Animal Health Board Project No. R-10630

Efficacy of an Oral Tb Vaccine on Wild Possum Populations

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Contents

Summary

Project and Client

The efficacy of an oral BCG vaccine bait in protecting free-living wild possum populations exposed to natural infection with bovine tuberculosis was evaluated by Landcare Research, AgResearch and Otago University for the Animal Health Board (AHB) between November 2003 and March 2007 (Project number R-10630).

Objective

To determine the efficacy of an oral BCG vaccine bait for protection of individual possums in free-living populations from Tb infection, by:

- undertaking a clinical field trial on populations at two field sites with a known history of Tb infection.

Results

- Vaccination treatments were applied in November and early December 2004 to 32 possums at one site in the Orongorongo Valley ('Stream Grid'), and 41 possums at another ('CK Grid'), matched to control groups of 32 and 41 possums at the two sites respectively. Additional animals were introduced to the treatment groups on the two sites at six and twelve months into the trial. A total of 51 possums were vaccinated on Stream Grid, with 71 designated as controls, while a total of 51 possums were vaccinated on CK Grid, with 63 designated as controls.
- Lymphocyte proliferation assays provided confidence that vaccination was inducing an immune response in possums, with Stimulation Index (SI) measures significantly higher for vaccinated animals than for control animals: Two months after initial vaccination (January 2005), 13 out of 35 vaccinated animals gave positive responses versus 3 out of 39 control animals ($\chi^2 = 9.44$, df = 1, $P < 0.01$).
- At the end of the 2-year trial, 123 independent possums (31 vaccinated, 27 control, and 65 'other' animals) were removed from Stream Grid and necropsied, while 129 independent possums (31 vaccinated, 29 control, and 69 'other' animals) were removed from CK Grid and necropsied. 'Other' animals were those not designated either control or vaccinated.
- Across all 2-month monitoring trips on Stream Grid (excluding the final trap-out), eight incident cases of culture-confirmed Tb infection were observed in experimental animals, all in the unvaccinated group. All infected animals had been designated as controls at the site for at least 6 months before external lesions were first observed, indicating most, if not all, infection must have occurred on site.
- An additional five further cases of culture-confirmed Tb infection were observed on Stream Grid in the final trap-out. Of these five cases, four were control animals that had been so designated for at least 12 months, while one was a vaccinated animal. Hence there was a highly significant protective effect of the vaccine on this site – 12 incident cases of infection in 71 control animals versus 1 in 51 vaccinated animals exposed to the same force of infection ($\chi^2 = 6.94$, df = 1, $P < 0.01$).

- While all infected control animals on Stream Grid had classical Tb lesions in the lungs and/or lymph nodes, the one infected vaccinated animal had only small lesions in the liver, and the pooled lymph node sample collected from this individual cultured negative for *M. bovis*.
- With only one incident case of Tb infection recorded in the control group on CK Grid during the course of the trial, Tb incidence at this site was insufficient to provide a statistically robust test of the vaccine.
- Possums with culture-confirmed palpable Tb lesions generally died within 4 months. However, there were notable exceptions, with one infected possum surviving for more than 2 years.

Conclusions

- Orally delivered BCG vaccine significantly protected wild possums against natural *M. bovis* infection on one of two sites in the Orongorongo Valley. Even though one vaccinated individual on this site was found to be infected by Tb on necropsy, the animal did not have clinical disease, and the form of infection was unusual. This animal was unlikely to be infectious. There was insufficient Tb infection at the second site for a statistically robust test of the vaccine.
- Vaccine efficacy was 88% against natural Tb infection and possibly 100% against disease. Even the lower level of efficacy is theoretically sufficient to eradicate Tb from possum populations.
- The apparent 100% protection against Tb disease provided by the vaccine, even though responses to bovine PPD in LP assays were not all positive post-vaccination, indicates that the presence of a peripheral blood immune response is not essential for protection against natural infection of possums with *M. bovis*. This suggests the period of effective protection offered by the vaccine to free-ranging animals will be longer than that concluded from cage trials. In addition, it is likely that repeated natural challenges of vaccinated animals in the wild with *M. bovis* will also prolong the period of immunity.
- Although the established general pattern of Tb infection in possums rapidly leading to clinical disease and death is confirmed in this study, observations of individual cases demonstrate that exceptions do occur. Not all exposure of possums to Tb in the wild becomes established infections and, more importantly in the context of pathogen persistence, not all diseased animals rapidly die. Such individuals may be infectious for long periods, and key drivers of disease persistence and spread ("super-spreaders").

Recommendations

- Further trials should be conducted to assess the duration of effective protection against Tb offered by oral BCG vaccination of possums in the wild.
- A suitable carrier for dissemination of the oral BCG vaccine in the field, for both bait-station and aerial delivery, should be investigated.
- Further trials should be conducted to assess the level of population vaccination achieved through both bait-station and aerial dissemination of oral BCG vaccine.
- Possums that act as "super-spreaders" of Tb in the wild should be characterized, potentially informing the best vaccination strategy.

1. Introduction

The efficacy of an oral BCG vaccine bait in protecting free-living wild possum populations exposed to natural infection with bovine tuberculosis was evaluated by Landcare Research, AgResearch and Otago University for the Animal Health Board (AHB) between November 2003 and March 2007 (Project number R-10630).

2. Background

Bovine tuberculosis (Tb; *Mycobacterium bovis)* in wild animals is a world-wide problem, with wildlife acting as a reservoir of infection for both domestic animals and humans (Biet et al. 2005; Baker et al. 2006), and the culling of wildlife proving an inadequate means of controlling the disease in many cases (Corner 2003; Donnelly et al. 2006; Woodroffe et al. 2006). In New Zealand, the principal wildlife reservoir of infection for cattle and farmed deer is the introduced Australian brushtail possum *Trichosurus vulpecula* (Caley et al. 1999; Kean et al. 1999; Coleman & Livingstone 2000). While an extensive programme of culling possums over the past decade has been a major factor in an 85% reduction in cattle herds infected with *M. bovis*, this strategy has not yet substantially reduced the total area in which Tb is endemic in wildlife (Livingston et al. 2006). Additional strategies of disease control are thus required.

