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Field Evaluation of a Novel Differential Diagnostic Reagent for Detection of *Mycobacterium bovis* in Cattle▽

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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 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. In a test of naturally infected cattle, this cocktail was significantly better than tuberculin was for identifying skin test-negative animals with confirmed bovine tuberculosis. In addition, the specificity of this cocktail was not compromised by *Mycobacterium bovis* BCG vaccination. In summary, our results prioritize this peptide-based, fully synthetic reagent for assessment in larger trials.

For the last two decades, cases of bovine tuberculosis (BTB) in Great Britain have been increasing at an annual rate of 16% (8). 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) enzyme-linked immunosorbent assay (Bovigam), also using tuberculin, has been used recently in the United Kingdom as an ancillary test to supplement the SICTT in specific situations. Vaccination has also been considered 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 (1, 3, 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 (6).

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 (6). Pools of overlapping peptides, representing the amino acid sequences of the deleted genes, were screened for their abilities 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 improve the specificity of tuberculin per se (6, 11) Interestingly, the immunogenicity of the most strongly recognized antigens was frequently concentrated in a single peptide pool rather than distributed among all of the 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 (6). 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 (5, 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 be addressed only by using naturally infected animals. For example, due to differences in responses between experimentally infected cattle and field reactors, it is important that assay cutoffs for positivity are defined by testing naturally infected cattle. Once such cutoffs have been set, the sensitivities of the reagents can be evaluated in populations of cattle with different infection and disease statuses.

The objective of this 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* Rv3873, Rv3879c, Rv3019c, Rv0288, ESAT-6, and CFP-10 genes and demonstrate the diagnostic potential of a cocktail composed of these dominant peptides using naturally infected field animals.

MATERIALS AND METHODS

Antigens and peptides. Bovine tuberculin (purified protein derivative B [PPD B]) and avian tuberculin (PPD A) were supplied by the Tuberculin Production

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Unit at the Veterinary Laboratories Agency-Weybridge, Surrey, United Kingdom, and used to stimulate whole blood at 10 μg/ml. Staphylococcal enterotoxin B was used as a positive control at 5 μg/ml. Peptides between 16 and 20 amino acids in length were synthesized, quality assessed, and formulated into a peptide cocktail (10 μg/ml/peptide) as previously described (6).

Cattle used in this study. All animal experiments were conducted within the limits of a United Kingdom Home Office license under the Animal (Scientific Procedures) Act 1986, which was approved by the local ethical reviews committee.

Uninfected controls. Heparinized 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 (AF 2122/97) (9) by intratracheal instillation of between 1×10^2 CFU and 1×10^3 CFU as previously described (7). Bovine tuberculosis was confirmed in these animals by the presence of visible lesions in lymph nodes and/or lungs found at postmortem examinations, by the histopathological examination of lesioned tissues, and by the culture of *M. bovis* from tissue samples collected from lymph nodes and lungs.

Cattle naturally infected with *M. bovis*. Heparinized blood samples were obtained from herds with a history of BTB infection, as determined by the State Veterinary Service, and that contained naturally infected, SICTT-positive reactors and SICTT-negative cattle ($n = 58$). The statuses of the animals were confirmed at postmortem by the examination of the animals for lesions and the culture of *M. bovis* from tissues.

BCG vaccination. Calves ($n = 10$) (6 months old, Friesian) were vaccinated with BCG Pasteur by subcutaneous injection of 1×10^6 CFU into the side of the neck as described previously (3, 26). The inoculum was prepared by the dilution of a frozen stock of BCG Pasteur of known CFU in phosphate-buffered saline. The vaccinating dose was confirmed by plating the inoculum on 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase and incubated for 4 weeks at 37°C. Blood was taken between 4 and 6 weeks postvaccination.

IFN-γ enzyme-linked immunosorbent assay. A total of 0.1 ml of heparinized blood was mixed with 0.1 ml of antigen solution, staphylococcal enterotoxin B, or medium (RPMI containing 5% complete processed serum replacement type 3; Sigma, Poole, Dorset) in 96-well plates and incubated for 48 h at 37°C and 5% CO_2. IFN-γ concentration in culture supernatants was measured using the Bovigam enzyme-linked immunosorbent assay kit (Prionics, Zurich, Switzerland) following the manufacturer's instructions. Optical density was determined at 450 nm (OD_{450}), and the response to medium control was subtracted from the response to antigen (ΔOD_{450}); positive response was ΔOD_{450} of >0.1.

