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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: CB0116 (OM0122)
2. Project title: Efficacy testing of BCG vaccine in badgers
3. Contractor organisation(s): Veterinary Laboratories Agency
4. Total Defra project costs (agreed fixed price): £ 1,192,328
5. Project: start date ..... 01 April 2006
   end date ..... 30 July 2010

6. It is Defra's intention to publish this form.
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(a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.
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*This report only contains information relating to the two objectives on injectable vaccines. Information relating to the third objective on oral vaccines will not be released at this time as it contains commercially sensitive information.

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Efforts to eradicate bovine tuberculosis (bTB) from cattle have been thwarted where badgers are a wildlife reservoir of M. bovis. Removal of infected badgers can significantly reduce the incidence of bTB in local cattle herds but can have complex epidemiological outcomes, including both positive and negative impacts on the incidence of TB in cattle. Furthermore, badger culling as an approach to disease control can be costly, practically difficult, and indiscriminate and remains controversial. Vaccinating badgers may be an alternative or complementary strategy that overcomes some of these challenges. As part of a programme of research into practical methods to control bTB, Defra commissioned the VLA to conduct a series of studies with the objective of seeking a Marketing Authorisation (MA) for an injectable form of the human TB vaccine, BCG, for use in badgers.

M. bovis strain BCG vaccination via the subcutaneous (SC) or combined intranasal/conjunctival routes confers a degree of protection to badgers against experimental challenge with M. bovis. However, neither route is currently practical for delivery to wild badgers. Until this project, BCG vaccination via the intramuscular (IM) route had not been considered, largely because the route is considered to be associated with adverse effects in humans. However, we have already demonstrated that IM administration of BCG to badgers is both safe and of equivalent immunogenicity to SC BCG and has the practical advantage of being easily administered to restrained wild badgers without recourse to chemical immobilisation.

The objectives of this project were three-fold: (1) to establish an experimental M. bovis infection model in captive badgers which could be used to evaluate the efficacy of BCG; (2) to use the model to evaluate the efficacy of two different doses of BCG delivered IM;

The first year of the project focussed successfully on the extensive preparations required for the experimental work, resulting in the appropriate licenses, resources, and expertise needed. In the second year, the efficacy of a high dose (10x prescribed human dose, 2-8x10^6) of IM BCG was determined in a small scale pilot study. These data were added to in the third year with a larger study of IM BCG at both the high dose and the dose used in human adults (2-8x10^5).

The first experiment (VES1) with IM BCG involved five badgers and the second experiment (VES2) 18 badgers. The third experiment (VES3) used 20 badgers. In each experiment a group of animals were kept non-vaccinated as controls. Seventeen weeks (for VES1 & 2) and thirteen weeks (for VES3) after vaccination of the remaining groups, all badgers were challenged by intrabronchial installation of

approximately 10^3 M. bovis. Once every two to three weeks throughout each experiment from a time point preceding vaccination, the badgers were anaesthetised and examined. Blood was collected by jugular venipuncture for immunological, haematological and biochemical analyses. Samples of tracheal mucus, urine and faeces were taken for culture of M. bovis. In all cases, the experiment was terminated 12 weeks after challenge and disease assessed following detailed post mortem examination to derive a gross lesion score and collection of tissues for M. bovis culture and semi-quantitative histological assessment of infection. In all three studies disease mainly occurred in the organs of the thoracic cavity, with limited dissemination elsewhere, no generalized TB, and the absence of visible lesions in some infected tissues.

BCG given IM generated a strong cell-mediated immune response characterised by the production of interferon-γ (IFNγ) and protection against artificial challenge with virulent M. bovis. Protection was seen as a significant reduction in visible lesions at post mortem examination and reduced bacterial excretion. Protection was dependent on BCG dose. The dose prescribed for adult humans (2-8x10^5) provided less protection than a ten-fold higher dose.

As IM BCG is both protective and safe for badgers and feasible to administer to restrained conscious animals, it makes field vaccination of badgers feasible for the first time; representing a new intervention tool for the control of bovine TB in badgers. On the basis of the efficacy data generated in this project, together with data on the safety and quality of the vaccine from other Defra-funded VLA projects, the VMD granted a Marketing Authorisation to the VLA for the HD IM BCG vaccine (called BadgerBCG) on 24th March 2010.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Scientific objectives

01 Obtain permissions, resources and protocols for the study (by 01/11/2006).
MET IN FULL

02 First Experiment: Determine protective efficacy of BCG vaccine injected intramuscularly (by 07/03/2008).
MET IN FULL

03 Second Experiment: Gather further data on the protective efficacy of BCG vaccine (by 12/02/2009).
MET IN FULL

Permissions, resources and protocols

The first year of the project focussed on the extensive preparations required for the experimental work (Objective 01). Only immunologically naïve badgers can be used in studies designed to demonstrate the efficacy of

vaccines. Such animals needed to be obtained from the wild under licence from Natural England. Successful application to Natural England was made first in 2006, and subsequently to cover the duration of the project.

The experimental work carried out on the badgers, including the preliminary screening of blood and clinical samples for M. bovis are regulated procedures under the Animals (Scientific Procedures) Act 1986. Permission was therefore sought from the Home Office to amend the existing Project and Personal licences held by staff at VLA to allow the work to proceed. The amendment was submitted to the Home Office in 2006, following extensive review by the internal Ethical Review Panel of the VLA, and after consent to proceed was sought from the directorship of the VLA. The revised licence was granted by the Home Office in August 2006.

The existing NEC facility at VLA was extensively modified in Spring 2006 to allow the badgers used for this project to be housed separately from the resident colony. Five large pens were constructed, each capable of subdivision into two, depending on the number of badgers housed. Each subdivision (10 in total) can hold up to four badgers, bringing the capacity up to 40 animals. The modifications to the NEC were based on the design used successfully by collaborators at University College Dublin (UCD) in the Republic of Ireland and at the VLA since 2001. The setts for this project were specifically designed so that they could be sealed off with the badgers inside during daylight and the entire sett transported to a high containment facility with as little disturbance to the badgers as possible. The animal facilities at the VLA for housing badgers have proved to be a key asset to Defra in realising this work.

