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LIPID-FORMULATED BCG AS AN ORAL-BAIT VACCINE FOR TUBERCULOSIS: VACCINE STABILITY, EFFICACY, AND PALATABILITY TO BRUSHTAIL POSSUMS (*TRICHOSURUS VULPECULA*) IN NEW ZEALAND

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ABSTRACT: Bovine tuberculosis (Tb), due to infection with virulent *Mycobacterium bovis*, represents a threat to New Zealand agriculture due to vectorial transmission from wildlife reservoir species, principally the introduced Australian brushtail possum (*Trichosurus vulpecula*). An oral-delivery wildlife vaccine has been developed to immunize possums against Tb, based on formulation of the human Tb vaccine (*M. bovis* BCG) in edible lipid matrices. Here BCG bacilli were shown to be stable in lipid matrix formulation for over 8 mo in freezer storage, for 7 wk under room temperature conditions, and for 3–5 wk under field conditions in a forest/pasture margin habitat (when maintained in weatherproof bait-delivery sachets). Samples of the lipid matrix were flavored and offered to captive possums in a bait-preference study: a combination of 10% chocolate powder with anise oil was identified as the most effective attractant/palatability combination. In a replicated field study, 85–100% of wild possums were shown to access chocolate-flavored lipid pellets, when baits were applied to areas holding approximately 600–800 possums/km^2. Finally, in a controlled vaccination/challenge study, chocolate-flavored lipid vaccine samples containing 10^8 BCG bacilli were fed to captive possums, which were subsequently challenged via aerosol exposure to virulent *M. bovis:* vaccine immunogenicity was confirmed, and protection was identified by significantly reduced postchallenge weight loss in vaccinated animals compared to nonvaccinated controls. These studies indicate that, appropriately flavored, lipid delivery matrices may form effective bait vaccines for the control of Tb in wildlife.

Key words: BCG, New Zealand, oral vaccine, possum, tuberculosis.

INTRODUCTION

Bovine tuberculosis (Tb), due to the bacterial pathogen *Mycobacterium bovis*, represents a threat principally to agriculture, but also to human health and to the conservation of endangered wildlife (Cleaveland et al., 2005, 2007). Although programs of surveillance, diagnostic testing, and selective culling have proved successful in eliminating bovine Tb from primary agriculture in many countries, the disease has proved refractory in cases where a significant wildlife reservoir coexists with livestock (Phillips et al., 2003; O'Brien et al., 2006). In New Zealand the introduced Australian brushtail possum (*Trichosurus vulpecula*) serves as a reservoir and vector of bovine Tb to farmed cattle and deer. Intensive poisoning and culling programs over the last 15 yr, in combination with managed livestock disease control programs, resulted in the national cattle herd Tb reactor prevalence falling from 2.4% in 1993 to 0.35% by 2004 (Ryan et al., 2006), with the period prevalence rate for cattle and deer herds standing at 0.39% in June 2007 (Anonymous, 2007). Despite this success, this strategy has not reduced the total area in which bovine Tb is endemic in wildlife, now extending over 39% of the country (Livingstone et al., 2006). Additional strategies for eliminating Tb from the vector population are urgently required.

Vaccination of wildlife is one means of controlling Tb, but vaccines applied to wildlife species are most expediently delivered as oral vaccines (Cross et al., 2007a). Examples where this approach has

proved successful in reducing disease prevalence include oral rabies vaccination programs among mesocarnivores in Europe and North America (Rupprecht et al., 2004) and oral vaccination against classical swine fever among wild boar in northern Europe (Blome et al., 2006). A key factor in the success of these programs has been the use of live, avirulent vaccine micro-organisms, which retain immunogenicity via oral delivery; the corollary to this is that any baiting system designed to deliver a live, attenuated micro-organism in the field must also be nontoxic to the live vaccine and must not detrimentally affect its immunogenicity. The existing Tb vaccine (*M. bovis*, strain BCG) is similarly a live, attenuated micro-organism, and oral vaccination studies have shown that it is necessary for BCG to be delivered live in order to generate effector immunity (Aldwell et al., 2006; Cross et al., 2008).