ROC curve analysis. Receiver operator characteristic (ROC) curve analysis was performed using Analyse-It software (Leeds, United Kingdom).

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 to 188), Rv3879c (representing amino acid residues 1 to 92), Rv0288, and Rv3019c, individual peptides were assayed in cattle experimentally infected with *M. bovis*. As shown in Fig. 1, dominant peptides could be readily identified. For example, peptide 9.3 and 9.9 from Rv3879c were predominantly recognized by 80 and 50%, respectively, of cattle tested, which responded to the total pool of 11 peptides, while the other 9 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 these 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).

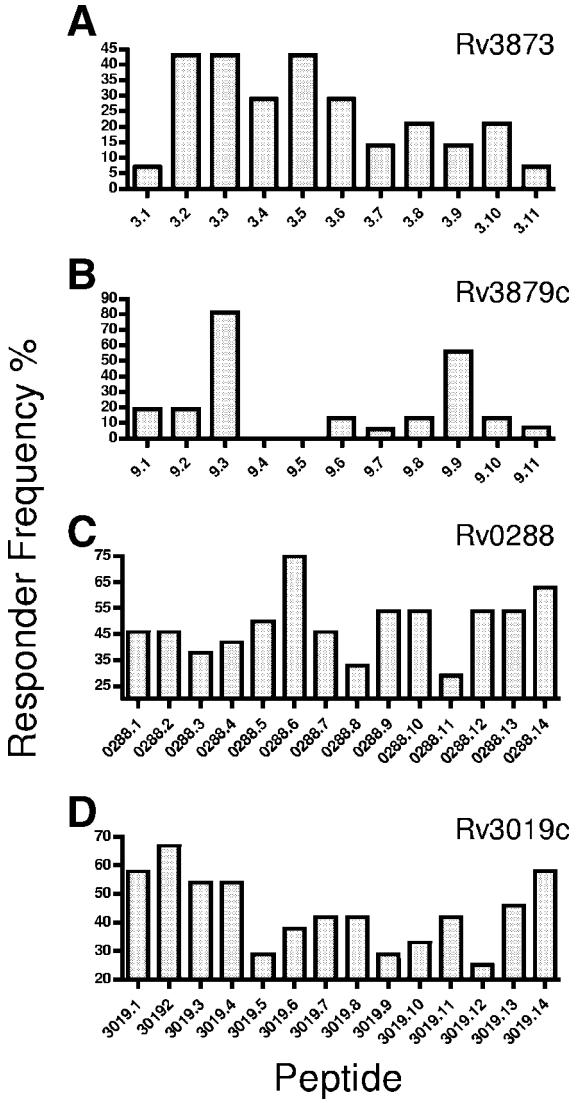

FIG. 1. Identification of frequently recognized peptides. Positive response was ΔOD_{450} (OD_{450} with peptide minus OD_{450} medium control) of >0.1. Results are expressed as responder frequencies (proportion of animals tested responding to a particular peptide). Calves were infected with a field strain of *M. bovis*, and blood was sampled 16 to 21 weeks postinfection. (A) IFN-γ responses induced by individual peptides from the second pool of Rv3873 (representing amino acid residues 89 to 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 to 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 ($n = 24$).

TABLE 1. Amino acid sequences of peptides selected for peptide cocktail 1

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

[a] CDS, coding sequence.

Receiver operator characteristic curve analysis. Test cutoff values for the peptide cocktail were determined by 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 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 tuberculins (PPD-B and 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 2 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 of these peptide cocktails were more accurate than the bovine tuberculin-biased response, as was indicated by their superior area under the ROC curves (Table 2), although the differences in the diagnostic accuracy were not statistically different.

TABLE 2. ROC curve analysis statistics[a]

Curve	Area	SE	P^b	95% CI of area
PPD-B and PPD-A	0.893	0.0358	<0.0001	0.823 to 0.963
ESAT-6/CFP-10	0.960	0.0203	<0.0001	0.920 to 1.000
Cocktail 1	0.980	0.0114	<0.0001	0.958 to 1.000

[a] Area under a ROC curve and its statistical analysis using data from *M. bovis*-infected (n = 58) and negative/environmentally sensitized cattle (n = 55). 95% CI, 95% confidence interval.
[b] Area values significantly greater than that under the bisecting line.