The sourcing of suitable badgers for this project was subcontracted to experienced staff of the Food and Environment Research Agency (Fera) who acted as accredited agents of the VLA under the terms of the Natural England licence. Fera staff identified suitable groups of badgers from the county of Suffolk to use for this project. Suffolk was an appropriate choice since the county has no history of TB in badgers (and only very little in cattle), the badger density is sufficiently high that badgers could be removed with only minimum disturbance to the remaining populations, and Fera staff have experience and contacts with the appropriate local interest groups. Before any fieldwork took place, representatives of Fera and VLA met with a Defra security advisor and discussed the intended operations with the local police in both Surrey and Suffolk. A plan of operation and appropriate contingency plans were put into place.

To facilitate the experimental infection of badgers, a fibrescope was used to locate and deliver the M. bovis challenge inoculum to a defined region of the lower respiratory tract. This method was used successfully in similar experiments in Ireland, resulting in a uniform and controlled pattern of pulmonary pathology. Suitable equipment was purchased by the VLA and staff trained in its use in the first year of the project.

At the time of post-mortem examination (PME) it was essential that a pre-agreed SOP was followed so that the measures used for determining vaccine efficacy (pathology and bacterial culture) were standardised and the staff conducting the PME blinded to the vaccination status of the animals. This was crucial to the legitimacy of analysing the data from different experiments collectively. In order to reach a consensus on the most suitable protocols to follow for the PME, culture, and subsequent semi-quantification of the pathology, a two-day 'Badger Pathology Workshop' was held at the VLA in February 2006. The workshop involved all those with experience of TB pathology in the badger and culture of M. bovis, and included representatives from Defra, VLA, UCD, overseas, and a private pathology consultant. The meeting was viewed as a success and a unique opportunity to pool experience and findings. As an outcome from the Workshop, a number of SOPs were drafted and circulated to the workshop attendees for comment and refinement. The SOPs used in this project therefore represent what is viewed as 'best practice' in the eyes of the relevant scientific peer-group.

Protective efficacy of BCG vaccine injected intramuscularly

The second year of the project saw the completion of the first vaccine efficacy study (VES1) to determine the protective efficacy of intramuscular (IM) BCG vaccine in badgers (Objective 02), and the initial steps to prepare for a repeat study (VES2) with more badgers. VES2 was completed in the third year of the project (Objective 03), building on the data already generated in VES1 and extending study to comparison of two doses of IM BCG.

The full results from studies VES1 & VES2 were compiled in a detailed study report made available to Defra in July 2009 and used as part of a submission to the Veterinary Medicines Directorate (VMD) for a Marketing Authorisation for the IM BCG vaccine. A summary of the materials, methods, results and conclusions are presented here and are followed by a summary of vaccine efficacy taken from the detailed report.

Materials and methods

BCG Vaccine

BCG Danish strain 1131 vaccine was supplied by the Statens Serum Institut (SSI), Denmark at 2-8x10^6 CFU per vial. The higher dose of vaccine was prepared by adding 1ml of Sauton diluent (provided by SSI) to each vial. Sufficient reconstituted vials were pooled in order to provide a uniform BCG dose to all animals. To prepare the low dose BCG, one BCG vial was reconstituted in 10 ml of Sauton diluent. At the end of each day's vaccination session, residual vaccine was cultured on modified Middlebrook 7H11 agar plates to determine the viable count and titre of the vaccine (Table 1).

TABLE 1. Vaccination of badgers with BCG: Number of animals in each treatment group, and the vaccine and challenge doses

Experiment	Treatment group(a)	Dose of BCG (CFU(b)/ml)	Dose of M. bovis (CFU/ml)	Number of badgers
VES1	HD BCG	5.4x10^6	4.8x10^3	3
	Non-vaccinated	NA	4.8x10^3	1
			3.7 x10^3	1
VES2	HD BCG	3.2x10^6	2.6 x10^3	4
			2.8 x10^3	2
	LD BCG	3.3x10^5	2.6 x10^3	4
			2.8 x10^3	4
	Non-vaccinated	NA	2.8 x10^3	4

(a)HD = high dose; LD = low dose.
(b)CFU = colony forming units.

Mycobacterium bovis

The M. bovis strain used for challenge was originally isolated from an infected wild badger in the UK in 1997 (isolate 74/0449/97). It had been stored as a first passage stock culture until expanded and stored as frozen aliquots (-80°C) for badger infection studies at the VLA. The clonality of the culture was confirmed by demonstrating the spoligotype (type 9) and VNTR type (8 5 5 5* 3 3.1) of 10% of colonies grown from a culture at ~10^5 CFU/ml. The stock vials used for the challenge had not been passaged further. Each vial contained ~10^7 CFU/ml viable M. bovis. On the day of challenge, one aliquot was thawed and serially diluted in sterile water + 0.05% (v/v) tween 80 to contain approximately 5x10^3 CFU/ml. The dilutions were vortexed to diminish the risk of bacterial clumping. The final dilution (challenge inoculum) was made in sterile PBS + 0.05% tween 80. The titre of the challenge inoculum was determined by plating a sample from a syringe, kept in the same conditions as those used for challenge, on Middlebrook 7H11 agar. For both experiments challenge occurred over two separate days, and the viable count of the inoculum was determined on each day (Table 1).