Previous research has demonstrated that edible lipid matrices provide an effective delivery vehicle for live BCG bacilli (Aldwell et al., 2003b), as the basis of an oral vaccine against Tb in possums (Aldwell et al., 2003a; Buddle et al., 2006; Collins et al., 2007). Edible lipids are thought to protect BCG during transit through the gastrointestinal tract, thus delivering live bacilli to intestinal sites of immune induction. The lipid matrices have been shown to be amenable to flavoring with nonaqueous additives (Aldwell et al., 2003a; Wedlock et al., 2005). In this study, we additionally demonstrate that a pharmaceutical-grade edible lipid matrix can constitute a predeployment storage vehicle for the vaccine that is nontoxic to BCG. Further, we identify a flavoring regime for the matrix that is palatable to possums, and that invokes a high uptake rate when applied to field populations of wild possums. Finally, we report a study that demonstrates that the flavored vaccine matrix retains immunogenicity and efficacy of BCG when used to deliver live bacilli as an oral vaccine to possums.

MATERIALS AND METHODS

Possums

For captive animal experimentation (bait preference and vaccine immunogenicity studies), adult possums were trapped from the wild and acclimatized to caged conditions for at least 4 wk, as described previously (Aldwell et al., 2003a). Animals weighed between 1.2 and 4.1 kg at commencement of studies and were caged individually, but in proximity. For immunologic studies, heparinized blood samples were first drawn and a lymphocyte proliferation (LP) assay was conducted, as described (Skinner et al., 2002), in order to eliminate any experimental animals with preexisting antimycobacterial immune reactivity. For field experiments involving wild possums (bait uptake studies), two study sites were grid-set within a region of mixed native and exotic forest near Darfield, southern New Zealand (latitude 172.13°E, longitude 43.48°S, elevation 250 m above sea level); preliminary trap catch-rate studies estimated the possum density within this region to be 600–800 animals per km^2 (R. J. Henderson, unpubl.). Both sites were bounded by open farmland on three sides but had forested land on the fourth, representing a typical forest/pasture habitat for possums that have frequent interactions with livestock.

In vitro vaccine formulation and testing

BCG bacteria and live vaccine formulations: Mycobacterium bovis BCG (Danish 1331) bacilli were grown to mid-log phase in Tween/albumin–supplemented Middlebrook 7H9 broth; bacilli were subsequently harvested, sedimented, and formulated into the pharmaceutical-grade vaccine matrix Lipid-PK as described (Aldwell et al., 2003b; Cross et al., 2008). Postformulation bacterial viability counts were undertaken retrospectively by first extracting bacilli from the lipid matrix by dispersal of the aqueous phase in nonionic detergent (Aldwell et al., 2005) and then serial dilution and plating onto Middlebrook 7H11 agar. Viable BCG colonies were counted after 3 wk growth.

Laboratory and field vaccine stability studies: Lipid-formulated BCG samples were maintained in sealed 10 cc thin-walled polypropylene cylinders. For laboratory studies samples were held either at ambient room temperature (18–24 C) or frozen at −20 C; 1 cc subsamples were taken weekly from ambient temperature samples, and monthly from the frozen samples, in order to calculate ongoing BCG

viability in the lipid matrix. For field studies, samples were placed (either free or maintained in polypropylene cylinders) inside weatherproof possum bait-delivery sachets (Connovation Ltd, Auckland, New Zealand), and several sachets were stapled to an upright post; the post was placed in a shaded bush setting (comprising mixed native low-canopy shrubs) immediately adjacent to an area of open pasture land (this site was chosen for its representation of typical New Zealand bush/pasture margin habitat). Similarly to the laboratory-held samples, 1 cc subsamples were taken from these sachets weekly and returned to the laboratory for extraction to determine ongoing BCG viability in the lipid matrix.

