More intensive action is needed to control and ultimately eliminate TB

Untitled – Ivo Saglietti
Photography

'My heart waits
also, towards light and towards life
for another miracle of spring'

On the Old Elm **(Poem) – Antonio Machado**

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CHAPTER 20

Development of Cattle Tuberculosis Vaccines

Martin Vordermeier

Abstract

Development of a TB vaccine for cattle is a research priority in Great Britain. A number of challenges need to be addressed. Firstly, vaccine strategies enhancing the efficacy of *M. bovis* BCG, currently the only potentially available TB vaccine, need to be developed; and secondly, the development of a diagnostic test to be used alongside vaccination to differentiate vaccinated and infected animals (DIVA test) is desirable. Significant progress in developing TB vaccines for cattle has been made over the last seven years. Specifically: (i) DNA, protein, or viral subunit vaccines used in combination with BCG have been shown to give superior protection against experimental challenge in cattle than BCG alone (heterologous prime-boost); (ii) neonatal BCG vaccination provides protection; (iii) prototype reagents that allow discrimination between vaccinated and infected animals have been developed; and (iv) correlates of disease severity have been identified that can predict the success or failure of vaccination.

Introduction

M. bovis is the causative agent of bovine TB which is a significant economic burden to the agricultural industries worldwide. It has been estimated that more than 50 million cattle are infected with *M. bovis* resulting in economic

losses of approximately $3 billion annually. Over the last two decades, in Great Britain, failure of the (tuberculin) test and slaughter strategy has resulted in a dramatic rise in the incidence of TB in cattle (http://www.defra.gov.uk/animalh/tb/stats/county.htm). The urgency for new and improved cattle vaccines and diagnostic reagents has been acknowledged by the British government, and development of wildlife and cattle vaccines is a research priority (http://www.defra.gov.uk/animalh/tb/vaccination/index.htm). The development effort for improved cattle TB vaccines faces two challenges that need to be addressed: firstly, vaccine strategies enhancing the efficacy of BCG, currently the only potentially available TB vaccine and secondly, the development of a diagnostic test to be used alongside vaccination to differentiate vaccinated and infected animals (DIVA test). In addition, correlates or predictors of protective immunity are ill-defined, and their identification would greatly accelerate the development process.

Vaccinating cattle against bovine TB could also be an attractive and cost-effective control strategy in developing countries where other control strategies are difficult and expensive to implement. The development of novel vaccines against bovine TB has to some degree closely followed that of the human TB vaccine effort and there is significant alignment between the human and bovine TB vaccine programmes (1). For example, promising vaccine candidates based on recombinant viruses that have been developed to combat human TB vaccines have already been tested in cattle (see below).

Control of bovine TB has been particularly difficult in countries with wildlife reservoirs of *M. bovis*, like the possum in New Zealand, deer in the USA, and the badger in the Republic of Ireland and UK (2). Vaccine development programmes for these wildlife reservoirs are also under way, with significant progress being made in all these countries. The wildlife vaccine of choice in this scenario is BCG, with an emphasis on oral delivery of a baited vaccine formulation (3–9).

M. Bovis BCG

BCG is the most widely used human vaccine in the world. It was derived from a strain of *M. bovis*, which was isolated from a cow with tuberculous mastitis. Challenge experiments and field trials of BCG in cattle since 1919 have resulted in data showing a high degree of variability in its ability to protect cattle against infection with *M. bovis*, the causative agent of bovine TB, although in most of these studies some degree of protection was induced by BCG vaccination (reviewed in 10–12). Importantly, BCG vaccination sensitizes animals to the

TST, and vaccinated animals will therefore, at least for a significant period post-vaccination, test positive in the classical skin test. For this reason, test and slaughter-based control strategies based on TST were favoured above BCG vaccination. More recent experimental studies with BCG have confirmed its potential to protect cattle to some degree against bovine TB by reducing disease severity and pathology (e.g. 13–17). In addition, BCG vaccination was more effective when delivered to neonatal calves than to older animals (18, 19). BCG can also be delivered orally to cattle, which may decrease the level and duration of skin test-positivity induced by BCG vaccination (20, 21). The BCG strain used most frequently since the mid-1980s was BCG Pasteur. However, mainly due to licensing issues, recently, the BCG strain of choice has become a freeze-dried preparation of BCG Danish produced by the Staten Serum Institute (Copenhagen, Denmark). A recent study has demonstrated equal protective efficacies of these two strains (22).

In summary, BCG has some of the qualities required for a veterinary vaccine (low costs, excellent safety profile) and remains the prototype, gold standard vaccine against which to judge the efficacy of any novel vaccine. However, it does not confer complete protection and therefore the aim of TB vaccine programmes is to improve its efficacy. Nevertheless, the most promising vaccination strategies identified to date have mostly involved improving upon BCG vaccination rather than replacing it (see below).

Cattle Models to Test TB Vaccines

The efficacy of a cattle TB vaccine can be tested directly in the target species, which is a considerable advantage over human vaccine development. To be able to compare vaccines tested in different laboratories it is important to use standardized infection models. The most commonly used direct experimental infection model is infection of calves using the intratracheal route (Table 20.1) (13, 14). Pathology develops mainly and highly reproducibly in the lower respiratory tract which is reflective of the pathology seen in the majority of infected cattle in Great Britain and in other developed countries. Almost 100 per cent of animals infected via this route display productive disease with reproducible location and severity of lesions thus requiring relatively small groups (<10/group) to detect significant protection. The relatively short experimentation period (3–4 months postinfection) makes it further attractive. Potential disadvantages are that, due to the relatively high infection doses required to achieve infection and disease in most animals, the immune system can be overwhelmed and potentially effective vaccines

could be classified as non-effective. Other direct experimental infection routes are also used (e.g. aerosol models, reviewed in 23). To overcome some of these limitations of direct infection systems, vaccination models, where transmission of disease is facilitated by in-contact with naturally infected cattle, are also under development (Table 20.1). The advantages are that a natural route and infective dose is used, and that data generated in this model will be highly relevant to the actual field situation to guide the design of field trials as the read-out for this model is the ability of the vaccine to prevent transmission of *M. bovis* to other animals. The disadvantages are relatively low transmission rates (<20 per cent) which necessitate very much larger group sizes (at least 60/group) to achieve the required statistical power. Vaccines giving promising results may then be tested in larger field trials, which would likely require large numbers of cattle and would run for a considerable time.