Animals and sampling

The first experiment (VES1) involved five badgers and the second experiment (VES2) 18 badgers (Table 1). The badgers were housed in groups of up to four animals, corresponding as much as possible to their originating social group at capture. Badgers were identified by subcutaneous microchip with a unique number and a cutaneous tattoo. The badgers received a diet of dog food, peanuts and occasionally eggs. Tap water was supplied ad libitum in large trays that allowed both bathing and drinking. Food uptake, weight variations, and blood chemistry and haematology parameters were used to monitor their welfare. The badgers were moved to an ACDP Containment Level 3 facility approximately 5-6 weeks before challenge.
Once every two to three weeks, the badgers were anaesthetised and examined. Blood was collected by jugular venipuncture into Vacutainer tubes for immunological, haematological and biochemical analyses. Tracheal mucus was collected by aspirating with a flexible urinary catheter and dispensed into Middlebrook 7H9 broth. Laryngeal and rectal swabs were collected and placed into 7H9 broth and PBS, respectively. Urine was collected into sterile 15ml plastic tubes following compression of the bladder.

Vaccination

Allocation of groups to treatments was by randomisation. The vaccine was injected in the left lumbar muscle, following shaving and cleaning of the overlying skin. All vaccinated animals received 1ml of vaccine that had been reconstituted for less than four hours (as per SSI recommendations). In VES1 only high dose vaccine was used and VES2 both high and low dose was used (Table 1).

Experimental infection with M. bovis

Seventeen weeks post-vaccination, all badgers were infected under anaesthesia, by endobronchial instillation of 1ml of M. bovis suspension using a 70cm fibroscope and targeting the bronchus of the right middle lobe. The M. bovis suspension was inoculated via a sterile plastic catheter and the catheter was flushed with 1ml PBS. Between animals the fibroscope was disinfected with ortho-phthalaldehyde and 70% ethanol, then rinsed with sterile water.

Post-mortem examination

Twelve weeks after challenge (29 weeks after vaccination), the badgers were killed humanely with an intravenous overdose of Sodium Pentobarbitone and immediately subjected to post-mortem examination. A pre-determined set of 27 tissues were collected at post-mortem and examined for gross/visible lesions (Tables 2-4). Gross lesions were detected by finely slicing lymph nodes (LNs) and organs. Samples of the sliced tissues were collected for histopathology and culture: each LN was divided between histology and culture; for the larger organs such as spleen, approx. 3cm of tissue was submitted for culture and the rest for histology. Histologically, a TB lesion consisted of one or more granulomas containing acid-fast bacteria (AFB) in Ziehl-Neelsen (ZN) stained sections. A visible lesion score was derived using a standardised ordinal scoring system of 1-4 (few foci or slight swelling to extensive caseation or areas of coalesced foci) [1,2]. Only visible lesions subsequently confirmed as tuberculous by either isolation of M. bovis from the tissue by culture or the appearance of AFB in ZN stained histological sections counted towards the final score. The score was derived from the sum of the highest scoring lung lobe plus the scores from all other tissues.

Culture of M. bovis

Clinical samples (tracheal aspirate, laryngeal swab, urine and rectal swab) were taken every 2-3 weeks from the day of challenge to detect M. bovis excretion. All samples were cultured on the day of collection, except for faecal swabs which were cultured on the following working day. The laryngeal swab and tracheal aspirate were placed in 7H9 broth and then cultured on Middlebrook 7H10 slopes and incubated for 12 weeks at 37°C. Except for faecal swabs, the samples were not decontaminated before culture. Faecal swabs were soaked overnight in 0.85% saline solution. The following day the swab was discarded and the sodium chloride saline solution decontaminated with 5% final volume oxalic acid for 10 minutes at room temperature. Material for sowing was retrieved by centrifugation and the oxalic acid removed by a wash step using saline. A sample of positive cultures were identified by spoligotyping [3] and VNTR [4] to confirm that the isolates were the same as the challenge strain.

Tissue samples collected at post-mortem were taken aseptically, weighed and frozen at -20°C. Tissues for culture were thawed to room temperature and each tissue sample was cultured separately. Tissues were homogenised in 10ml 0.85% saline using IKA® tubes. Samples (100μl) of tissue homogenate were plated onto each of four plates of Middlebrook 7H11 and each of four plates of Middlebrook 7H11 containing 60μg/ml cycloserine [5,6] to preferentially select for BCG growth. If contamination occurred, stored homogenate was re-cultured. Plates were examined after six and 12 weeks of incubation. Up to 20 colonies from one plate per tissue were typed by spoligotyping and VNTR.

Immunological assays

The kinetics of the immune responses were monitored by measuring the frequency of peripheral blood mononuclear cells (PBMC) producing IFNγ by ELISPOT assay [7], and by measuring specific antibodies against MPB83 in serum using the Brock TB Stat-Pak test [8]. Antigens used to stimulate PBMC cultures were PPD-B and a cocktail of ESAT-6 and CFP-10. The mitogen concanavalin A was used as a positive control.

Data analysis

Analyses were undertaken on lesion scores using general linear modelling with factors treatment group and experiment to provide evidence of any significant difference between treatment groups and between experiments. In view of visible lesion scores being measured on an ordinal scale and as there was no significant influence of experiment as a factor when comparing the same treatments across experiments, data from the same treatment were pooled and the final analysis undertaken using the non-parametric Kruskal Wallis test followed by Dunn's Multiple Comparison Test to identify significant differences ($p<0.05$). The proportions of culture positive tissues were compared for significant differences ($p<0.05$) between treatment groups using Fisher's Exact Test. The distribution of times post-challenge until a positive culture was obtained was compared between treatment groups and the mean time to a positive culture of clinical samples post-challenge was estimated with 95% confidence

interval using Kaplan-Meier estimates. A nonparametric distribution analysis approach was undertaken using the log-rank test. All analyses were undertaken by an independent, qualified statistician using Minitab Version 15.1 (2007) and NCSS Version 7.1.5 (2008).