The effective control or eradication of Tb from possum populations requires that the rate of Tb transmission among possums be reduced. This can be achieved by reducing the abundance of susceptible individuals in the host population, as projected by models of host/pathogen dynamics (Barlow 1991; Roberts 1996; Barlow 2000). This logically follows from the idea of a threshold density for the persistence of disease (Kermack & McKendrick 1927; Anderson & May 1979; May & Anderson 1979). One means of reducing susceptible host abundance, instead if or in addition to lethal control, is vaccination (Heesterbeek & Roberts 1995; Cleaveland et al. 2002).

Vaccination against disease using the tuberculosis vaccine, *M. bovis* Bacille Calmette-Guérin (BCG), is an attractive option for reducing the Tb burden in possum populations (Buddle et al. 2000; Skinner et al. 2001). However, while BCG is currently administered to humans via intradermal injection to control human Tb, vaccination via oral bait is the only feasible means of disease management in wildlife reservoirs. Such a route of delivery is more challenging than intradermal, given the need for sufficient live bacilli to reach sites of mucosal immune induction in the gastrointestinal tract in the face of degradation by gastric hydrolysis. Previous trials have demonstrated that an orally delivered BCG vaccine based on lipid encapsulation of live bacilli induces a significant level of protection against Tb in caged possums (Aldwell et al. 2003; Wedlock et al. 2005; Buddle et al. 2006). In addition, a field trial has demonstrated that such a vaccine reduces the death rate of free-living possums artificially inoculated with *M. bovis* via the intra-tracheal route (Ramsey et al. 2006).

Here we extend this programme of research to assess the efficacy of orally delivered BCG vaccines at protecting free-living possum populations from natural infection by Tb. In addition to providing essential information for assessing the utility of the vaccine as an additional tool for controlling Tb in possum populations in New Zealand, this trial is, to the best of our knowledge, the first such test globally of the efficacy of an orally delivered BCG vaccine against natural *M. bovis* infection in any wild reservoir of infection.

3. Objective

To determine the efficacy of an oral BCG vaccine bait to protect individual possums in free-living populations from Tb infection, by:

- undertaking a clinical field trial on populations at two field sites with a known history of Tb infection.

4. Methods

4.1 Study site selection

Two sites were required for the field trial with (1) sufficient possum densities to allow sample sizes of at least 30 animals per treatment group, and (2) sufficient natural Tb infection in resident possum populations to enable a valid test of the BCG vaccine. Based on discussions with Regional Councils, the AHB, local possum trappers, and other researchers, three locations were considered – the Orongorongo Valley, the Hochstetter Range, and the central West Coast.

Orongorongo Valley – initial assessment

An initial assessment of two sites ('CK Grid' and 'Stream Grid') in the Orongorongo Valley was carried out in November 2003, in an area where Tb had been recorded in possums. A grid of approximately 200 cage traps set at 30-m spacings was established at each site and covered approximately 20 ha. All traps were baited with apple lured with flour and aniseed, and set over 4 consecutive nights. During each trapping session, all captured possums were anaesthetised with ketamine hydrochloride at an average dose of 25 mg/kg. Once sedated, possums were individually marked with either a metal ear tag and tattoo, or two metal ear tags, had their Tb status ascertained by external examination and palpation of the major superficial lymph nodes, sexed, examined for tooth wear (an index of age; Winter 1980), and released. For possums with suspect Tb lesions (i.e. open lesions or palpable lumps), either swabs (for open lesions) or aspirated fluid (for closed lesions) were collected for bacteriological confirmation of infection. Bacteriology was carried out at the Infectious Disease Laboratory, AgResearch, Wallaceville, with samples processed and cultured following methods described by Aldwell et al. (1995).

Hochstetter Range

Due to a lower than desired level of Tb infection being detected in possums at the Orongorongo Valley sites (see Results), site selection surveys were carried out in central Westland. Preliminary investigations via widely dispersed cyanide baited lines were conducted at five promising locations on the Hochstetter Range in February and March 2004, to enable large areas to be rapidly assessed at minimal cost and minimal impact to potential study populations. An intensive follow-up trapping programme was undertaken at one location, where preliminary investigation indicated both relatively high possum densities and high Tb prevalence (see Results). From 30 March to 8 April 2004, 2457 trap-nights were monitored using a grid of leghold trap on scott-boards at three sites adjacent to the original cyanide line. All possums caught were dealt with as described for the Orongorongo Valley and released.

West Coast – Hohonu and Lake Kaniere

With survey work on the Hochstetter Range being unable to identify sites more suitable than those initially considered in the Orongorongo Valley (see Results), further exploratory work was carried out on the West Coast. Through discussion with the AHB and the West Coast Regional Council (WCRC), it was agreed that the area between the Eastern Hohonu River and Mitchells Flat would also be worth investigating. However, changes in the planned area for vector ground control by the WCRC ruled out this location as a long-term study site. Furthermore, work conducted by Jim Coleman on a separate AHB project (No. R-10536) highlighted a very low possum density in this general locality.

Further discussion with the WCRC identified the area immediately north of Lake Kaniere as the only other suitable location in Westland where possum numbers and Tb prevalence was likely to be high enough, and control operations wouldn't intervene in the two years required for the trial. A preliminary survey was thus carried out at this location from 16 to 23 June 2004, with cage-traps placed alongside roads and tracks across the total area and 410 trap-nights monitored. Cyanide bait lines were not used, since we did not wish to kill any of the Tb possums we found. All possums caught were dealt with as described above and released.

Orongorongo Valley – additional surveys

Surveys were unable to identify sites more suitable for the trial than those initially considered in the Orongorongo Valley. Although Tb prevalence in possums at the initial November 2003 assessment of this location was lower than was considered optimal, it was deemed sufficient. This decision led to additional surveys (in July, September and November 2004) to better characterize natural Tb infection on the two sites at this location. As before at these sites, surveys comprised the setting of cage-trap grids for four consecutive nights, with all possums trapped dealt with as previously described. To assess the degree of non-palpable Tb infection in possums on the two sites, 2-ml blood samples were collected from the tail or jugular vein of each animal where possible for Lymphocyte Proliferation (LP) assays carried out at AgResearch, Wallaceville. In addition, all animals suspected of being infected, based on external examination and palpation, and/or LP response at prior trapping occasions, were fitted with mortality-sensing radio-collars before release.