In vivo bait preference and uptake studies using the lipid matrix

Bait preference studies—captive possums: Bait preference and palatability studies were undertaken, using flavored lipid matrix samples presented to individual possums in the presence of a flavored food item, a slice of carrot. Bait preference studies were undertaken in two phases: the first phase assessed the best flavoring additive for the lipid matrix; the second phase determined the optimal lipid volume that a possum would consume in a single feeding bout. In the first phase, 16 cage-acclimatized possums were presented test and control (unflavored) lipids daily, with the order that different test lipids were presented over a 4-day period randomized in a block design. Sequentially, possums were presented with flavored or unflavored 1 cc Lipid-PK pellets alongside a slice of carrot on core-flute trays inserted into their cages. The order of olfactory responses to test lipid, control lipid, and carrot was recorded and assigned a value (1=1st, 2=2nd, 3=3rd, 4=not smelled), and similarly the sequence of lipid consumption and number of test and control lipid pellets eaten were recorded during a 10 min observation period. Lipid samples were flavored with either of the following palatability agents blended into the lipid matrix: 10% chocolate powder (the powder comprising 20:80 ratio of ground cocoa:sugar; Cadbury's Confectionary, Dunedin, New Zealand) plus 0.67% anise oil (PSM Healthcare, Manukau, New Zealand; this is a standard olfactory attractant for possums: Morgan et al., 1995); 2.5% chocolate powder plus anise oil; 10% fine ground sugar plus anise oil; or 10% smooth peanut butter.

The second phase of the study determined how much lipid possums would consume in a 10 min feeding period. The same 16 possums were sequentially presented with 5, 10, or 20 1-cc flavored Lipid-PK pellets in the presence of carrot (the flavoring of choice having been identified from phase 1 studies), and 20 pellets were also presented in the absence of carrots. Possums were observed closely, and the number of flavored lipid samples consumed during a 10 min feeding period was recorded.

Bait uptake studies—wild possums: Two thousand 5-cc lipid pellets were flavored with the additives identified from earlier palatability studies; additionally, 1% w/v rhodamine-B dye (Animal Control Products, Wanganui, New Zealand) was incorporated into the matrix as a biomarker. Each lipid sample was placed in a weather-resistant possum bait-delivery sachet (Connovation Ltd., Auckland, New Zealand); these sachets were subsequently deployed in two forested study sites (as outlined above). Sachets were stapled to trees approximately 25 cm above ground level and applied at 10 m intervals on transects 50 m apart. At site 1 an additional sachet was applied to each initial site after 3 days (representing a target baiting density of 40 sachets/ha); at site 2 the same configuration of transects was used except two sachets were attached to each tree, and an additional two sachets were attached to the same tree after 3 days (i.e., 80 sachets/ha). A 300 m buffer between the core research area and the remaining forest land (i.e., an area where dyed lipid was applied but possums were not trapped) was established to prevent immigration of nondyed possums from surrounding habitat. Possums were given 5 days to feed on lipid, and then 200 leg-hold traps were set at each site to catch as many resident possums as possible during the following 2–3 days. Possums caught in traps were humanely killed and transported to the laboratory. Possums were systematically checked around the mouth and the anus with the aid of a UV-emitting lamp. The gastrointestinal tract was excised and placed on a bench, and similarly scanned for fluorescence. Possums were scored as being marked with rhodamine as indicated above (i.e., had eaten lipid) or free of rhodamine (i.e., had not eaten lipid).

Captive possum vaccine efficacy study

Cage-acclimatized possums were assigned to groups comprising six or seven possums/group. At time 0, the test vaccine group ($n=6$) received two 1-cc pellets of Lipid-PK, each containing approximately 10^8 viable BCG, administered approximately 20 hr apart (the pellets being flavored with 10% chocolate powder as identified from the earlier palatability study). A control group of possums