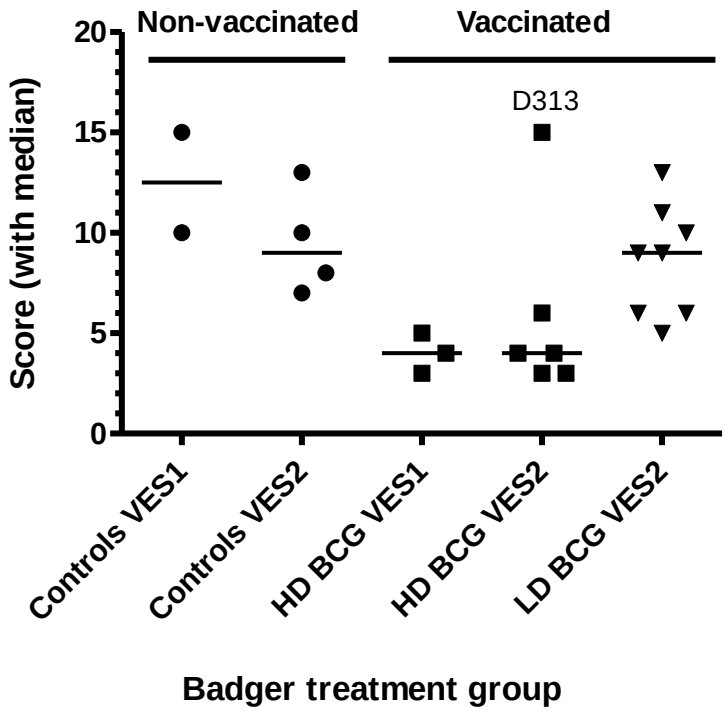
Results

The M. bovis challenge strain was recovered from all badgers. Tables 2-4 presents details of where gross lesions were observed and which tissues were culture or histologically positive for M. bovis for each animal in each experiment.

Tuberculous lesions post mortem

The number of visible lesion sites and lesion scores for each animal in both experiments, based on visible lesions confirmed to be of M. bovis origin, are shown in Tables 2, 3 and 4. The scores were similar for the same treatment across experiments as determined by GLM. Combining the results of experiments VES1 and VES2 increased statistical power to detect treatment effects. Analysis of the combined data across experiments for the same treatment revealed significant differences between treatments: non-vaccinated vs LD BCG ($p = 0.431$); non-vaccinated vs HD BCG ($p = 0.004$); LD BCG vs HD BCG ($p = 0.025$). One HD vaccinated badger in VES2 (D313) was not protected, and in fact exhibited the greatest pathology (Fig. 1).
In most animals, the right middle lobe contained the most severe lesions, but in four, the right caudal lobe was infected with no visible lesions in the middle lobe. The left side of the lung contained visible lesions in three badgers vaccinated with LD BCG, and in one HD BCG badger (D313). Left side lesions were always in association with lesions on the right side. Pleurisy on the mediastinum was observed across all groups and no statistically significant difference between groups was detected in this respect. The most affected thoracic draining LNs were the right bronchial LN and posterior mediastinal LN. The most affected LN outside of the thoracic cavity was the hepatic LN. M. bovis was isolated from the spleen of four non-vaccinated badgers, one HD vaccinated badger (D313), and four LD vaccinated badgers. No lesions were found in the kidneys of any animal.
The distribution of histological lesions and bacteriologically positive tissues are also shown in Tables 2, 3 and 4.

Fig. 1. Vaccination of badgers with BCG and challenged 17 weeks later with endobronchial M. bovis. Lesion scores at post-mortem (12 weeks post-challenge) in experiments VES1 and VES2. Individual animal results are shown together with the group median. Badgers were vaccinated with either a high or low dose of BCG (HD, LD, respectively). The score of one animal (D313) is indicated where vaccinated failed to protect.



Distribution of infection

As there was some discordance between culture positive and histologically positive tissues, to describe the dissemination of infection an affected site was defined as either culture positive or histologically positive or both. The median number of tissues affected by M. bovis was nine in the non-vaccinated group, five in the HD BCG group and eight in the LD BCG group. These differences were not significant.

Excretion of M. bovis

M. bovis was detected intermittently in the larynx/trachea of non-vaccinated badgers from four weeks post-challenge in VES1 (Table 5) and from two weeks post-challenge in VES2 (Table 6). All six controls and 7/8 badgers vaccinated with LD BCG excreted M. bovis at some point in the experiment, while only 3/9 animals vaccinated with HD BCG had positive samples (Tables 5 and 6). The controls were positive on 17 of 36 possible occasions, the LD BCG group positive 14 of 48 occasions and the HD BCG group 7 of 54 occasions. One of the HD BCG badgers, D313, which had extensive lesions in the lung at post-mortem, was positive on 5/6 occasions, and two HD BCG badgers (C071 in VES1 and D591 in VES2) were positive on one occasion only.
Fewer badgers excreted M. bovis in the group vaccinated with BCG than in the control. The proportion of animals in a treatment group yielding an M. bovis positive clinical sample and the time until the first positive culture result was obtained were examined (Fig. 2). Pair-wise comparison of proportions between the three groups showed no significant differences between the non-vaccinated and LD BCG groups ($p = 1.00$, Fisher's exact test). However, the non-vaccinated and the LD BCG groups had significantly higher proportions of positive cultures than the HD BCG group ($p = 0.028$ and 0.050, respectively, by Fisher's exact test). Similarly, the time taken for the HD BCG group to provide a positive sample was significantly longer than the times for the non-vaccinated and LD BCG groups ($p = 0.002$ and $p = 0.006$, respectively, by log-rank test). M. bovis was not detected in the urine of any badger, and only a single faeces sample yielded M. bovis: the post-mortem sample from HD BCG vaccinated badger D313.