LP responses to *M. bovis* purified protein derivative (Bovine PPD; Pfizer Limited, Australia) were measured in blood depleted of red blood cells (see Skinner et al. 2005). The stimulation

index (SI) was calculated by dividing the mean counts per minute (cpm) from triplicate cultures stimulated with bovine PPD, by the mean cpm from triplicate cultures with phosphate-buffered saline. Based on previous studies (e.g., Buddle et al. 2006), the cut-off for a positive response in the LP assay to bovine PPD was set at a conservative SI level = 5.00 units. Results from samples with a low response to the non-specific mitogen Concanavalin A (Con-A; Sigma, St Louis, MO, USA), indicating insufficient viable lymphocytes, were excluded.

4.2 Vaccine field trial

Initial treatments

The field trial was carried out at the two sites previously surveyed in the Orongorongo Valley, CK Grid (centred on E2672410, N5979660) and Stream Grid (centred on E2671471, N5978702), from November 2004 to November 2006. Control and vaccinated groups of animals were set up on each grid, with initial vaccinations being applied in November and early December 2004. Matched pairs of animals were identified on each site (matched on trap location within the grid, sex, and age), with one animal from each matched pair designated to receive the vaccine, and the other to be sham-handled as a control. This ensured no bias with respect to age, sex, or location (and, hence, potential exposure to natural infection) between treatment groups. These animals were located within a central core of the 150 most effective traps on each grid, with the other 50 traps not being monitored further. Animals with suspected Tb infection, based on external examination and palpation, and positive LP assays from the previous surveys, were excluded from the matched pairs.

Oral BCG vaccine, using the Danish 1331 *M. bovis* BCG strain, was supplied by Immune Solutions Limited. The mycobacteria was grown to mid-log phase in Middlebrook 7H9 broth (Difco Laboratories, USA) supplemented with albumin-dextrose-catalase (ADC; BBL, Becton Dickenson, USA) and 0.01% Tween 80. Initial dilutions of mycobacteria were made in TAB, and the number of colony forming units (cfu) was determined retrospectively (see Buddle et al. 1994). The vaccine was formulated in a lipid matrix, as described by Aldwell et al. (2003). The lipid was an animal-derived fractionated complex lipid previously used in oral BCG vaccination studies in possums (Aldwell et al. 2003; Buddle et al. 2006; Ramsey et al. 2006). Pelleted BCG was resuspended in the molten lipid medium at 37ºC to achieve a final concentration of approximately 1 x 10^7 cfu/ml, with 5% v/v unsaturated vegetable oil added so that the formulation cooled to a paste at room temperature (Aldwell et al. 2003). For vaccination, 1 ml of the vaccine formulation was administered by syringe directly into the mouth of anaesthetised animals.

Monitoring and re-vaccinations

Possum populations at the two sites were monitored at 2-month intervals over the course of the 2-year trial. Monitoring trips consisted of trapping at both sites for 4 consecutive nights, as in the pre-trial surveys, unless weather conditions dictated otherwise. In particular, extreme rainfall in March 2005, which destroyed the access road to the Orongorongo Valley, meant traps could only be opened for 3 consecutive nights on that and the following two trips. Extreme weather also limited opening the traps at CK Grid to just 2 consecutive nights in March 2006.

Apart from the final monitoring trip, all possums trapped were dealt with as described above and released. Blood samples were collected from the tail or jugular vein of each animal where possible at all trips until May 2006, apart from May 2005 when there were again problems with the access road to the field sites. As in the pre-trial surveys, all animals suspected to be infected based on external examination and palpation and/or LP response were fitted with mortality-sensing radio-collars prior to release. All collared possums were located at 2-month intervals, with dead animals being retrieved when mortality switches were triggered.

Cage trials with possums had indicated that there may be some loss of protective immunity from BCG to bovine Tb after six months (Buddle et al. 2006). Hence, at the 6- (May 2005), 12- (November 2005) and 18- (May 2006) month monitoring trips after initial vaccination, those animals in the vaccinated groups trapped were re-vaccinated. In addition, new control and vaccinated animals were designated at both sites in both May and November 2005 to maintain sample sizes. At the final monitoring trip, in November 2006, possum populations on both study sites were trapped out. To achieve this, cage traps were opened at each site for 5 consecutive nights, In addition, two lines of leg-hold traps (at 30 m intervals) were placed around the perimeter, and 20 leg-hold traps dispersed among the cage traps at each site, for the same time period. All possums caught on the final trip were euthanized and subjected to post-mortem examination to identify any visible tuberculous lesions. Pooled lymph node samples (retropharyngeal, axillary, inguinals, bronchial, hepatic and mesenteric) were collected from all individuals for bacteriology, in addition to samples from any suspected Tb lesions.

Artificial infections

At the three survey trips before the initial application of vaccination treatments, the occurrence of natural Tb infection was markedly lower on CK Grid than on Stream Grid (Table 1). Hence, to attempt to increase the force of Tb infection to which the experimental groups were exposed on the CK Grid, adult possums (that were not allocated to either the control or vaccinated treatment groups) were directly inoculated with *M. bovis* in March–May 2005. The *M. bovis* strain used was *M. bovis* 83/6235, a strain that was originally isolated from a tuberculous possum in the central North Island, and can be differentiated from strains isolated from the lower North Island by REA strain typing. This strain has also been used in previous inoculation studies in possums (Buddle et al. 1994, 2006), and was grown to mid-log phase as described above. Only adult animals were inoculated, as they were considered less likely to disperse (see Cowan & Clout 2000).

Anaesthetised animals were inoculated via conjunctival instillation, with approximately 50 µl of a 100 000 cfu/ml *M. bovis* suspension being instilled into the conjunctival sack of each eye using an eye dropper. This infection model has been shown to most closely mimic the natural progression of Tb infection in possums in the wild (Corner et al. 2003). In March 2005, only two animals were inoculated due to the poor weather conditions affecting trapping rates. An additional 12 animals were inoculated in May 2005. With 20% of animals inoculated with a lower dose by this route becoming infected in previous work (Corner et al. 2003), it was expected this manipulation would result in at least 3 infected animals to which experimental groups would be exposed at this site. All inoculated animals were fitted with mortality-sensing radio-collars before release.