Table 20.1 Examples of Cattle Models to Test Vaccines

Model	Advantages	Disadvantages
Direct experimental infection (e.g. intratracheal route)	• 'Few' animals required (n <10–12/group): • 100 % infection rates of control animals • Short duration (3–4 months): highly standardized, synchronized infection, defined infection strain, defined disease kinetics and pathology)	• Challenge may be too strong, therefore promising vaccine candidates could be screened out
Experimental infection by in-contact with infected donor animals	• Natural route and infective dose: data highly relevant for trial designs	• More animals required (>60/group): potentially low infection rates of controls • Long in-contact period (12 months): no synchronized infection, disease kinetics not defined, pathology less defined
Field trial	• 'Real-life' situation in respect to routes, doses, management	• Very large numbers required (n = 100–1000s) • Long and expensive (years), may be illegal in some countries

Recent Progress in Developing Cattle Tuberculosis Vaccines that Improve the Efficacy of BCG

Several strategies have been implemented to improve the efficacy of BCG (Table 20.2), namely, the use of subunit vaccines in the form of DNA vaccines, protein subunit vaccines administered with a suitable adjuvant, live recombinant vaccines such as attenuated recombinant viruses expressing mycobacterial antigens, or over-expressing genes in BCG that are deleted or are under-expressed in BCG (Table 20.2). Another possible strategy involves the development of rationally attenuated *M. bovis* strains (Table 20.2). The practicality of this strategy has been greatly facilitated by the elucidation of the genome sequences of *M. bovis*, MTB, and *M. bovis* BCG (Pasteur) (24–26).

Whilst some of these approaches gave rise to some degree of protection in cattle, used individually, none of them induced immunity levels that approached or surpassed protection imparted by BCG, or were not protective at all (Table 20.2). It is, however, of interest to point out that a number of these approaches such as DNA vaccines, recombinant viruses, and auxotrophic BCG did not lead to TST sensitization (see Table 20.2), which constitutes an advantage over BCG, as this would in principle allow the continuation of the existing test and slaughter control strategy.

Recent results in cattle have shown that the most effective vaccination strategies against bovine TB to date are based on using combinations of BCG with subunit vaccines containing antigens that are present in BCG to enhance immunity conferred by BCG (heterologous prime-boost strategies, see Tables 20.2 and 20.3), or to use subunits containing antigens not part of BCG to supplement its antigen complement (Table 20.3). Heterologous prime-boost immunization strategies involve the use of two different vaccines, each expressing the same antigen. One vaccine primes the immune response against a protective antigen and the other serves to boost the immune response against the same antigen (27). An obvious drawback of using BCG in these scenarios is that animals will become, at least for some time, positive to the TST and other assays that rely on tuberculin as the antigen (like the IFN-γ in vitro test, see Tables 20.2 and 20.3). This drawback can, however, be overcome by using antigens that can differentiate between infection and vaccination (DIVA reagents) based on antigens such as ESAT-6 and CFP-10 (see section on DIVA reagents).

Table 20.2 Comparison of Different Types of Vaccines to Protect against Experimental Challenge of Cattle with *M. Bovis*

Vaccine	Protection [a]	Induction of TST Reactivity[b]	DIVA based on ESAT-6/ CFP-10 Possible[c]	Reference
BCG[d]	Yes	Yes	Yes	e.g. (13, 14, 16, 19, 22, 27, 28)
Auxotrophic MTB	No	Yes	No	(28)
Attenuated *M. bovis*	Yes (>BCG)	Yes	No	(29, 30)
Auxotrophic BCG	Not assessed	No	Yes	Vordermeier, Hewinson, Jacobs, unpublished data
Live vectors (e.g. viruses like MVA, adenoviruses)	Immunogenic, NT	No	Yes	(31–33)
DNA vaccines	No (17) or Yes (<BCG) (34, 35)	No	No/Yes (in cases where ESAT-6/CFP-10 were used as vaccine antigens (35, 36)	(17,34,35)
Protein subunits	Yes (<BCG)	No	Yes	(17)
Heterologous prime-boost				
DNA/protein	No	No	Yes	(37)
DNA/BCG	Yes (>BCG)	No	Yes	See Table 20.3
BCG/proteins	Yes (>BCG)	No	Yes	See Table 20.3
BCG/MVA	Yes (>BCG)	No	Yes	See Table 20.3

[a]Vaccine-induced protection after *M. bovis* challenge as measured by reduction of pathology or bacterial loads. Brackets: Protective efficacy in relation to BCG (< lower efficacy than BCG; > better efficacy than BCG).

[b]Vaccination procedure will induce TST positivity. Yes = induction of skin test responses; No = vaccine does not sensitize tuberculin reactivity.

[c]DIVA reagents based on ESAT-6 and CFP-10 can be used in combination with vaccine: Yes = can be used; No = compromised by vaccine used.

[d]Vaccination with BCG Pasteur and Danish, subcutaneous and oral routes, vaccination of neonatal or older calves, see text for details.

NT= not tested.