TABLE 2. Distribution and score of gross lesions (L), tissues that yielded an M. bovis culture (C), or contained acid-fast bacilli on histology (H) in non-vaccinated badgers in both studies

	VES1						VES2											
	C037			C029			D118			D123			D547			D811		
TISSUE(a)	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H
L cranial					X			X										
L caudal																		X
R cranial					X		1	X	X			X				2	X	X
R middle			X	4	X	X	3	X	X	4	X	X	4		X	3	X	X
R caudal	4	X	X				1	X					1	X		2	X	X
Accessory		X		2	X				X								X	
Ant med					X												X	X
L bronchial	1	X		1	X						X						X	
Post med	3	X	X	1	X		2	X	X				1	X		4	X	X
R bronchial	3	X	X	3	X	X	3	X	X	3	X	X	4	X	X	4	X	X
L mandib																		
R mandib																		X
L parotid																	X	
R parotid																	X	
L retrophar																		
R retrophar																		
L&R axillary					X												X	
L&R inguin																		
L&R pop																		
HLN	2	X	X		X			X	X		X	X		X	X		X	X
MLN					X												X	
Tonsils																		
Mediastinum	2	X		1	X						X					2	X	
Spleen		X						X					1	X			X	
Liver																	X	
Heart																		
Kidney																		

(a)Ant med = anterior mediastinal LN; Post med = posterior mediastinal LN; mandib = mandibular LN; retropar = retropharyngeal LN; inguin = inguinal LN

TABLE 3. Distribution and score of gross lesions (L), tissues that yielded an M. bovis culture (C), or contained acid-fast bacilli on histology (H) in badgers vaccinated with HD BCG in both studies

	VES1									VES2																
	C071			C067			C094			D101			D313			D546			D564			D591			D862		
TISSUE(a)	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H
L cranial													3	X	X												
L caudal													2	X	X												
R cranial			X										2	X	X												
R middle	2	X	X	1	X					1	X		3	X	X				1	X	X	1	X	X	1	X	X
R caudal					X								3	X	X	1	X										
Accessory				1	X	X							2	X	X				1	X							
Ant med																											
L bronchial							2	X	X					X													
Post med		X			X	X	1	X					1	X	X		X		1	X		1	X				
R bronchial	2	X	X	2	X	X				2	X	X	4	X	X	2	X	X	2	X	X	2	X	X	1	X	X
L mandib																											
R mandib																											
L parotid																											
R parotid																											
L retrophar																											
R retrophar																											
L&R axillary																											
L&R inguin																											
L&R pop														X													
HLN		X						X			X			X	X		X	X		X						X	
MLN													1	X													
Tonsils																											
Mediastinum	1	X		1	X						X		4	X					2	X					1	X	
Spleen														X													
Liver													2	X	X		X			X							
Heart																											
Kidney																											

(a)Ant med = anterior mediastinal LN; Post med = posterior mediastinal LN; mandib = mandibular LN; retropar = retropharyngeal LN; inguin = inguinal LN

TABLE 4. Distribution and score of gross lesions (L), tissues that yielded an M. bovis culture (C), or contained acid-fast bacilli on histology (H) in badgers vaccinated with LD BCG

	VES2																							
	D126			D264			D304			D343			D346			D619			D779			D816		
TISSUE(a)	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H	L	C	H
L cranial										1		X							3	X				
L caudal	1	X																	2	X				
R cranial										3	X	X							3	X	X	3	X	X
R middle		X		3	X	X	3	X	X	3	X	X	3	X	X	1	X	X				4	X	X
R caudal	2		X						X	3	X								2	X		1	X	
Accessory									X										1	X				
Ant med																								
L bronchial	1	X									X													
Post med	1	X	X					X	X	1	X	X		X		1	X	X					X	X
R bronchial	1	X	X	2	X	X	2	X	X	1	X	X	4	X	X	2	X	X	4	X	X	2	X	X
L mandib																								
R mandib																								
L parotid																								
R parotid																								
L retrophar																								
R retrophar																								
L&R axillary														X										
L&R inguin																								
L&R pop																								
HLN		X			X	X		X		1	X	X		X	X		X	X		X	X		X	X
MLN																								
Tonsils																								
Mediastinum	1	X						X		4	X		3	X		2	X		2	X		4	X	
Spleen					X						X		1	X						X				
Liver											X			X									X	
Heart																							X	
Kidney																								

(a)Ant med = anterior mediastinal LN; Post med = posterior mediastinal LN; mandib = mandibular LN; retropar = retropharyngeal LN; inguin = inguinal LN

TABLE 5. Culture of clinical samples for M. bovis in experiment VES1

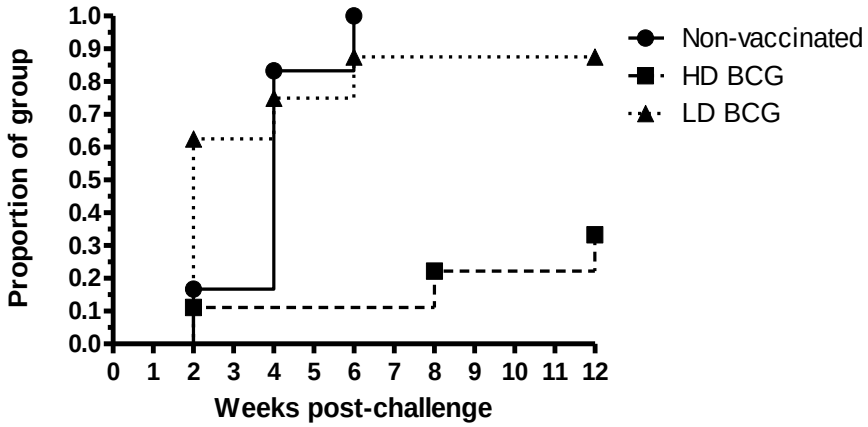
		Weeks after M. bovis challenge						
Treatment(a)	Badger	0	2	4	6	8	10	12
Non-vaccinated	C029			LS	LS			
Non-vaccinated	C037			LS/TA(b)	LS/TA		LS	
HD BCG	C094							
HD BCG	C071							LS
HD BCG	C067							