5. Results

5.1 Study site selection

Orongorongo Valley – initial assessment

Out of 76 possums trapped on CK Grid, one animal had suspected Tb by palpation (1.3% prevalence). Out of 61 possums trapped on Stream Grid, two animals had suspected Tb by palpation (3.3% prevalence). An approximate estimate of the annual incidence of Tb in possums at the two sites was calculated from these point prevalence estimates by dividing them by the average duration of survival by clinically infected possums (0.4 years). This gave estimates of 0.03/year and 0.08/year for CK Grid and Stream Grid respectively. Using recent estimates of 4-night capture probabilities from other sites in the Orongorongo Valley (0.85) gave approximate estimates of population sizes of 89 and 72 possums for CK Grid and Stream Grid respectively. Extrapolating from these figures gave *a priori* estimates of approximately 5 and 12 incident cases of tuberculous possums during the 2-year vaccine trial for CK Grid and Stream Grid respectively. These numbers were considered marginal for providing a valid test of the vaccine.

Hochstetter Range

Results of preliminary surveys in the Hochstetter Range were:

Site 1: 27 possums recovered from 12 km of line; 0 Tb possums (0% prevalence)
Site 2: 15 possums recovered from 15 km of line; 3 Tb possums (20% prevalence)
Site 3: 15 possums recovered from 15 km of line; 0 Tb possums (0% prevalence)
Site 4: 7 possums recovered from 7 km of line; 0 Tb possums (0% prevalence)
Site 5: 70 possums recovered from 20 km of line; 3 Tb possums (4% prevalence)

Although there was high Tb prevalence in animals examined from site 2, possum density at this site was deemed insufficient for the purposes of the vaccine field trial. Cyanide baiting at site 5 (Flagstaff Flat, Hochstetter Forest) indicated both high possum density and a relatively high level of Tb incidence. It was considered potentially the best location for the study and thus warranted further inspection. However, 2457 trap-nights with leg-hold traps on scott-boards subsequently carried out at three sites adjacent to the original cyanide line caught only 38 different animals, of which none were positive for Tb by external examination and palpation. Even if Tb-positive animals were missed, their density at Flagstaff Flat was deemed insufficient for the vaccine trial.

West Coast – Lake Kaniere

From 410 cage-trap nights monitored in the vicinity of Lake Kaniere, only 17 different possums were caught, none of which were positive for Tb by external examination and palpation. As with the Flagstaff Flat site, the possum density at this location was deemed insufficient for the needs of the vaccine trial.

Orongorongo Valley – additional surveys

Although Tb prevalence in possums in the Orongorongo Valley at the initial November 2003 assessment was lower than desired, it was deemed sufficient to warrant further investigation. In a power test analysis, the estimates of Tb incidence and possum density from Stream Grid in November 2003 were calculated as sufficient for a trial to detect a vaccine efficacy of 95% over 2 years.

In the additional surveys, carried out from July to November 2004, Tb incidence as estimated by external examination and palpation alone was markedly higher than in the earlier November 2003 survey (Table 1). Furthermore, Tb incidence was even greater when ascertained by LP assay in addition to external examination and palpation (Table 1). As before, approximate estimates of the annual incidence of Tb in possums at the two sites were calculated from point prevalence estimates. For Stream Grid the average population size across this period was estimated as 63 animals, with the average point prevalence of suspected infection being 15% when assessment included LP assays. For CK Grid the average population size across this period was estimated as 80 animals, with the average point prevalence of suspected infection being 5% when assessment included LP assays. Based on these new point prevalence figures, estimates of the annual incidence of Tb in possums were calculated as 0.38/year and 0.13/year for Stream Grid and CK Grid respectively, and equated to *a priori* estimates of approximately 48 and 21 incident cases of tuberculous possums during the 2-year vaccine trial for Stream Grid and CK Grid respectively.

Table 1 Suspected cases of natural Tb infection in possums at the two Orongorongo Valley field trial sites from July to November 2004, based either on external examination and palpation alone or on examination, palpation, and LP assays.

Survey data	Stream Grid			CK Grid		
	No. trapped	**Infected** Palpation alone	Palpation and LP	**No. trapped**	**Infected** Palpation alone	Palpation and LP
July 2004	29	1	2	76	2	2
September 2004	68	9	15	55	0	3
November 2004	63	6	10	72	1	4

There was clearly a large degree of temporal variation in both the possum densities revealed through trapping and the estimated Tb incidence at the two Orongorongo Valley sites, illustrating that the force of natural Tb infection at the two sites is highly variable over time. However, the initial and additional surveys combined indicated that Tb incidence should be sufficient at Stream Grid to provide a valid field test of the vaccine in protecting possums against Tb infection, and marginal at CK Grid.

5.2 Vaccine field trial

Initial treatments

Vaccination treatments were applied in November and early December 2004 to 32 possums at Stream Grid, and 41 possums at CK Grid, matched to control groups of 32 and 41 possums at the two sites respectively. All these possums had been caught at least once in the July and September surveys.

Monitoring and re-vaccinations

Over the course of the 2-year trial, Tb infection was largely absent on CK Grid, and occurred sporadically on Stream Grid (Figs 1 & 2). At 6–8 months into the vaccine trial (May–July 2005), 32 of the original 41 vaccinated animals on CK Grid were re-vaccinated, as were 27 of the original 32 vaccinated animals on Stream Grid. In addition, 4 animals were vaccinated for the first time and 10 animals were designated as new controls on CK Grid, while 16 animals were vaccinated for the first time and 20 animals were designated as new controls on Stream Grid. New vaccine and control animals were distributed across the same spatial area as each other, and as existing experimental animals within the two sites. The inclusion of new animals into experimental groups was necessary on both sites, with sample sizes of experimental animals being re-trapped falling dramatically after the extreme weather of March 2005 (Figs 1 & 2). The inclusion of more animals as new controls, as opposed to being vaccinated for the first time, was necessary on both sites due to significantly lower proportions of control animals than vaccinated animals being re-trapped following March 2005 (Figs 1 & 2). For example, across both grids at the May 2005 monitoring trip, only 34 of the 73 control animals were re-trapped, compared with 47 of the 73 vaccinated animals ($\chi^2 = 4.68$, df = 1, $P < 0.05$). The inclusion of more animals into experimental groups on Stream Grid as opposed to CK Grid was necessary since fewer matched pairs were initially available at this site for allocation to experimental groups.