Strategies Improving BCG Based on DNA Vaccines

DNA vaccines can be useful as part of heterologous prime-boost protocols. We tested heterologous prime-boost protocols in cattle based on priming the immune response with a cocktail of three DNA vaccines encoding the mycobacterial proteins hsp65, hsp70, and APA (which were not protective by themselves), followed by boosting with BCG (15) (Table 20.3). This induced significant enhancement of protection in all six parameters used to determine vaccine efficacy, compared to BCG which induced significant protection in only 2/6 of these parameters (15). Subsequent experiments showed that superior protection to BCG could be achieved with this combination of vaccines irrespective of whether the DNA vaccines or BCG were used for the priming immunization (38) (Table 20.3). A similar approach was used by Cai et al. (39) who demonstrated improved protection of calves when they were primed with a DNA vaccine cocktail composed of MPB64, MPB83, and Ag85B followed by BCG boosting (Table 20.3). An alternative approach to improve BCG efficacy was used in the study of Maue et al. (36), who supplemented BCG vaccination by co-vaccination with DNA vaccines encoding ESAT-6 and CFP-10. They furthermore enhanced DNA vaccine efficacy by co-administering plasmids encoding GM-CSF and CD80/86 to enhance antigen presentation and T cell–APC interactions. However, one disadvantage of using ESAT-6 and CFP-10 as vaccine antigens is that it will compromise their role as diagnostic reagents to discriminate vaccinated from infected animals (Table 20.3).

Heterologous Prime-Boost Strategies Based on Protein Subunits

Conceptually, protein subunits are very attractive. However, in contrast to DNA vaccines, protein subunits are unlikely to induce cellular immune responses in the absence of an adjuvant. Therefore, a high priority for the development of protein subunit vaccines is the identification of adjuvants that enhance the development of cellular immune responses in cattle. A recent important development has been the definition of CpG motifs as adjuvant units within DNA vaccines (40). Synthetic oligonucleotides containing such CpG motifs can be synthesized to produce short immunostimulatory sequences (CpG oligodeoxynucleotide (CpG-ODN)), which can be added to vaccine formulations to enhance immunogenicity. Using this approach, *M. bovis* CFP were used in conjunction with CpG-ODN as cattle TB vaccines and they significantly enhanced the cellular immune responses of CFP. Importantly, significant protection was also seen in animals vaccinated with CFP plus CpG-ODN, although the protective efficacy was inferior to that observed after BCG vaccination (37).

Based on these findings, further prime-boost experiments were performed in cattle using CFP delivered in the presence of CpG containing ODN to boost primary immune responses induced by BCG. Groups of cattle were vaccinated with either BCG or with BCG and CFP plus CpG; at the same time followed by two CFP/CpG boosts. The results indicated that boosting BCG with CFP in CpG gave superior protection to that obtained by BCG alone (Table 20.3) (41). However, *M. bovis* CFP was used, which contains ESAT-6 and CFP-10, and these antigens can therefore not be used as DIVA reagents (Table 20.3). Experiments are underway to determine whether BCG CFP will be equally effective, whose use would then allow the application of ESAT-6 and CFP-10.

Heterologous Prime-Boost Strategies Based on Recombinant Viruses

Some of the advantages of live attenuated viruses over protein subunit vaccines are better induction of strong CMI, and potentially lower production costs and simplified batch release test protocols. The first Phase I human trial of a new TB vaccine was based on a heterologous prime-boost strategy involving boosting BCG-mediated immunity with an attenuated Vaccinia virus expressing Ag85A of MTB (MVA85A) (42).

In a collaborative study with the group of Professor Adrian Hill at Oxford University, we have performed immunogenicity studies of the BCG/MVA85A heterologous prime-boost regimen in cattle. Prime-boost protocols using recombinant MVA85A and BCG in either combination resulted in significantly higher frequencies of Ag85-specific IFN-γ secreting cells than the viral vectors or BCG used alone. The most promising combination was BCG priming followed by one MVA85A boost (33). Similarly, we have shown that a prime-boost protocol applied to cattle that consisted of BCG priming followed by heterologous boosting with a recombinant adenovirus (Ad85A) expressing the same antigen, Ag85A, developed by Professor Xing's group at McMaster University, Toronto, Canada (32) resulted in superior antigen-specific IFN-γ responses as well as improved central T cell memory compared to BCG vaccination alone (32). Most encouragingly, in an *M. bovis* challenge experiment using the intratracheal infection route, we could demonstrate that both MVA85A and Ad85A when used to boost BCG-induced immunity conferred significant protection, which was superior to BCG vaccination alone (41a, Table 20.3).

Thus, significant advances have been made to develop prototype vaccine strategies that can enhance BCG vaccination efficacy based on DNA, protein, and viral subunit vaccination. Further work is required to determine which of these approaches is the most effective, if and which additional subunit antigens will improve efficacy, to determine the duration of immunity and

Table 20.3 Examples of Vaccination Strategies Improving Compared to BCG

Vaccine Strategy	BCG Strain Used	Subunit Antigen(s)	Adjuvant/ Live Vector	DIVA ESAT6/ CFP10 Applicable?	Reference
DNA prime and BCG boost	Pasteur	hsp65, hsp70, APA	In-build CpG motifs of DNA vaccine	Yes	(15)
BCG prime and DNA boost	Pasteur	As above	As above	Yes	(38)
DNA prime and BCG boost	Not specified	MPB64, MPB83, Ag85B	Plasmid DNA in DDA	Yes	(39)
BCG + DNA prime followed by DNA boost	Pasteur	ESAT-6/CFP-10	Plasmid DNA: GM-CSF+CD80/86	No	(36)
BCG plus protein prime and protein boost	Pasteur	*M. bovis* CFP	TLR ligands (CpG ODN, lipopeptides)	No	(41)
BCG prime and MVA85A	Danish	Ag85A	MVA (modified Vaccinia Ankara strain)	Yes	(41a)
BCG prime and Ad85A	Danish	Ag85A	Attenuated human adenovirus type 5	Yes	(41a)

to determine the minimum effective vaccine doses required. For example, in our experiments, BCG Danish is being delivered to cattle at five times the human dose. However, in a recent experiment, we could demonstrate that 0.5 of a human dose is equally effective in protecting calves against *M. bovis* (Buddle, Vordermeier, Hewinson, unpublished data). Using such a dose would significantly reduce the cost of vaccine production.