($n=7$) comprised nonvaccinated animals from the same cohort. Heparinized blood was drawn from all possums under anesthesia at time 0, and at 6 and 8 wk postvaccination. Peripheral blood lymphocyte reactivity to mycobacterial antigens was determined via lymphocyte proliferation assay, as described (Skinner et al., 2002), utilizing *M. bovis* purified protein derivative (PPD-B; Prionics, Switzerland) as the in vitro stimulation antigen at 60 μg/ml final concentration and measuring mean responses from triplicate wells of each sample (Skinner et al., 2002). Concanavalin A (Con A, Sigma, St Louis, Missouri) was used as the T cell mitogen control (25 μg/ml final concentration). At 8 wk postvaccination, all possums were sedated and their respiratory tracts exposed to an estimated dose of 10–20 virulent *M. bovis* strain 83/6235 bacilli/animal, via an aerosol-generating apparatus as described (Aldwell et al., 2003a; Buddle et al., 2006). Postchallenge weight changes were monitored regularly, and possums were subsequently euthanized 8 wk postchallenge and subjected to detailed postmortem examination. Occurrence and distribution of tuberculous lesions were recorded. Since *M. bovis* pulmonary infection causes lesions primarily in the lungs and reticuloendothelial organs, samples of lung and spleen tissue were excised, homogenized, and plated onto Middlebrook 7H11 agar for the enumeration of pathogen loads per gram of tissue (Buddle et al., 2006); an approximately 1 g tissue section was sampled from a representative lesion when lesions were observed, or, in the absence of lesions, a similar size biopsy was taken from a predetermined site of each tissue.

Data analysis

For vaccine stability studies, predictive decay lines of BCG viability were fitted to time-series data as second-order polynomial curves. Previous studies with lipid-based possum vaccines have indicated that it is possible to formulate up to 10^8 viable BCG bacteria per dose (Wedlock et al., 2005; Buddle et al., 2006), while laboratory-based studies have suggested that a minimal effective dose for the lipid-formulated vaccine is approximately 10^7 viable bacilli per dose (Cross et al., 2007b): hence, a theoretical drop in bacterial viability of 1 $\log_{10}$ from the original (time 0) value was set as the determination point for vaccine stability. For bait preference studies, statistical significance was determined by rank test analysis of the order in which possums accepted each flavored bait. Acceptance was recorded as the percentage of occasions on which some lipid was eaten by 16 possums, and indicative palatability was estimated as the percentage of test sample eaten in relation to total sample consumption (i.e., test+positive control), where lipid containing 10% chocolate and anise oil was used as the positive control. For field studies, bait uptake was expressed as a percentage with 95% confidence intervals based on a binomial distribution. For possum immunologic studies, LP responses were expressed as a stimulation index (mean counts/min of beta emission from tritiated thymidine-pulsed samples cultured with PPD-B divided by mean count/minute of samples cultured without antigen); median SI values were compared between groups by Mann Whitney U tests. For *M. bovis* challenge studies, the incidences of tuberculous lesions were compared between groups by Fisher's exact test. The pathogen loads per gram of tissue were compared by one-way ANOVA on $\log_{10}$–transformed data, while the mean body weight losses and lung weight/body weight ratios were compared by one way ANOVA on raw data.

RESULTS

Laboratory and field stability of BCG in lipid matrix formulation

Because the efficacy of the BCG vaccine is reliant upon retention of microbial viability, studies were undertaken to determine the survival of bacilli over time and under various environmental conditions, inside the matrix Lipid-PK. Cold-chain storage of a vaccine (such as refrigeration or freezing) represents long-term predeployment storage, while room temperature storage represents the shelf life of a vaccine once it is removed from long-term storage and is prepared for deployment. The vaccine was shown to be stable for at least 8 mo when stored at −20 C (Fig 1a), with a predicted time to 1 $\log_{10}$ decline in bacterial viability of 17.3 mo. Under conditions of room temperature storage, the predictive stability period for the vaccine was 7 wk (Fig. 1b). When unsealed vaccine samples were maintained in weather-resistant bait-delivery sachets in New Zealand field conditions, the stability period was 3 wk (Fig. 2a), although this could be increased