At 12 months into the vaccine trial (November 2005), 28 animals were re-vaccinated on CK Grid, 6 new animals were vaccinated for the first time (bringing the number vaccinated at least once on this site to 51), and 12 animals were designated as new controls (bringing the number designated as controls on this site to 63). On Stream Grid, 34 animals were re-vaccinated, 3 new animals were vaccinated for the first time (bringing the number vaccinated at least once on this site also to 51), and 19 animals were designated as new controls (bringing the number designated as controls on this site to 71). As before, new vaccine and control animals were distributed across the same spatial area as each other, and as existing experimental animals, within the two sites. More animals were again introduced to control groups than to vaccinated groups to ensure that sufficient control animals remained through to the end of the trial.

At 18 months into the vaccine trial (May 2006), 18 and 33 animals were re-vaccinated on CK Grid and Stream Grid respectively. With only 6 months of the trial remaining, no new individuals were introduced to experimental groups at this late stage. At the end of the vaccine trial (November 2006), 129 independent possums (31 vaccinated, 29 control, and 69 'other' animals) were removed from CK Grid and necropsied, while 123 independent possums (31 vaccinated, 27 control, and 65 'other' animals) were removed from Stream Grid and necropsied. 'Other' animals were those not designated either control or vaccinated and, at this point of the trial, were generally individuals recently trapped for the first time.

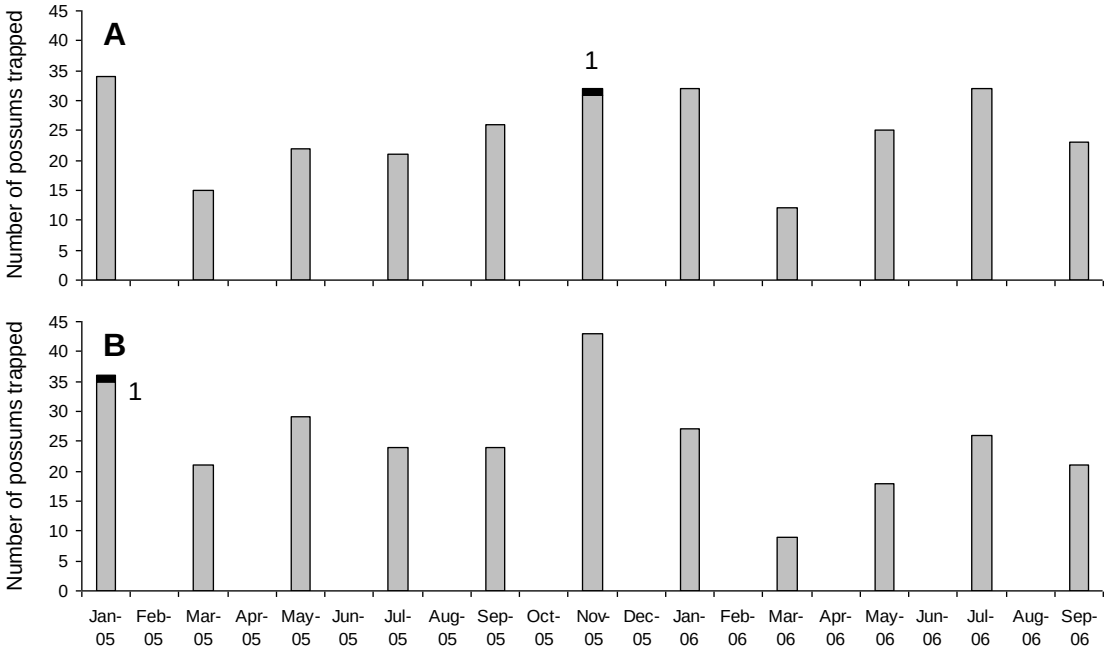

Fig. 1 Number of possums with (black) and without (grey) culture-confirmed Tb lesions in (A) control and (B) vaccinated animals on CK Grid, from January 2005 to September 2006. Numbers indicate infected individuals.

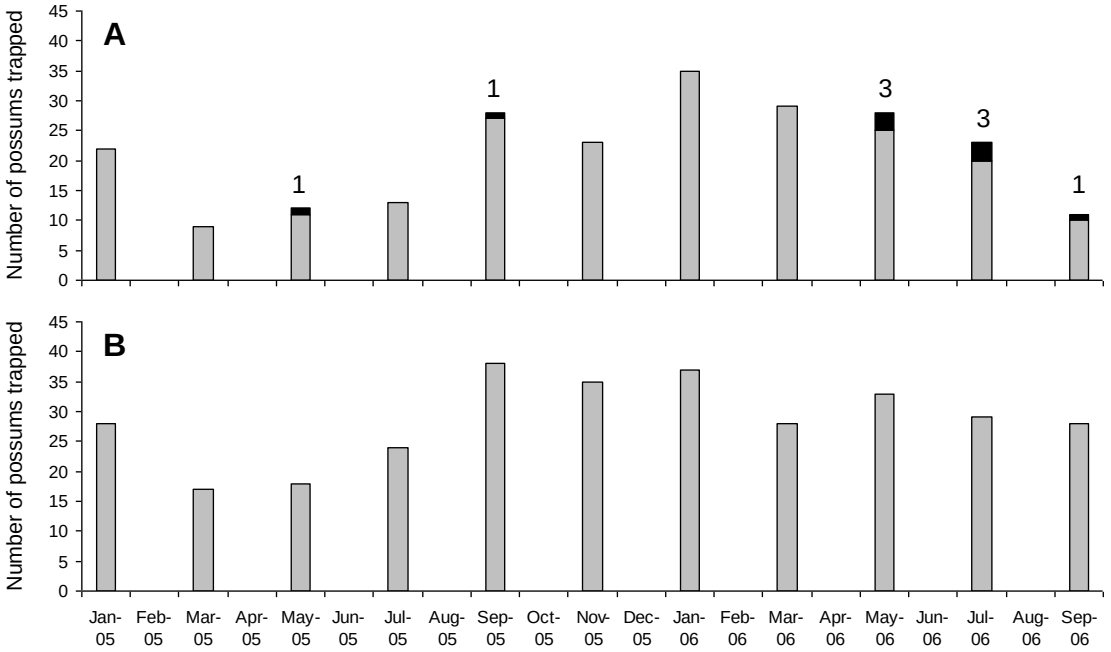

Fig. 2 Number of possums with (black) and without (grey) culture-confirmed Tb lesions in (A) control and (B) vaccinated animals on Stream Grid from January 2005 to September 2006. Numbers indicate infected individuals.