Predictors and Correlates of Vaccine Efficacy and Protection

TB vaccine development would be greatly facilitated by the definition of immunological predictors and correlates of protection which would accelerate vaccine development by relieving pressure on limited cattle Biosafety 3 facilities. An additional advantage would be the reduction in the cost of large animal experiments. In the context of the following discussion of such immunological surrogates, 'predictors of vaccine efficacy' are defined as immunological markers

detectable after vaccination that predict whether a vaccine strategy is protective BEFORE challenge (i.e. without *M. bovis* infection). In contrast, we will use the term 'correlate of protection' when we refer to markers that predict vaccine success after vaccinated animals have been challenged with *M. bovis*. Such correlates could also constitute correlates of disease progression/pathology or bacterial burden, which would be elevated in infected/non-protected animals compared to infected/vaccinated ones. Some progress has been made over recent years to defining either predictors of protection or correlates of protection and pathology, and we will discuss this progress in the following section. These parameters are also listed in Table 20.4.

IFN-γ induced after vaccination is an important parameter of vaccine success or failure. However, on its own it is insufficient as a predictor of protection because not all vaccines able to induce IFN-γ have been found to be protective. In contrast, vaccines that fail to induce any IFN-γ almost invariably fail to protect. Thus, whilst not positively predicting success, IFN-γ responses will allow for screening out from further testing vaccines that are unlikely to protect cattle (43, 44). Therefore, further markers need to be defined.

In 2002, we defined the in vitro IFN-γ production induced by RD1-region antigens (ESAT-6 and CFP-10) as a correlate of pathology and bacterial burden (Table 20.4) (16, 45). This therefore constitutes an inverse correlate of protection, as vaccinated/*M. bovis*-infected animals will present low response levels compared to naïve/infected ones (15). Measuring this parameter can therefore predict relatively soon after experimental challenge (about two months post-challenge) the outcome of vaccination determined at post-mortem, which in our model is performed about four months post-challenge. Similarly, IgG1 serum responses specific for the antigen MPB83, which are boosted after TST, also correlate with bacillary loads, and can also serve as an inverse correlate of protection (45). Another parameter that has been described to correlate with the presence of disease (in this case: presence of lung lesions) is IL-4 mRNA levels in MPB70 DNA/protein subunit-vaccinated calves after in vitro stimulation with bovine tuberculin (17).

Whilst such correlates of protection or disease severity can be useful, they still require *M. bovis* infection to proceed. Of more practical advantage would be reliable predictors of protection that can be measured after vaccination and do not necessitate infection to determine vaccine efficacy. Recently, our laboratory has described several such potential predictors of protection, namely, the expression of the IL-4 splice variant IL-4δ3 (Table 20.4), central memory responses measured by cultured ELISPOT, and antigen-specific IL-17 production (Table 20.4). Splice variants of IL-4 have been described in humans, mice, and cattle, and have been shown to act as antagonists to inhibit undesirable IL-4-mediated cellular responses that may down-regulate Th1

responses. Work in human TB has highlighted a relation between the IL-4δ2 splice variant expression and lack of progression to clinical TB (46, 47). Cattle express two IL-4 splice variants, IL4-δ2 and IL-4δ3, and we demonstrated in a recent study that *ex vivo* expression of IL-4δ3 was elevated following BCG vaccination compared to naïve calves, making measurement of this marker a potential predictor of protection (48).

Measuring *ex vivo* IFN-γ responses assesses effector cell responses and effector memory cells; yet studies of viral and parasitic infections in mice and humans have suggested that central memory responses rather than effector/effector memory T cell responses correlated with pathogen clearance and protection (49, 50). Central memory cells are able to migrate through lymph nodes, which is facilitated by expression of the chemokine receptor CCR7, whilst effector cells and effector memory cells reside in peripheral tissues and, due to the absence of CCR7 expression, are unable to migrate into lymphatic organs. However, bovine CCR7 detecting reagents are unavailable, and we therefore adapted a cultured IFN-γ ELISPOT system to measure central memory responses. Such cultured ELISPOT protocols have been shown to measure human central memory responses (51). Our results demonstrated that subunit vaccine antigen-specific central memory responses measured by cultured ELISPOT analysis before *M. bovis* infection correlated directly with the protection determined by post-mortem analysis after challenge (Table 20.4), and constitutes therefore a predictor of vaccine efficacy (41a). This could not only be shown using Ag85A as a recall antigen, after Ag85A viral subunit vaccination to boost BCG (41a), but also when the antigens part of BCG (TB10.4 and Ag85A) were used following BCG or attenuated *M. bovis* construct vaccination (52).

Further, we have also recently shown that in vitro production of IL-17 following in vitro tuberculin or subunit antigen (Ag85A) stimulation of cells isolated from vaccinated calves also directly correlated with vaccination success when measured after vaccination but before infection (Table 20.4) (41a). The role of IL-17 producing cells in TB has been studied in mouse models recently (53, 54). Whilst these studies did not demonstrate a role for IL-17 in primary TB infections, in vaccinated animals absence of IL-17 producing memory cells resulted in the loss of Th1 responses and protection. Thus Th1 and Th_{17} responses seem to cross-regulate themselves, and are both important for protective anti-tuberculous responses. Our results are therefore confirmatory of these findings.

In conclusion, the definition of practical immunological parameters or biomarkers predicting or correlating with vaccine success would substantially accelerate vaccine development. Significant progress has been made to prioritize a number of such markers that await validation in further vaccination experiments.