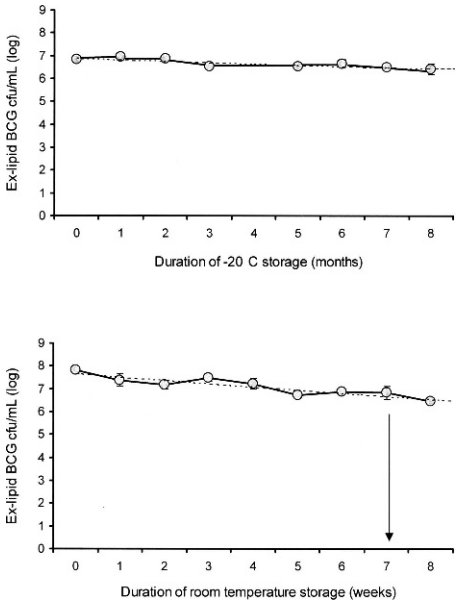

FIGURE 1. Laboratory storage stability of lipid-formulated BCG vaccine. Symbols and solid lines represent time-series data for viable BCG bacilli extracted from Lipid-PK matrix, following varying periods of time under storage conditions at −20 C (upper graph) or room temperature (lower graph). Data represent means±SE of the mean for quadruplicate sample readings at each time point. Dashed lines represent second-order polynomial decay curves fitted to the data (r^2 values 81 and 73%, respectively), which predict 1 log declines in bacterial viability at 17.3 mo for −20 C storage, and at 7.0 wk for room temperature storage (indicated by a drop arrow in lower figure).

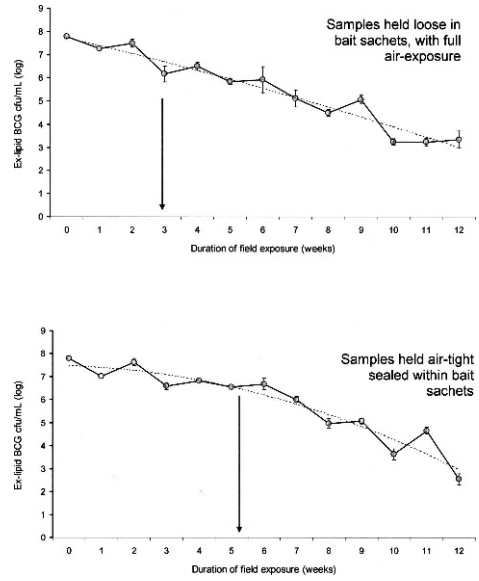

FIGURE 2. Field exposure stability of lipid-formulated BCG vaccine. Symbols and solid lines represent time-series data for viable BCG bacilli extracted from Lipid-PK matrix, following varying periods of time after exposure to New Zealand field conditions (forest/pasture margin habitat). Vaccine samples were either held loose (upper graph) or sealed air-tight (lower graph) and were maintained in weatherproof bait-delivery sachets (Connovation Ltd, Auckland, New Zealand). Data represent means ±SE of the mean for triplicate readings at each time point. Dashed lines represent second-order polynomial decay curves fitted to the data (r^2 values 94 and 91% for air-exposed and air-tight samples, respectively), which predict 1 log declines in bacterial viability at 2.9 and 5.3 wk (respectively) as indicated by drop arrows.

to 5 wk by retention of the vaccine sample inside an air-tight polypropylene cylinder (Fig. 2b); both of these latter cases are representative of potential field-deployment conditions for a live Tb vaccine.

Bait preference studies (captive possums)

When captive possums were presented with different flavored lipid samples on successive days (in the copresence of a flavored food item, carrot), both the samples containing 10% chocolate powder and those containing 10% peanut butter proved attractive to possums (Table 1). On average, samples flavored with 10% chocolate were the first smelled and first eaten and had an indicative palatability of 50%; in contrast, overall bait acceptance among the trial flavorings was highest using 10% peanut butter. Both 10% chocolate powder and 10% peanut butter–flavored lipid samples had mean acceptance rates of over 90% (Table 1). Lipid-PK, flavored with 10% chocolate powder and 0.67% anise oil, was selected as the lead flavoring formulation to take into further in vitro and in vivo studies.