TABLE 6. Culture of clinical samples for M. bovis in experiment VES2

		Weeks after M. bovis challenge						
Treatment(a)	Badger	0	2	4	6	8	10	12
Non-vaccinated	D118			TA/LS(b)	LS	LS		
Non-vaccinated	D123				TA	LS		
Non-vaccinated	D811		LS	TA/LS	LS	TA/LS	TA	
Non-vaccinated	D547			LS	TA			
LD BCG	D126		LS					
LD BCG	D284				TA	TS	TA/LS	
LD BCG	D304		TA	TA				
LD BCG	D343		LS	TA	TA			
LD BCG	D346			LS				
LD BCG	D619							
LD BCG	D779		LS		LS			
LD BCG	D816		TA		TA			
HD BCG	D546							
HD BCG	D862							
HD BCG	D101							
HD BCG	D313		LS	LS		LS	LS	Fb
HD BCG	D564							
HD BCG	D591					TA		

(a)HD = high dose; LD = low dose.
(b)LS = laryngeal swab; TA = tracheal aspirate; F = faecal sample collected at post-mortem.

Fig. 2. Vaccination of badgers with BCG and challenged 17 weeks later with endobronchial M. bovis: Proportion of treatment group that yielded M. bovis growth from a clinical sample. Data from both experiments were combined. Badgers were vaccinated with either a high or low dose of BCG (HD, LD, respectively).



Immunology

IFNγ ELISPOT

The IFNγ ELISPOT assay measures the number of PBMC responding to antigenic stimulation. An IFNγ response was detected by ELISPOT in all vaccinated and all challenged badgers (Figs 3 and 4). The magnitude of the responses was greater in VES1 than in VES2 but the overall pattern was similar in the two experiments. The vaccinated animals were responsive to PPD-B but not to a combination of ESAT-6 and CFP-10 antigens, consistent with these latter antigens being expressed by M. bovis but not BCG [9]. The magnitude of response following HD BCG vaccination was lower in VES2 (Fig. 4A) than in VES1 (Fig. 3A). The response to LD BCG was

lower still, but still clearly above the background responses seen in the non-vaccinated controls (Fig. 4A), providing evidence that the vaccine generated T-cell memory in these animals. No correlation was found between the pre-challenge IFNγ response and the final lesion score.

Fig. 3. Vaccination of badgers with BCG and challenged 17 weeks later with endobronchial M. bovis: IFNγ ELISPOT results from experiment VES1 expressed as the net spot forming units (SFU) calculated by subtracting the response in the absence of antigen from the response to stimulation with PPD-B (A) or ESAT-6/CFP-10 (B). Vaccination with high dose (HD) BCG occurred on day 8 and challenge with M. bovis on day 126 following commencement of the study.

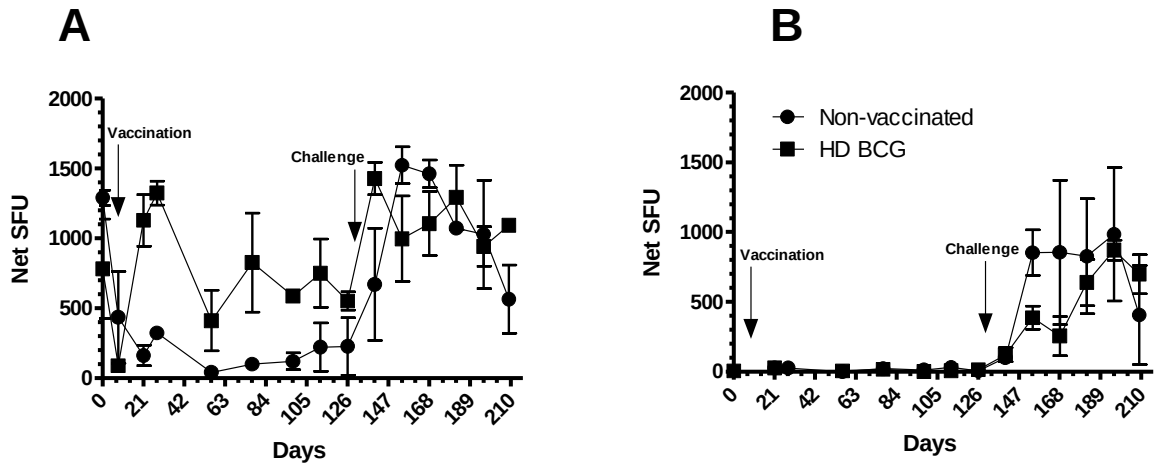
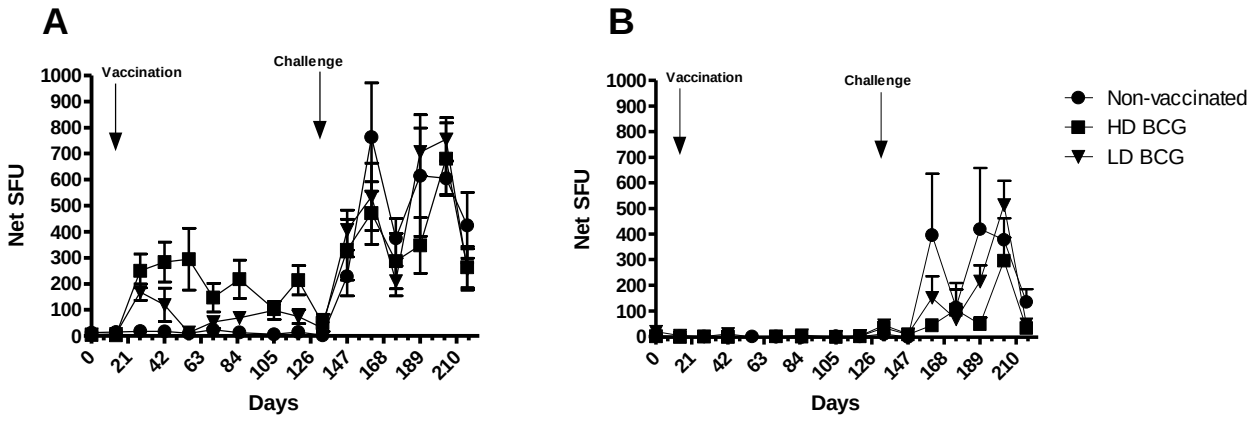