Artificial infections

Fourteen possums at CK Grid, not included in either the control or vaccinated groups, were directly inoculated with *M. bovis* in March–May 2005, to increase the force of Tb infection to which the experimental groups were exposed on this site. Animals occurred across the same spatial area as experimental animals within the site. Of these animals, 10 were not re-trapped. Of the 4 re-trapped animals, 2 had positive responses to bovine PPD on LP assay 2–4 months post-challenge, while the other 2 were negative. Of the 2 positive animals, only one developed disease (culture confirmed open lesions observed in January 2006), while the other had no sign of disease or infection even on necropsy at the end of the trial. The *M. bovis* strain type in the one diseased animal was identified as that used in the inoculation.

Tb Incidence

Even though artificial inoculations of non-experimental animals were carried out at CK Grid, only two incident cases of culture-confirmed Tb infection were observed in experimental animals during monitoring trips on this site (excluding the final 'trap-out' in November 2006) – a vaccinated animal with a draining lesion in January 2005, and a control animal with a draining lesion in November 2005 (Fig. 1; Table 2). The infected vaccinated animal had only received the vaccine 1 month before diagnosis. Hence, given that disease progression to open external lymph node lesions of naturally occurring Tb infection in possums takes a minimum of 2 months, this animal must have been infected before receiving the vaccine. Moreover, it is generally accepted that BCG-mediated protection against Tb is not fully expressed until 4–8 weeks post-vaccination. The infected control animal was one of the initial animals assigned as a control in November 2004. With only one incident case of Tb infection recorded in the control group during the course of the trial, Tb incidence on CK Grid was obviously unable to provide a statistically robust test of vaccine efficacy.

Across all monitoring trips on Stream Grid (again, excluding the final 'trap-out'), eight incident cases of culture-confirmed Tb infection were observed in experimental animals, all in the control group (Fig. 2; Table 2). All infected animals had been designated controls at the site for at least 6 months prior to external lesions first being observed (Table 2), indicating that most, if not all, infection occurred on site. Hence, during the monitoring phase of the vaccine trial on Stream Grid, significantly more control animals were naturally infected with Tb than vaccinated individuals exposed to the same force of infection (8 out of 71 control animals infected versus 0 out of 51 vaccinated animals infected; $\chi^2 = 6.14$, df = 1, $P < 0.05$).

Other possums (not designated as either control or vaccinated animals) were caught on both CK Grid and Stream Grid during the monitoring visits. These were generally animals that were not part of a matched pair at the beginning of the trial, animals that had not been present on the study sites for long, or animals (at CK Grid) that had been artificially challenged with *M. bovis* (see above). Few incident cases of culture-confirmed Tb were observed in these animals. Excluding the one artificially challenged animal that was subsequently observed to develop disease, no cases occurred on CK Grid, while just three cases occurred on Stream Grid – a female in May 2005, a male in January 2006, and a male in July 2006.

Table 2 Culture-confirmed incident cases of Tb in vaccine trial animals during the January 2005–September 2006 monitoring trips on CK Grid and Stream Grid. 'NC' indicates 'Not Caught'. 'M' and 'F' denote male and female.

Ear tag	Sex	Treatment	Date entered trial	Date Tb confirmed	Clinical symptoms	Symptoms before	Symptoms after
CK GRID							
X1918	M	Vaccinated	December 2004	January 2005	Open lesion right axillary	November 2005 – None	March 2005 – NC
X4982	M	Control	November 2004	November 2005	Open lesion right axillary	September 2005 – None	January 2006 – NC
STREAM GRID							
X1936	F	Control	November 2004	May 2005	Open lesion left inguinal	March 2005 – None	July 2005 – NC
X4206	F	Control	November 2004	September 2005	Closed lesion left axillary	July 2005 – None	November 2005 – NC
X2258	M	Control	November 2005	May 2006	Closed lesion left axillary	March 2006 – None	July 2006 – NC
X4290	M	Control	November 2004	May 2006	Closed lesion left parotid	March 2006 – None	July 2006 – Open lesion
X4349	M	Control	May 2005	May 2006	Closed lesions right parotid and right axillary	March 2006 – NC	July 2006 – NC
X4775	M	Control	November 2004	July 2006	Closed lesion left axillary	May 2006 – Equivocal lump	September 2006 – NC
X1906	M	Control	May 2005	July 2006	Closed lesion right parotid	May 2006 – Equivocal lump	September 2006 – NC
X4448	F	Control	November 2005	September 2006	Open lesion right axillary	July 2006 – None	November 2006 – Open lesion

No further culture-confirmed incident cases of Tb infection in experimental animals were observed on CK Grid during necropsy at the end of the trial, but five further cases were observed on Stream Grid (Tables 3 & 4). Of these five cases, four were control animals that had been so designated for at least 12 months, while one was a vaccinated animal.

Combining the results from the final 'trap-out' with those from the monitoring trips, the culture-confirmed incidence of Tb in possums on CK Grid was still insufficient for a statistically robust test of the vaccine – 1 incident case in 63 control animals versus 1 in 51 vaccinated animals (with infection acquired by this animal before vaccination). Combining the results from the final trap-out with those from the monitoring trips for Stream Grid, a highly significant protective effect of the vaccine was apparent – 12 incident cases of infection in 71 control animals versus 1 in 51 vaccinated animals exposed to the same force of infection ($\chi^2 = 6.94$, df = 1, $P < 0.01$). Furthermore, while all infected control animals on this site had classical Tb lesions in the lungs and/or lymph nodes, the one infected vaccinated animal only had small lesions in the liver (Table 4); none of the infected control animals had similar liver lesions. In addition, the pooled lymph node sample collected from this individual on necropsy cultured negative for *M. bovis*. This suggests that although this vaccinated animal became infected with *M. bovis* at this site, infection was held in check by the vaccination and restricted to just small lesions in the liver – the animal did not develop more traditional symptoms of disease, and hence was likely not infectious.