Table 20.4 Potential Correlates of Protection and Pathology

Parameter	Category[a]	Comment	Reference
RD1 antigen-induced IFN-γ	Correlate of pathology/protection	Inverse correlation with protection/direct correlation with pathology and bacterial load	(15, 16, 45)
MPB83-specific IgG1	Correlate of pathology/protection	As above, when measured post-TST	(45)
(MPB70)-specific IL-4 expression	Correlate of protection/pathology	Direct correlation with lung pathology in MP83 protein subunit vaccinated calves	(17)
Ex vivo IL-4δ3 expression	Predictor of protection	Elevated after BCG challenge compared to naïve cattle	(48)
Central memory measured by cultured ELISPOT	Predictor of protection	Subunit antigen, or BCG-derived antigen specific re-call IFN-γ responses	(41a, 52)
Subunit antigen-specific IL-17 expression	Predictor of protection	Ag85A-induced in vitro responses after BCG or BCG/viral subunit boost	(41a)

[a]Predictor of protection = parameter of vaccination success patent after vaccination but BEFORE *M. bovis* infection.
Correlate of protection = Parameter of vaccine success patent after vaccination AND *M. bovis* challenge.
Correlate of pathology = Parameter associated with disease progression or bacterial load; can be used as inverse correlate of protection after infection.

Differential Diagnosis of Infected from Vaccinated Individuals (DIVA)

BCG vaccination results in the sensitization of animals to antigens present in bovine tuberculin and therefore compromises tuberculin-based diagnosis strategies in BCG vaccinated animals (55, 56). In the absence of a vaccine that results in 100 per cent protection, or alternatively, a vaccine that does not sensitize for TST reactivity, the development of more specific diagnostic reagents capable of discriminating between infected and uninfected vaccinated animals (DIVA reagent) is therefore an important component of cattle TB vaccine development so that test and slaughter control strategies can be carried out alongside vaccination regimens.

Conceptually, antigens whose genes are expressed in *M. bovis* yet absent from environmental mycobacterial species and BCG (57–59) constitute candidates for inclusion as diagnostic reagents, which are both more specific and more defined than PPD. Two of the major antigenic targets identified in both cattle

and humans are ESAT-6 and CFP-10 (60, 61). CFP-10 and ESAT-6 are encoded by genes located on the RD1 region of the *M. bovis* genome that is deleted from the genome of all BCG strains (26, 57–59). When used as diagnostic antigens, ESAT-6 and CFP-10 have been shown to be able to discriminate between infected and BCG-vaccinated cattle (55, 56, 62). Frequently recognized peptides derived from the sequences of the two proteins were readily identified using blood from *M. bovis*-infected cattle and gave equivalent responses to the recombinant proteins in naturally infected cattle. In contrast, BCG-vaccinated cattle did not respond to this peptide cocktail (62).

Comparative Genomics

To increase test sensitivity by complementing ESAT-6 and CFP-10, further antigens need to be defined that are recognized in infected animals that did not respond to CFP-10 or ESAT-6. The genome sequences of *M. bovis* (26), MTB (25), *M. bovis* BCG Pasteur (24), *M. avium* subsp. *paraTB* (63), *M. avium* subsp. *avium,* and *M. marinum* (64) have been completed. This allows the search for further differential diagnostic candidates based on comparative genome and transcriptome analyses. Antigens that were prioritized by *in silico* analysis have to be assessed experimentally both for immunogenicity and specificity in cattle. We applied a high-throughput immunological screening system based on pools of chemically synthesized peptides representing the complete amino acid sequences of target proteins to identify highly immunogenic antigens from three genomic regions of *M. bovis* that are absent in BCG Pasteur (RD1, RD2, RD14) (24, 26). Seven of these 28 antigens (Rv1769, Rv1979c, Rv1986, Rv3873, Rv3873, Rv3878, Rv3879c) were recognized at high frequencies (41–86 per cent) (65). Interestingly, despite the deletion of their genes from the genome of BCG, only Rv1986, Rv3872, and Rv3878 were not recognized by T cells from BCG vaccinated cattle, and are therefore candidates for differential diagnosis (65). Two of the other four proteins were recognized by BCG-vaccinated animals but not by environmentally sensitized cattle (Rv3873 and Rv3879c). When we mapped individual epitopes within these proteins, we found that small sequence regions with high degrees of homology to these epitopes could be found in unrelated and otherwise non-homologous proteins encoded in genomic regions not deleted from BCG (65), thus likely accounting for the observed cross-reactivity. Other immunogenic antigens identified that could be useful for either diagnosis or as vaccine antigens include proteins belonging to the important ESAT-6 protein family (66) such as Rv3019c or Rv0288 (67–69). In a further refinement, we identified the major immunodominant (i.e. most frequently) recognized peptides from these antigens (Rv3873, Rv3879c, Rv0288, and Rv3019c) and together with peptides from the current lead diagnostic

antigens, ESAT-6 and CFP-10, formulated them into a peptide cocktail (65). This cocktail was significantly better than tuberculin for identifying skin test-negative animals with confirmed bovine TB and the specificity of this cocktail was not compromised by *M. bovis* BCG vaccination.

Comparative Transcriptome Analysis

In an extension of the *in silico* mining effort based on comparative genome analysis, we used an approach that integrated genome data and DNA microarray technology to assess the transcriptome of BCG and *M. bovis* after infection of bovine macrophages (*comparative transcriptome analysis*), the transcriptome being the complete gene expression profile of an organism under defined conditions. To provide a comparison data set, the transcriptomes of bacilli grown in vitro under different growth conditions were also determined and the combined data used to prepare a list of potential antigens. One antigen thus identified, Rv3615c, was recognized at a frequency of about 55 per cent by blood cells from *M. bovis*-infected animals (Table 20.5) (70). Encouragingly, Rv3615c was not recognized by uninfected or BCG-vaccinated cattle thus highlighting its potential as a DIVA reagent (70). In this context, it is worth noting that Rv3615c has been shown to be secreted by the RD1 locus (71); as BCG lacks the RD1 secretion locus, it explains why Rv3615c was not recognized in vaccinated animals. Further analysis of the Rv3615c responses observed in the field reactor animals revealed that it was recognized predominantly in the subset of *M. bovis*-infected animals that tested ESAT-6/CFP-10-negative (70). Rv3615c was therefore able to complement ESAT-6 and CFP10 to increase test sensitivity when applied as part of a combined diagnostic reagent (Table 20.5).