In phase 2 of this study, possums were presented with graded volumes of chocolate-flavored Lipid-PK samples. As shown in Figure 3, when possums were presented with five 1-cc flavored pellets, they would on average consume at least four,

TABLE 1. Results of bait preference, palatability, and acceptance studies in captive brushtail possums (*T. vulpecula*).[a]

Bait type	Order smelled (ranking)	Order eaten (ranking)	Acceptance (%)	Indicative palatability (%)
Carrot	1.38±0.20	1.48±0.17	93.8	N/A
Lipid-PK flavored with:				
10% chocolate+anise	1.88±0.22	2.12±0.20	90.6	50.0
10% peanut butter	2.50±0.20	2.18±0.22	93.8	48.9
2% chocolate+anise	2.75±0.28	2.50±0.18	87.5	44.4
1% sugar+anise	2.75±0.29	2.56±0.24	68.8	38.6
Plain Lipid-PK	3.00±0.18	2.62±0.15	87.5	42.5

[a] Individually caged possums were offered 1-cc Lipid-PK samples with various flavorings in bait preference studies alongside a preferred food item (carrot) and an unflavored lipid sample (control). Order in which flavored samples were smelled and eaten was averaged over 16 possums and reported as a mean ranking (±SEM). Mean percentage acceptance of each bait and indicative palatability were calculated, in relation to carrot. Samples are listed in order of decreasing overall attractiveness to possums.

and when presented with 10 pellets, would consume on average at least six; but when presentation was increased to 20 pellets, average consumption increased to only seven pellets per animal (although this latter figure could be raised to an average of 10 pellets per animal, if possums were presented with a total of 20 pellets in the absence of carrot).

Bait uptake studies (wild possums)

With the information gathered from bait palatability and uptake studies in captive possums, 5-cc lipid samples, flavored with 10% chocolate powder and anise oil, were taken through to field trials of bait uptake. Following deployment of 1% rhodamine-dyed flavored lipid samples, a total of 124 possums were trapped from the two study sites for post mortem examination (Table 2). At site 1, where baits had been deployed at an actual density of 35 per hectare, 99.3% of the baits had been taken over the deployment period; of the 66 possums trapped, 56 were rhodamine-positive, indicating a bait uptake rate of 85%. At site 2, where the actual baiting density was increased to 76 baits per hectare, 94% of the baits had been taken over the deployment period; all 58 possums trapped from this site were rhodamine-positive.

Vaccination and virulent *M. bovis* challenge experiment

Captive possums were vaccinated by feeding them with chocolate-flavored Lipid-PK samples containing live BCG. Retrospective bacterial counts on the vaccine samples indicated that possums had each received an immunizing dose of 2.2×10^8 live BCG. Six and 8 wk postvaccination, median lymphocyte stimulation indices were significantly higher among vaccinated possums compared to control (nonvaccinated animals; Table 3). At postmortem, lung lesions (indicative of pulmonary infection) were observed among all animals regardless of vaccination status; macroscopically, these presented as necrotic lesions in six of seven control (nonvaccinated) animals, while none of the vaccinated animals had macroscopically necrotic lesions. Three of the six control possums had macroscopic, extrapulmonary, tuberculous-like lesions (1–2 mm in diameter) in the spleen (two possums) or liver (one possum), while no such lesions were observed in the vaccinated possums. The mean lung:body weight ratio (indicating the pathologic severity of lung lesions) was lower for the vaccinated group than that for the control group, although this difference was not significant ($P=0.08$; Table 4). The mean body weight loss for