Fig. 4. Vaccination of badgers with BCG challenged 17 weeks later with endobronchial M. bovis: IFNγ ELISPOT results from experiment VES2 expressed as the net spot forming units (SFU) calculated by subtracting the response in the absence of antigen from the response to stimulation with PPD-B (A) or ESAT-6/CFP-10 (B). Vaccination with either high dose (HD) or low dose (LD) BCG occurred on day 14 and challenge with M. bovis on day 133 following commencement of the study.



In both experiments all animals showed an ELISPOT response to challenge. In both experiments, vaccinated badgers had a greater IFNγ response than non-vaccinated at two weeks post vaccination; evidence of priming caused by vaccination (Fig. 3A and 4A). At 4 and 6 weeks after challenge the mean response of non-vaccinated animals in both VES1 and VES2 exceeded that of the vaccinated animals. From 8 to 12 weeks after challenge, there were no distinct pattern in the responses of the groups but the responses remained elevated in all groups. The earliest distinct response to ESAT-6/CFP-10 antigens was seen at four weeks after challenge (Figs. 3B & 4B). In both experiments the mean response to the combined antigens was greater in the non-vaccinated groups compared with the vaccinated groups between weeks 4 and 8 after challenge; after which there were no consistent differences between groups.

Serology

The reactivity of serum in the Brock TB Stat-Pak test are shown in Table 7. One animal (C071) was reactive in the Stat-Pak prior to vaccination and remained so for the duration of the experiment. No animal became reactive in the Stat-Pak as a consequence of vaccination. After challenge with M. bovis, all badgers in VES2 were reactive in the Stat-Pak on two or more sampling occasions. For VES1, both of the non-vaccinated animals and 1/3 animals in the HD BCG vaccinated group remained non-reactive after challenge.

TABLE 7. Serological responses after vaccination and challenge measured by Brock TB Stat-Pak(a)

				V	Weeks post-vaccination							C	Weeks post-challenge					
Expt.	Treatment	ID	-2	0	2	4	6	8	10	13	15	17	2	4	6	8	10	12
VES1	Non-vacc	C029																
	Non-vacc	C037																
VES2	Non-vacc	D118											■	■	■	■	■	■
	Non-vacc	D123															■	■
	Non-vacc	D811												■	■	■	■	■
	Non-vacc	D547												■	■	■	■	■
VES1	HD BCG	C094												■	■	■	■	■
	HD BCG	C071	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	HD BCG	C067																
VES2	HD BCG	D546												■	■	■	■	■
	HD BCG	D862												■	■	■	■	■
	HD BCG	D101												■	■	■	■	■
	HD BCG	D313												■	■	■	■	■
	HD BCG	D564													■	■	■	■
	HD BCG	D591													■	■	■	■
VES2	LD BCG	D126												■	■	■	■	■
	LD BCG	D284												■	■	■	■	■
	LD BCG	D304														■	■	
	LD BCG	D343												■		■	■	■
	LD BCG	D346													■	■	■	■
	LD BCG	D619												■	■	■	■	■
	LD BCG	D779												■	■	■	■	■
	LD BCG	D816													■	■	■	■

(a)Shaded cell indicates a positive test result.

Animal welfare

The badgers adapted well to captivity with food intake and body weights considered normal. Haematological and biochemical parameters remained within the normal ranges and did not vary between vaccinated and non-vaccinated groups, or between pre- and post-challenge periods (data not shown). The badgers did not show a reduction in the number of leukocytes or reduced activation of neutrophils (data not shown) that are signs of stress in badgers [10], and in possums after capture [11].

Discussion

BCG administered IM to badgers elicited an IFNγ response and was able to reduce the severity and progression of experimentally-induced TB in badgers. Vaccination with BCG induced a protective response, with the high dose (10 times the human dose) being more immunogenic and giving superior protection than the low dose (the normal human dose).

A target challenge dose of 10^3-10^4 CFU M. bovis was used (actual dose, 2.6-4.8x10^3 CFU) and badgers were killed 12 weeks after challenge for assessment of disease severity. Disease severity parameters were based on studies published previously [1,12] that showed the disease induced by endobronchial inoculation displayed the characteristics of disease observed in naturally infected badgers [12]: disease mainly occurred in the organs of the thoracic cavity, with limited dissemination elsewhere, and no generalized TB. In addition, experimental infection did not generate visible lesions in every affected tissue, as seen in wild badgers [2,13]. The