Table 20.5 DIVA Reagents: Rv3615c Complements ESAT-6 and CFP-10

Antigen	*M. bovis*-Infected Cattle (naturally infected)[a] (%)	BCG-Vaccinated Cattle (experimental vaccination)[a] (%)
PPD-B minus PPD-A	90–95	40–75
ESAT-6/CFP-10 peptide cocktail	77–82	1
Rv3615c peptide cocktail	55	1
Rv3615c plus ESAT-6/CFP-10 peptide cocktails	90	1

[a]Results are expressed as percentages of animals responding to these antigens in the Bovigam whole blood IFN-γ assay. Results represent values from a number of published (62, 65, 68, 70), and unpublished studies from our laboratory.

Conclusion

Significant progress has been made in the development of TB vaccines for cattle. Subunit vaccines based on DNA, proteins, or viral subunits used in combination with BCG have resulted in better protection against experimental challenge with *M. bovis* than BCG vaccination on its own. BCG vaccination of neonates has also proved to be highly protective. DIVA reagents that allow discrimination between vaccinated and infected animals have been developed. Finally, correlates of disease severity are being actively sought that can predict the success or failure of vaccination hopefully in future shortening experimental protocols.

Acknowledgements

The author was funded by the Department for Environment, Food and Rural Affairs, United Kingdom. I also thank Dr Gareth Jones, VLA, for his comments to this manuscript.

❒ References

1 Hewinson RG, Vordermeier HM, and Buddle BM. Use of the bovine model of tuberculosis for the development of improved vaccines and diagnostics. *Tuberculosis (Edinb)*, 2003; 83: 119–30.

2 Buddle BM, Wedlock DN, and Denis M. Progress in the development of tuberculosis vaccines for cattle and wildlife. *Vet Microbiol*, 2006; 112: 191–200.

3 Aldwell FE, Keen DL, Parlane NA, Skinner MA, de Lisle GW, and Buddle BM. Oral vaccination with Mycobacterium bovis BCG in a lipid formulation induces resistance to pulmonary tuberculosis in brushtail possums. *Vaccine*, 2003; 22: 70–6.

4 Corner LA, Costello E, Lesellier S, O'Meara D, and Gormley E. Vaccination of European badgers (Meles meles) with BCG by the subcutaneous and mucosal routes induces protective immunity against endobronchial challenge with Mycobacterium bovis. *Tuberculosis (Edinb)*, 2008; 88: 601–9.

5 Lesellier S, Palmer S, Dalley DJ, Dave D, Johnson L, Hewinson RG, et al. The safety and immunogenicity of bacillus Calmette-Guerin (BCG) vaccine in European badgers (Meles meles). *Vet Immunol Immunopathol*, 2006; 112: 24–37.

6 Nol P, Palmer MV, Waters WR, Aldwell FE, Buddle BM, Triantis JM, et al. Efficacy of oral and parenteral routes of Mycobacterium bovis bacille Calmette-Guerin vaccination against experimental bovine TB in white-tailed deer (Odocoileus virginianus): a feasibility study. *J Wildl Dis*. 2008; 44: 247–59.

7 Palmer MV, Thacker TC, and Waters WR. Vaccination of white-tailed deer (Odocoileus virginianus) with Mycobacterium bovis bacillus Calmette Guerin. *Vaccine*, 2007; 25: 6589–97.

8 Southey A, Sleeman DP, Lloyd K, Dalley D, Chambers MA, Hewinson RG, et al. Immunological responses of Eurasian badgers (Meles meles) vaccinated with Mycobacterium bovis BCG (bacillus Calmette Guerin). *Vet Immunol Immunopathol*, 2001; 79: 197–207.

9 Wedlock DN, Aldwell FE, Keen D, Skinner MA, and Buddle BM. Oral vaccination of brushtail possums (Tichosurus vulpecula) with BCG: immune responses, persistence of BCG in lymphoid organs and excretion in faeces. *NZ Vet J*, 2005; 53: 301–6.

10 Francis J. TB. *Nature*, 1950; 165: 102–4.

11 Francis J. Control of infection with the bovine tubercle bacillus. *Lancet*, 1950; 1: 34–9.

12 Skinner MA, Wedlock DN, and Buddle BM. Vaccination of animals against Mycobacterium bovis. *Rev Sci Tech*, 2001; 20: 112–32.

13 Buddle BM, de Lisle GW, Pfeffer A, and Aldwell FE. Immunological responses and protection against Mycobacterium bovis in calves vaccinated with a low dose of BCG. *Vaccine*, 1995; 13: 1123–30.

14 Buddle BM, Keen D, Thomson A, Jowett G, McCarthy AR, Heslop J, et al. 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*, 1995; 59: 10–16.

15 Skinner MA, Buddle BM, Wedlock DN, Keen D, de Lisle GW, Tascon RE, et al. A DNA prime-Mycobacterium bovis BCG boost vaccination strategy for cattle induces protection against bovine tuberculosis. *Infect Immun*, 2003; 71: 4901–17.

16 Vordermeier HM, Chambers MA, Cockle PJ, Whelan AO, Simmons J, and Hewinson RG. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following Mycobacterium bovis BCG vaccination against experimental bovine tuberculosis. *Infect Immun*, 2002; 70: 3026–32.

17 Wedlock DN, Skinner MA, Parlane NA, Vordermeier HM, Hewinson RG, de Lisle GW, et al. Vaccination with DNA vaccines encoding MPB70 or MPB83 or a MPB70 DNA prime-protein boost does not protect cattle against bovine tuberculosis. *Tuberculosis (Edinb)*, 2003; 83: 339–49.

18 Buddle BM, Wedlock DN, Parlane NA, Corner LA, De Lisle GW, and Skinner MA. Revaccination of neonatal calves with Mycobacterium bovis BCG reduces the level of protection against bovine tuberculosis induced by a single vaccination. *Infect Immun*, 2003; 71: 6411–19.