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 OD_{450} cutoff values (Table 3). 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 increased sensitivities at all three specificity levels chosen compared with that of tuberculin. With specificity set at 91%, cocktail 1 detected 5.2% more infected cattle than did ESAT-6/CFP-10, and with specificity set at 93%, cocktail 1 detected 12.1% more infected cattle than did avian and bovine tuberculin. ESAT-6/CFP-10 peptides and cocktail 1 demonstrated equal sensitivities when the specificity was set at 98% (Table 3).

Sensitivities 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 tuberculin skin test-positive (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 skin test-negative (SICTT −ve) animals, as measured by the areas under the ROC curve, was better after the application of cocktail 1 compared to that after the application of ESAT-6/CFP-10 peptides and tuberculin, although the difference reached only statistical significance relative to tuberculin (P = 0.0372) (Fig. 2). In addition,

TABLE 3. Sensitivity of antigens at given specificities and the required cutoff

Antigens	Sensitivity level at a specificity (%) of[a]:		
	91	93	98
PPD-B and PPD-A	84.5 (0.11)	84.5 (0.13)	81.0 (0.32)
ESAT-6/CFP-10	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] Sensitivity of antigens expressed as a percent, with the required cutoffs (ΔOD_{450}) in parentheses, using data from *M. bovis*-infected (n = 58) and negative/environmentally sensitized cattle (n = 55).

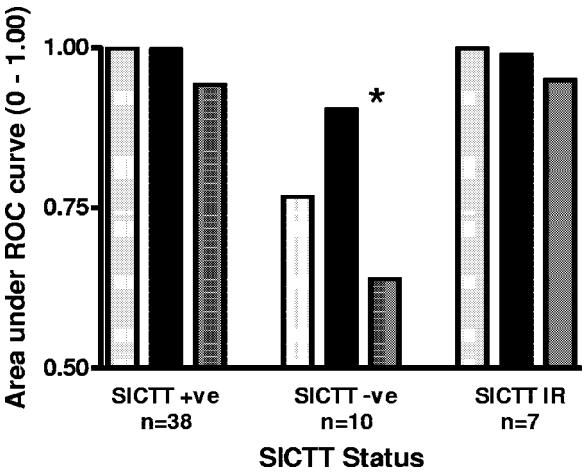

FIG. 2. Sensitivity of diagnostic reagents in relation to disease status. Bars represent the area under the ROC curve, demonstrating the ability of ESAT-6/CFP-10 peptides (darkly shaded), cocktail 1 (black), and the tuberculins (lightly shaded) to identify *M. bovis*-infected animals grouped by their skin test statuses: SICTT +ve, SICTT –ve, and SICTT IR. All animals were visibly lesioned and culture positive for *M. bovis*. ROC curve analysis and statistical calculations were performed using Analyse-It software (Leeds, United Kingdom). *, area under ROC curve generated using cocktail 1 is significantly different from that using PPD-B and PPD-A ($P = 0.0372$).

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, visibly 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 postinfection. A set of six experimentally infected calves were used in this time course experiment. The initial responses to cocktail 1 were 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 three animals developed at the same time as those towards ESAT-6/CFP-10 peptides (data not shown). Although not statistically conclusive, these data suggest that the improved performance of cocktail 1 for skin test-negative cattle could be due to its earlier recognition postinfection.

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 3 of the 10 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 abilities 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 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 bovine leukocyte antigen 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 postinfection. While 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 statuses, are 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 were isolated from the British herd and these strains may differ in their abilities to stimulate IFN-γ-mediated immune responses (25). For these reasons, therefore, it is important to validate these defined reagents in field experiments (4, 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 curve analysis. The area under the curve generated by peptide cocktail 1 was greater than those of both ESAT-6/CFP-10 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 ESAT-6/CFP-10 peptide cocktail (data not shown).

M. bovis-infected cattle that have been skin tested prior to subsequent slaughter and postmortem can be subdivided into several groups depending on their tuberculin skin test results and pathological statuses. 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 responses. 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 its capability to detect infected animals escaping skin testing (false negatives) at a level exceeding those 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 postinfection than skin test-positive ani-

mals 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 (2, 13, 17). It is therefore interesting to speculate on 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 suggest 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). We recently employed cattle models of *M. bovis* infection and BCG vaccination to identify highly immunogenic antigens from genomic regions absent in BCG Pasteur, and 7 of 28 tested antigens were recognized at high frequencies by T cells from infected cattle (6). Liu and coworkers tested four out of seven 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 (6, 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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