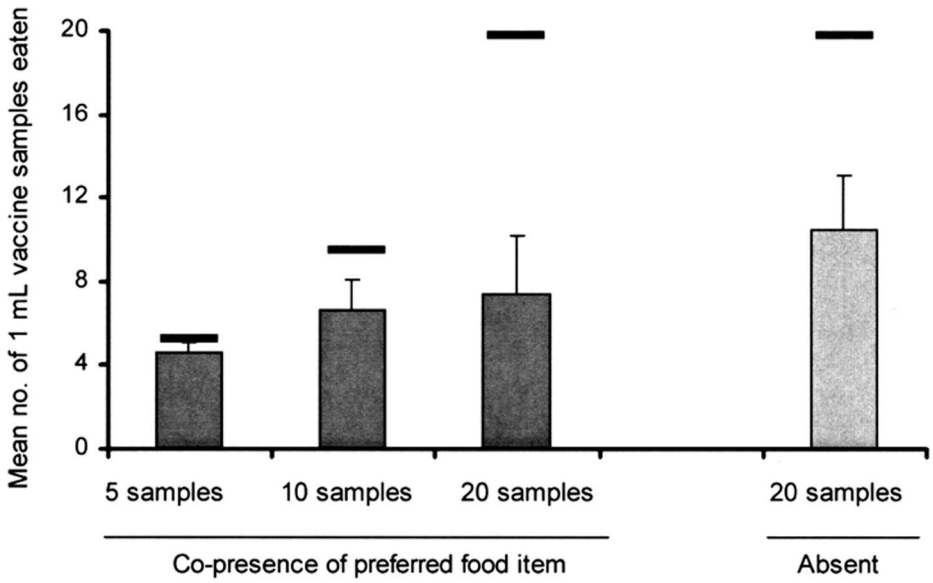

FIGURE 3. Ten-minute feeding bout study to determine optimal volume of chocolate-flavored sample uptake by possums. Data represent the mean (±SE of the mean) number of 1-cc chocolate-flavored lipid samples eaten by possums in a 10-min feeding bout, when these animals were presented with 5, 10, or 20 samples in the copresence of a preferred food item (carrot) or in the absence of preferred food. Horizontal bars represent the theoretical upper limit of samples that could be eaten, assuming 100% consumption of all samples by all possums.

the vaccinated group was significantly lower than that for the control group ($P=0.05$). Lung and spleen *M. bovis* tissue burdens indicated mean $\log_{10}$ reductions in the pathogen loads for the vaccinated group of 0.67 and 0.68, respectively (Table 4); however, these reductions were not statistically significant.

DISCUSSION

Ideal qualities for a practical wildlife vaccine include that the vaccine is stable, the delivered material is safe and remains effective in conjunction with the baiting system of choice, and the deployed baited vaccine has a high uptake rate in the target species (Cross et al., 2007a). Most commonly for oral wildlife vaccines, the vaccine itself is delivered inside a separate bait, as is the case with existing rabies and classical swine fever wildlife vaccines (Kaden et al., 2003; Blackwell et al., 2004), as well as prototypical vaccines for plague (Creekmore et al., 2002). In the present study we have evaluated the novel

TABLE 2. Field studies of uptake of chocolate-flavored lipid samples by wild brushtail possums (*T. vulpecula*).[a]

Site	Estimated possum density (animals/km^2)	Effective trapping area/estimated baiting density	No. baits taken/no. placed (%)	No. possums trapped/ no. dyed (%)
1	600	13 ha (35.1 baits/ha)	453/456 (99.3)	56/66 (85)
2	800	9 ha (75.9 baits/ha)	640/683 (93.7)	58/58 (100)

[a] Five-cc chocolate-flavored Lipid-PK samples were dyed with 1% rhodamine B powder and maintained in weather-resistant bait-delivery sachets (Connovation Ltd, Auckland, New Zealand). In two separate studies, baits were applied at a density of 35 baits/ha (site 1) and 76 baits/ha (site 2) over five consecutive nights, in order to determine field acceptance and uptake by wild possums. Possums were subsequently trapped from these two sites, euthanized, and their GI tracts examined for signs of bait uptake via the presence of UV-fluorescence.

TABLE 3. Median lymphocyte proliferation responses to bovine PPD following vaccination of brushtail possums (*T. vulpecula*).