19 Hope JC, Thom ML, Villarreal-Ramos B, Vordermeier HM, Hewinson RG, and Howard CJ. Vaccination of neonatal calves with Mycobacterium bovis BCG induces protection against intranasal challenge with virulent M. bovis. *Clin Exp Immunol*, 2005; 139: 48–56.

20 Buddle BM, Aldwell FE, Skinner MA, de Lisle GW, Denis M, Vordermeier HM, et al. Effect of oral vaccination of cattle with lipid-formulated BCG on immune responses and protection against bovine tuberculosis. *Vaccine*, 2005; 23: 3581–9.

21 Buddle BM, Denis M, Aldwell FE, Vordermeier HM, Hewinson RG, Wedlock DN. Vaccination of cattle with Mycobacterium bovis BCG by a combination of systemic and oral routes. *Tuberculosis (Edinb)*, 2008; 88: 595–600.

22 Wedlock DN, Denis M, Vordermeier HM, Hewinson RG, and Buddle BM. Vaccination of cattle with Danish and Pasteur strains of Mycobacterium bovis BCG induce different levels of IFNgamma post-vaccination, but induce similar levels of protection against bovine tuberculosis. *Vet Immunol Immunopathol*, 2007; 118: 50–8.

23 Pollock JM, Rodgers JD, Welsh MD, and McNair J. Pathogenesis of bovine tuberculosis: the role of experimental models of infection. *Vet Microbiol*, 2006; 112: 141–50.

24 Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. *Proc Natl Acad Sci USA*. 2007; 104: 5596–601.

25 Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, 1998; 393: 537–44.

26 Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, et al. The complete genome sequence of Mycobacterium bovis. *Proc Natl Acad Sci USA*. 2003; 100: 7877–82.

27 Gilbert SC, Moorthy VS, Andrews L, Pathan AA, McConkey SJ, Vuola JM, et al. Synergistic DNA-MVA prime-boost vaccination regimes for malaria and tuberculosis. *Vaccine*, 2006; 24: 4554–61.

28 Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CF, Estes DM, et al. Failure of a Mtb DeltaRD1 DeltapanCD double deletion mutant in a neonatal calf aerosol M. bovis challenge model: comparisons to responses elicited by M. bovis bacille Calmette Guerin. *Vaccine*, 2007; 25: 7832–40.

29 Buddle BM, Wards BJ, Aldwell FE, Collins DM, and de Lisle GW. Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine*, 2002; 20: 1126–33.

30 Wedlock DN, Aldwell FE, Collins DM, de Lisle GW, Wilson T, and Buddle BM. Immune responses induced in cattle by virulent and attenuated Mycobacterium bovis strains: correlation of delayed-type hypersensitivity with ability of strains to grow in macrophages. *Infect Immun*, 1999; 67: 2172–7.

31 Taracha EL, Bishop R, Musoke AJ, Hill AV, and Gilbert SC. Heterologous priming-boosting immunization of cattle with Mycobacterium tuberculosis 85A induces antigen-specific T-cell responses. *Infect Immun*, 2003; 71: 6906–14.

32 Vordermeier HM, Huygen K, Singh M, Hewinson RG, and Xing Z. Immune responses induced in cattle by vaccination with a recombinant adenovirus expressing mycobacterial antigen 85A and Mycobacterium bovis BCG. *Infect Immun*, 2006; 74: 1416–18.

33 Vordermeier HM, Rhodes SG, Dean G, Goonetilleke N, Huygen K, Hill AV, et al. Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Guerin. *Immunology*, 2004; 112: 461–70.

34 Cai H, Tian X, Hu XD, Li SX, Yu DH, and Zhu YX. Combined DNA vaccines formulated either in DDA or in saline protect cattle from Mycobacterium bovis infection. *Vaccine*, 2005; 23: 3887–95.

35 Maue AC, Waters WR, Davis WC, Palmer MV, Minion FC, and Estes DM. Analysis of immune responses directed toward a recombinant early secretory antigenic target six-kilodalton protein-culture filtrate protein 10 fusion protein in Mycobacterium bovis-infected cattle. *Infect Immun*, 2005; 73: 6659–67.

36 Maue AC, Waters WR, Palmer MV, Nonnecke BJ, Minion FC, Brown WC, et al. An ESAT-6:CFP10 DNA vaccine administered in conjunction with Mycobacterium bovis BCG confers protection to cattle challenged with virulent M. bovis. *Vaccine*, 2007; 25: 4735–46.

37 Wedlock DN, Skinner MA, de Lisle GW, Vordermeier HM, Hewinson RG, Hecker R, et al. Vaccination of cattle with Mycobacterium bovis culture filtrate proteins and CpG

oligodeoxynucleotides induces protection against bovine tuberculosis. *Vet Immunol Immunopathol*, 2005; 106: 53–63.

38 Skinner MA, Wedlock DN, de Lisle GW, Cooke MM, Tascon RE, Ferraz JC, et al. The order of prime-boost vaccination of neonatal calves with Mycobacterium bovis BCG and a DNA vaccine encoding mycobacterial proteins Hsp65, Hsp70, and Apa is not critical for enhancing protection against bovine TB. *Infect Immun*, 2005; 73: 4441–4.

39 Cai H, Yu DH, Hu XD, Li SX, and Zhu YX. A combined DNA vaccine-prime, BCG-boost strategy results in better protection against Mycobacterium bovis challenge. *DNA Cell Biol*, 2006; 25: 438–47.

40 Klinman DM, Ishii KJ, and Verthelyi D. CpG DNA augments the immunogenicity of plasmid DNA vaccines. *Curr Top Microbiol Immunol*, 2000; 247: 131–42.

