rv0288 responsive *M. bovis* infected cattle (n=24).

D) IFN-γ responses induced by individual peptides from Rv3019c, tested with Rv3019c responsive *M. bovis* infected cattle (n=24).

Figure 2

Sensitivity of diagnostic reagents in relation to disease status.

Bars represent the area under the ROC curve, demonstrating the ability of ESAT-6/CFP-10 peptides (shaded), Cocktail 1 (black) and the tuberculins (white), to identify *M. bovis* infected animals grouped by their skin-test status: SICTT +ve, SICTT –ve or SICTT IR presenting with inconclusive/suspect skin-test reaction (IR). All animals were visibly lesioned and culture positive for *M. bovis*. ROC analysis and statistical calculations were performed using Analyse-it™ software (Leeds, UK).

*Area under ROC curve generated using Cocktail 1, is significantly different than that using B – A (PPD-B – PPD-A) (p=0.0372).

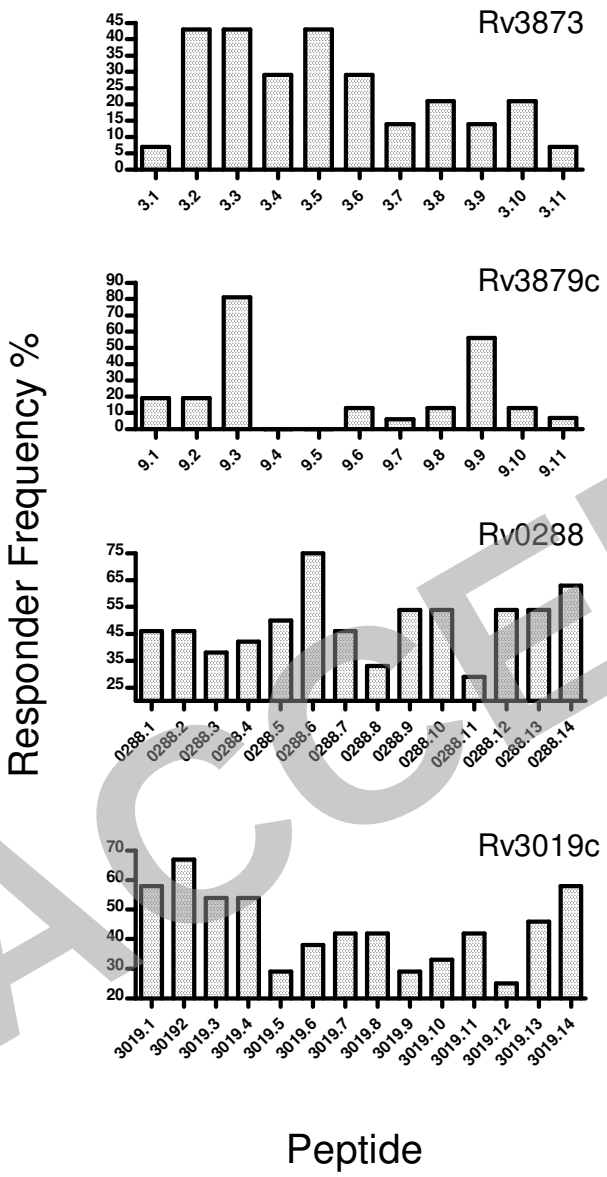
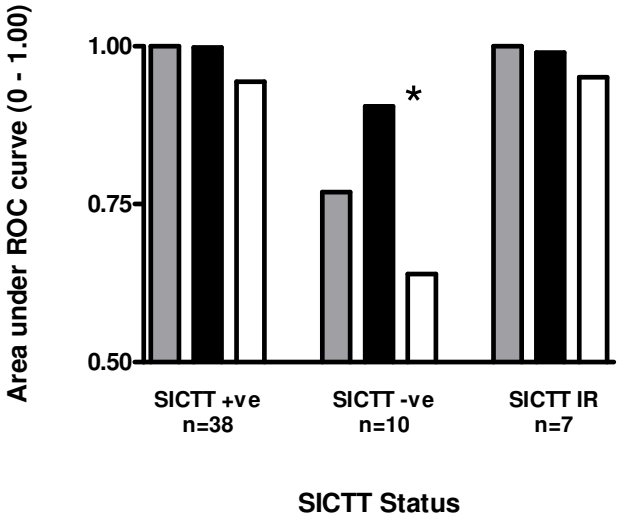


Figure 1.



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2 **Figure 2.**

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