Apart from the pooled lymph node samples and tissue samples collected from those animals listed in Table 4, none of the other samples collected from necropsied animals cultured positive for *M. bovis.* Three of the positive cultures were strain-typed, with isolates all identified as 'Wairarapa Type 21'. This type is one of the most widespread in New Zealand, and was the type identified in the three isolates that have been typed from the Orongorongo Valley in the past.

Note that all the analyses undertaken do not consider possums excluded from either the control or vaccinated treatment groups, since these animals may not have been exposed to the same force of Tb infection as the animals in the treatment groups.

Table 3 Necropsy results from the November 2006 trap-out at the end of the 2-year vaccine trial in the Orongorongo Valley. Numbers reported are independent possums (i.e. not including pouch young or back riders). Only those animals not confirmed as Tb positive at an earlier date are included. All incident cases of Tb infection were culture-confirmed. Large numbers of 'other' animals were trapped due to trapping extending beyond the borders of the trial sites.

SITE	TREATMENT	TB STATUS		TOTAL
		Positive	Negative	
CK Grid	Control	0	29	29
	Vaccinated	0	31	31
	Other	1	68	69
Stream Grid	Control	4	22	26
	Vaccinated	1	30	31
	Other	1	64	65

Table 4 Culture-confirmed incident cases of Tb in vaccine trial animals necropsied in the November 2006 trap-out of CK Grid and Stream Grid. 'NC' indicates 'Not Caught'. Only those animals not confirmed Tb positive at an earlier date are included. 'M' and 'F' denote male and female.

Ear tag	Sex	Treatment	Date entered trial	Tb symptoms on necropsy	Symptoms before
Stream Grid					
X2206	M	Control	November 2005	1 mm haemorraghic spots in lungs, 8 mm caceous lesion right retropharyngeal	September 2006 – None
X4190	F	Control	November 2005	12 mm white lesions in lung, 4 smaller lesions in lung, swollen bronchial lymph nodes	September 2006 – None
X4473	M	Vaccinated	November 2004	3 mm white lesion in liver, several 1 mm white lesions in liver, kidneys nephritic	September 2006 – None
X4499	F	Control	May 2005	3 mm white nodules in lungs, 30 mm draining lesion in right inguinal	September 2006 – None
X4935	M	Control	November 2005	20 mm lesion in apex of left lung, several other <5 mm lesions in lung, 20 mm caceous lesion left superficial axillary	September 2006 – NC

LP assays

LP assays were of three key uses through the course of the vaccine trial. First, possibly infected animals were less likely to be included initially in the control or vaccinated groups. Second, lack of LP response in control animals through most of the trial provided confidence that external examination and palpation was not missing most of the clinical cases. Third, LP assays also provided confidence that vaccination was inducing an immune response in possums and there were no technical reasons (e.g. no live bacteria in the vaccine) for the vaccine not to work. Although not all vaccinated animals tested gave positive responses (SI > 5.0) to the LP assay, SI measures were significantly higher for vaccinated animals than for control animals. Two months after initial vaccination (January 2005), 13 out of 35 vaccinated animals gave positive responses versus 3 out of 39 control animals (Fig. 3; $\chi^2 = 9.44$, df = 1, $P < 0.01$). This effect was still observable in March 2005, with 8 out of 24 vaccinated animals giving positive responses (SI > 5.0) versus 1 out of 19 control animals ($\chi^2 = 5.05$, df = 1, $P < 0.05$).

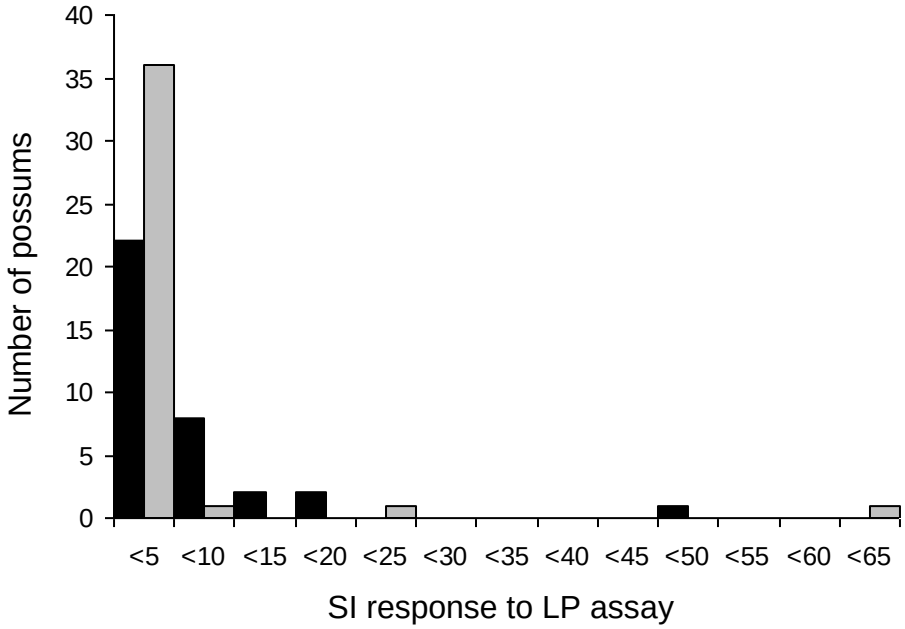

Fig. 3 Responses to the LP assay against bovine PPD for control (grey bars) and vaccinated (black bars) possums 2 months after initial vaccination treatments. Assay results from the two study sites are presented together. Note that the cut-off for a positive response in the assay is an SI response of 5.0.