41 Wedlock DN, Denis M, Skinner MA, Koach J, de Lisle GW, Vordermeier HM, et al. Vaccination of cattle with a CpG oligodeoxynucleotide-formulated mycobacterial protein vaccine and Mycobacterium bovis BCG induces levels of protection against bovine tuberculosis superior to those induced by vaccination with BCG alone. *Infect Immun*, 2005; 73: 3540–6.

41a Vordermeier HM, Villarreal-Ramos B, Cockle PJ, McAulay M, Rhodes SG, Thacker T, et al. Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis. *Infect Immun*, 2009; 77: 3364–73.

42 McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med*, 2004; 10: 1240–4.

43 Vordermeier HM, Chambers MA, Buddle BM, Pollock JM, and Hewinson RG. Progress in the development of vaccines and diagnostic reagents to control TB in cattle. *Vet J*, 2006; 171: 229–44.

44 Vordermeier M and Hewinson RG. Development of cattle TB vaccines in the UK. *Vet Immunol Immunopathol*, 2006; 112: 38–48.

45 Lyashchenko K, Whelan AO, Greenwald R, Pollock JM, Andersen P, Hewinson RG, et al. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with Mycobacterium bovis BCG and infected with *M. bovis*. *Infect Immun*, 2004; 72: 2462–7.

46 Demissie A, Abebe M, Aseffa A, Rook G, Fletcher H, Zumla A, et al. Healthy individuals that control a latent infection with Mycobacterium tuberculosis express high levels of Th1 cytokines and the IL-4 antagonist IL-4delta2. *J Immunol*, 2004; 172: 6938–43.

47 Wassie L, Demissie A, Aseffa A, Abebe M, Yamuah L, Tilahun H, et al. Ex vivo cytokine mRNA levels correlate with changing clinical status of Ethiopian TB patients and their contacts over time. *PLoS ONE*, 2008; 3: e1522.

48 Rhodes SG, Sawyer J, Whelan AO, Dean GS, Coad M, Ewer KJ, et al. Is interleukin-4delta3 splice variant expression in bovine tuberculosis: a marker of protective immunity? *Infect Immun*, 2007; 75: 3006–13.

49 Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, et al. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease. *Nat Med*, 2004; 10: 406–10.

50 Todryk SM, Bejon P, Mwangi T, Plebanski M, Urban B, Marsh K, et al. Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans. *PLoS ONE*, 2008; 3: e2027.

51 Godkin AJ, Thomas HC, and Openshaw PJ. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. *J Immunol*, 2002; 169: 2210–14.

52 Waters WR, Palmer MV, Nonnecke BJ, Thacker TJ, Capinos Scherer CF, Estes DM, et al. Efficacy and immunogenicity of Mycobacterium bovis DeltaRD1 against aerosol M. bovis infection in neonatal calves vaccine. *Vaccine*, 2009.

53 Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during M. tuberculosis challenge. *Nat Immunol*, 2007; 8: 369–77.

54 Khader SA and Cooper AM. IL-23 and IL-17 in tuberculosis. *Cytokine*, 2008; 41: 79–83.

55 Buddle BM, Parlane NA, Keen DL, Aldwell FE, Pollock JM, Lightbody K, et al. Differentiation between Mycobacterium bovis BCG-vaccinated and M. bovis-infected cattle by using recombinant mycobacterial antigens. *Clin Diagn Lab Immunol*, 1999; 6: 1–5.

56 Vordermeier HM, Cockle PC, Whelan A, Rhodes S, Palmer N, Bakker D, et al. Development of diagnostic reagents to differentiate between Mycobacterium bovis BCG vaccination and M. bovis infection in cattle. *Clin Diagn Lab Immunol*, 1999; 6: 675–82.

57 Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*, 1999; 284: 1520–3.

58 Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, and Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Mol Microbiol*, 1999; 32: 643–55.

59 Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, and Stover CK. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. *J Bacteriol*, 1996, 178: 1274–82.

60 Pollock JM and Andersen P. Predominant recognition of the ESAT-6 protein in the first phase of interferon with Mycobacterium bovis in cattle. *Infect Immun*, 1997; 65: 2587–92.

61 Pollock JM and Andersen P. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of TB. *J Infect Dis*, 1997; 175: 1251–4.

62 Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, and Hewinson RG. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine TB in cattle. *Clin Diagn Lab Immunol*, 2001; 8: 571–8.

63 Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, et al. The complete genome sequence of Mycobacterium avium subspecies paratuberculosis. *Proc Natl Acad Sci USA*, 2005; 102: 12344–9.

64 Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PD, et al. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mtb. *Genome Res*, 2008; 18: 729–41.

65 Cockle PJ, Gordon SV, Lalvani A, Buddle BM, Hewinson RG, and Vordermeier HM. Identification of novel Mycobacterium tuberculosis antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect Immun*, 2002; 70: 6996–7003.

66 Pallen MJ. The ESAT-6/WXG100 superfamily — and a new Gram-positive secretion system? *Trends Microbiol*, 2002; 10: 209–12.

67 Aagaard C, Govaerts M, Okkels LM, Andersen P, and Pollock JM. Genomic approach to identification of Mycobacterium bovis diagnostic antigens in cattle. *J Clin Microbiol*, 2003; 41: 3719–28.

68 Cockle PJ, Gordon SV, Hewinson RG, and Vordermeier HM. Field evaluation of a novel differential diagnostic reagent for detection of Mycobacterium bovis in cattle. *Clin Vaccine Immunol*, 2006; 13: 1119–24.

69 Vordermeier M, Whelan AO, and Hewinson RG. 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*, 2003; 71: 1980–7.

70 Sidders B, Pirson C, Hogarth PJ, Hewinson RG, Stoker NG, Vordermeier HM, et al. Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the Mycobacterium tuberculosis complex. *Infect Immun*, 2008; 76: 3932–9.

71 MacGurn JA, Raghavan J, Stanley SA, and Cox JS. A non-RD1 gene cluster is required for Snm secretion in Mycobacteruim tuberculosis. *Mol Microbiology*, 2005; 57: 1653–63.