number of viable M. bovis organisms used in the challenge inoculum is likely to be substantially higher than that encountered in natural exposure. This provides a stringent test of vaccine induced protection.
BCG vaccination had a significant effect in reducing the severity of the experimental infection and the frequency of excretion of M. bovis. HD BCG vaccination was found to significantly reduce the severity of disease compared to the non-vaccinated controls, with the disease in the LD BCG group in between the two. Vaccination did not prevent infection, most likely because of the overwhelming size of the challenge dose. It is possible that in settings of natural infection, BCG vaccination may fare better than in experimental efficacy studies and the protection induced by the LD or HD BCG vaccination may provide complete or adequate protection, either indivudally or through a herd immunity effect.
BCG vaccination reduced the frequency of excretion in both vaccine groups and significantly delayed the onset in the HD BCG group. Excreta (urine, faeces, tracheal mucus) were collected from the badgers at regular intervals in order to establish when infected badgers commenced excreting M. bovis. Significantly fewer badgers excreted M. bovis in the HD BCG group than in the control. M. bovis was most frequently isolated from samples of tracheal mucus, consistent with the pulmonary infection.
Animals vaccinated with BCG were responsive in the ELISPOT with the number of IFNγ producing cells detected after vaccination being higher in those animals given the higher dose of BCG. However, we found no correlation between the pre-challenge IFNγ response and the final lesion score. Consistent with the observations that the magnitude of in vitro (PPD)-specific IFNγ production does not correlate with protection [14,15], BCG vaccination of badgers via different routes results in little to no peripheral IFNγ as measured by an ELISA method even though the vaccine confers protection [16]. We similarly found the amount of IFNγ produced to BCG using ELISA [17] was very small and unrelated to the vaccine dose or levels of protection (data not shown). Thus it seems that the ELISPOT method might be a more informative method than ELISA for measuring the IFNγ response to BCG vaccination in some species.
All animals in both experiments responded in the IFNγ ELISPOT after they were challenged with M. bovis. Whilst there was clear evidence of an anamnestic response to PPD-B after challenge in VES1, consistent with (HD) BCG-mediated immunological memory, this was less apparent in VES2. Nonetheless, HD BCG vaccination was as protective in VES2 as it had been in VES1. Responses to ESAT-6/CFP-10 antigens were seen after challenge but not before, consistent with these antigens being expressed by M. bovis but not BCG [9]. The extent of IFNγ production post-challenge has been correlated with disease severity, for example in experimental M. bovis infection of cattle [18]. The same association was found in these studies: lower responses to ESAT-6/CFP-10 were seen in the vaccinated groups compared to non-vaccinated; most notably in the HD BCG groups in both studies.
The failure of HD BCG to protect badger D313 was not due to a failure to deliver the vaccine successfully or for it to induce T-cell memory as the response of D313 by IFNγ ELISPOT was indistinguishable from the other animals in the same treatment group (data not shown). Based on the levels of haematological and biochemical parameters monitored throughout the experiment as markers for the clinical condition of badgers, there was no evidence that D313 had any underlying physiological or pathological condition that might have explained the failure of BCG vaccination.
After challenge with M. bovis, the majority of VES badgers became seropositive but there was no evidence that vaccinated badgers became seroreactive later than the non-vaccinated controls, despite this being observed in VES3 (see later) and in the studies conducted in Ireland [16]. A positive Stat-Pak result would not be expected to be 100% specific to M. bovis infection [19,20]. The Stat-Pak test relies on antibody recognition of one or more of the following antigens, MPB83, CFP-10, Mtb8.4, and 38kDa antigen. BCG Danish is a constitutively low expresser of MPB83 [21]. CFP10 is encoded by a gene in the so-called RD1 region that is deleted from all strains of BCG [22]. The 38Kda antigen (encoded by gene Rv0934) and Mtb8.4 (encoded by gene Rv3874) are expressed by BCG [23,24] and as such have the potential to be recognised in the Stat-Pak test by badgers vaccinated with BCG. However, both antigens appear poorly immunogenic in badgers [25]. The imperfect specificity explains why a single HD BCG vaccinated animal (C071) was positive in the Stat-Pak repeatedly before vaccination and challenge.

Summary of vaccine efficacy

The severity of TB in VES badgers was quantified at different levels:

Based on gross pathology
1. Number and severity of visible lesions – Total Visible Lesion Score (Fig. 1)

Based on histopathology
2. Severity of granulomata (data not shown, in detailed report)
3. Abundance of collagen within granulomata (data not shown, in detailed report)
4. Bacterial load scored in granulomata (estimated by ZN staining) (data not shown, in detailed report)
5. Dissemination of M. bovis infection (number of tissues in which M. bovis was detected by culture and/or ZN staining)

Based on culture alone
6. Bacterial load of M. bovis recovered per gram of tissue (data not shown, in detailed report)
7. Isolation of M. bovis from clinical samples (Fig. 2)

By five out of seven parameters, HD BCG vaccination gave statistically significant protection compared with non-vaccinated controls (Table 8). In contrast LD BCG only gave significant protection in terms of reducing the Thoracic LN score.

TABLE 8. Summary of significant p-values for each measure of vaccine efficacy (only HD BCG versus non-vaccinated controls shown)

Measure of vaccine efficacy (reduction in…)	p-value	Test
1. Total Visible Lesion Score	0.004	Dunn's
2. Granuloma severity	0.004	Dunn's
3. Amount of collagen within granulomata	0.014	Dunn's
4. Bacterial load within granulomata	0.003	Dunn's
5. Dissemination of M. bovis infection	NS	Tukey's
6. Bacterial load of M. bovis recovered	NS	Tukey's
7. Isolation of M. bovis from clinical samples	0.002	Log-rank

Conclusion

IM administration of BCG was shown to confer protection to badgers against experimental inoculation with M. bovis. As IM BCG is both protective and safe for badgers [7] and feasible to administer to restrained conscious animals, it makes field vaccination of badgers feasible for the first time; representing a new intervention tool for the control of bovine TB in badgers.
On the basis of these efficacy data, together with data on the safety and quality of the vaccine, the VMD granted a Marketing Authorisation to the VLA for the HD IM BCG vaccine (called BadgerBCG) on 24th March 2010.

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References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Efficacy data from VES1 & 2 were submitted as part of a successful Marketing Authorisation application to the VMD for HD IM BCG (BadgerBCG).

An abbreviated form of the data, together with results of the efficacy of HD IM BCG in a field study (project CB0115) is in draft form for the journal Current Biology. A more detailed manuscript relating to VES1 & 2 is in draft form for the journal Infection and Immunity.