During the course of the vaccine trial, all individuals with culture-confirmable Tb lesions also gave positive responses to *M. bovis* PPD when tested at the same time. In addition, in 50% of cases where the assay was carried out 2 months before Tb was confirmed, a positive response to the LP assay was observed. There was also one case where a positive LP response was observed 4 months before Tb was confirmed by bacteriology.

Interestingly, some unvaccinated individuals that had positive responses to bovine PPD in the assay during the course of the trial did not subsequently develop disease. Three individuals that were positive to the LP assay were not observed to develop disease *and* were negative to

the assay at a later date (i.e. transient LP positive and no disease). Three other positive individuals that were not subsequently tested had developed no disease symptoms up to 4 months later, after which they were not re-trapped (i.e. LP positive but no disease in the short- to medium-term). An additional three positive individuals that were subsequently necropsied at the end of the trial had no sign of disease *or* infection, even with bacteriology (i.e. LP positive but no disease or infection). In one further additional case of note, one of the animals artificially challenged with *M. bovis* at the 'CK Grid' site was sampled for LP assay 4 months later, when it gave a positive response (as reported above). However, 4 months later still, at a second sample for LP assay, it gave a negative response, and subsequently had no sign of disease or infection when necropsied at the end of the trial. These results indicate that not all exposure by possums to Tb in the wild results in established infections, even if a positive response to bovine PPD on LP assay is triggered.

Disease progression in infected possums

Possums with culture-confirmed Tb lesions either in the 6 months before the trial began at the Orongorongo Valley, or during the vaccine trial monitoring trips, generally died within 4 months. However, there were two notable exceptions. The first was an animal with a culture-confirmed lesion (also positive on LP assay) in September 2004, before the vaccine trial started, but had no detectable lesion (and was negative on LP assay) just 2 months later in November 2004. This animal then had an equivocal Tb lump on palpation (and was positive to LP assay again) after a further 2 months in January 2005, after which it was not re-trapped. The second diseased animal had a culture-confirmed Tb lesion (and was positive on LP assay) in both September and November 2004. This animal then had no detectable disease symptoms when re-trapped in January, May and July 2005, but was positive on LP assay in July 2005. This animal still had no detectable disease symptoms when re-trapped in November 2005, but had large (20 mm) culture-confirmed lesions in both the lung and in an axillary lymph node on necropsy a year later in November 2006. This demonstrates that even though the course of Tb disease in infected possums is characteristically fast, some infected individuals can survive in the wild for long periods of time.

6. Conclusions

- Orally delivered BCG vaccine significantly protected wild possums against natural *M. bovis* infection on one of two sites in the Orongorongo Valley (Stream Grid). There was insufficient Tb infection at the second site (CK Grid) for a statistically robust test of vaccine efficacy, but the only two animals infected there were unvaccinated at the time of infection. Only one vaccinated individual on Stream Grid was found to be infected by Tb on necropsy; the animal did not have clinical disease and the form of infection was unusual. This animal was unlikely to be infectious.
- Vaccine efficacy, defined as the disease rate in the control group minus the disease rate in the vaccinated group, divided by the disease rate in the control group (Martin et al. 1988), was 88% against natural Tb infection and possibly 100% against disease on Stream Grid. Even the lower level of efficacy is theoretically sufficient to eradicate Tb from possum populations (Roberts 1996).
- The potentially 100% protection against Tb disease provided by the vaccine in possums on Stream Grid, even though response to bovine PPD in LP assays were not all positive post-vaccination, indicates that the presence of a peripheral blood immune response is

not essential for protection against natural infection of possums with *M. bovis*. This is also the picture seen in cattle (Buddle et al. 2005). This suggests the period of effective protection offered by the vaccine to free-ranging animals will be longer than that concluded from cage trials. In addition, it is likely that repeated natural challenges of vaccinated animals in the wild with *M. bovis* will also prolong the period of immunity.

- Artificial challenge with *M. bovis* of 'other' possums on CK Grid failed to translate into incident cases of Tb in experimental animals (i.e. either vaccinated or control) at this site. This is in line with results from Corner et al. (2002), where artificial challenges similarly failed to increase the force of Tb infection to a wild possum population.
- Although the established general pattern of Tb infection in possums rapidly leading to clinical disease and death is confirmed in this study, observations of individual cases demonstrate that exceptions do occur. Importantly, our results suggest that not all exposures of possums to Tb in the wild become established infections and, more importantly in the context of pathogen persistence, not all diseased animals rapidly die. Such individuals may be infectious for long periods, and key drivers of disease persistence and spread ("super-spreaders"; Lloyd-Smith et al. 2005).
- The issues encountered around study site selection highlight the value of the Orongorongo Valley as a resource for carrying out field trials with wild possum populations in New Zealand. Few other high-density populations with the same level of accessibility remain. Such resources will be essential for future trials that target wild possums, such as trials of fertility control agents.

7. Recommendations

- Trials are now needed to determine what duration of effective protection against Tb can be achieved using oral BCG vaccination of possums in the wild, because the longevity of such protection crucially influences the cost-effectiveness of vaccination as a Tb control strategy. Studies on captive possums have indicated that the oral vaccine still confers some protection up to 12 months post-vaccination (Buddle et al. 2006); however, only field studies can confirm whether the level of extended protection is sufficient against natural infection.
- A suitable carrier for dissemination of the oral BCG vaccine in the field, for both bait-station and aerial delivery, should be investigated. A previous report to the AHB (Aldwell et al. 2006) identified (i) promising packaging materials for field delivery, and (ii) that the lipid matrix itself could be flavoured to form bait-cum-vaccine (thus negating the extra production costs associated with utilising a separate baiting system). We recommend studies to pinpoint the most effective means of vaccine flavouring and packaging to achieve this aim, along with studies on captive possums (or a limited scale field trial) to determine attractiveness and palatability of a flavoured lipid formulation to possums.
- Further trials should be conducted to assess the level of population vaccination achieved through both bait-station and aerial dissemination of oral BCG vaccine.
- The possibility that long-surviving infected possums may act as "super-spreaders" or "temporal bridging" hosts of Tb in the wild should be investigated. Such information is potentially crucial for determining the length of time that the protection from infection that is conveyed by vaccination has to remain effective.

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