	Weeks after vaccination[a]		
	0	6	8
Control animals (nonvaccinated)	1.0 (0.7, 2.0)	2.8 (0.6, 5.6)	2.4 (1.1, 3.8)
Vaccinated animals (BCG in Lipid-PK, oral)	1.0 (0.8, 2.2)	9.4* (2.5, 47.2)	8.2* (2.2, 38.5)

[a] Median LP (range) expressed as a stimulation index (LP response in counts per minute for bovine PPD/LP counts/min for cells with no antigen). The overall median counts/min for no antigen was 302, while the overall median stimulation index for the mitogen control (Con A) was 383.

* Significantly different compared to the nonvaccinated group ($P<0.05$).

approach of utilizing the lipid matrix as a combined vaccine delivery vehicle and bait.

For a BCG-based vaccine, one of the key criteria is retention of the bacilli in a viable state, in order to maintain vaccine stability. Previous studies have identified that live BCG bacilli can be maintained inside edible food-grade lipid matrices (Aldwell et al., 2003b), although with a finite period of ambient storage stability that can be measured in weeks. In the present study we have defined the storage stability of BCG inside Lipid-PK, which is an edible vaccine matrix comprising pharmaceutical-grade lipid (a necessary requirement for vaccine licensing). When stored frozen, the vaccine remained stable for at least 8 mo. Furthermore, an operational form of the vaccine (held at ambient room temperature) remained stable for 7 wk; thus the vaccine appears robust to both preusage storage and predeployment handling. When deployed in the field, it was noteworthy that the stability period of the vaccine could be increased from 3 to 5 wk via air-tight retention of the vaccine sample; this figure compares reasonably with a field study conducted on the stability of live attenuated rabies vaccine, where active viral titre was reported to decline by 0.5 $\log_{10}$ after 4 wk of field exposure of sealed vaccine samples (Lawson and Bachmann, 2001). Whether such an extended period of BCG vaccine stability in the field for several weeks is necessary, however, remains to be determined; generally in New Zealand bush conditions, wildlife baiting operations for possum control (i.e., poison delivery) achieve over 80–95% of target delivery within the first 2 days of deployment, particularly if a prebaiting strategy is applied to initiate familiarization (Morgan and Hickling, 2000).

Previous studies on attractants and palatability additives for possum baits have indicated that aromatic oil essences (such as cinnamon or anise) are ideal in the former role, while foodstuff-based flavorings (such as peanut butter or chocolate) are suitable in the latter (Morgan et al., 1995; Gillies et al., 2003). For wildlife vaccination purposes, this is in contrast to reported baiting additives that are employed for carnivorous species, such as the meat- or fish-flavored baits used for rabies vaccine delivery (Linhart et al., 2002), although more akin to a plague vaccine bait formulation that has been reported for prairie dogs (the latter utilizing alfalfa and molasses as bait flavorings; Creekmore et al., 2002). In the present study Lipid-PK samples flavored with 10% chocolate powder and anise oil proved to be highly palatable to captive possums, and so this flavoring was taken forward into field studies of bait acceptance. When applied at a density of 35 baits/ha, 85% of recaptured possums had indications of bait uptake; this figure was increased to 100% uptake by doubling the baiting density. Both of these baiting densities are markedly higher than those used in

TABLE 4. Effect of oral vaccination of captive brushtail possums (*T. vulpecula*) with BCG in chocolate-flavored Lipid-PK on protection against virulent *M. bovis* aerosol challenge.

	Effect of vaccination on:				
	Morphometric parameters		Proportion of animals with macroscopic extrapulmonary lesions[b]	Mean $\log_{10}$ *M. bovis* cfu/g of tissue	
	Mean % body weight loss[a]	Mean % lung weight/body weight at challenge		Lung tissue	Spleen tissue
Control animals (nonvaccinated)	14.54 (±2.27)	2.10 (±0.20)	3/7	7.794 (±0.314)	3.035 (±0.504)
Vaccinated animals (BCG in Lipid-PK, oral)	5.37* (±3.66)	1.62 (±0.14)	0/6	7.128 (±0.304)	2.359 (±0.383)

[a] % body weight loss: body weight at challenge minus body weight at necropsy divided by body weight at challenge and expressed as percentage (±SEM).

[b] Lesions in the spleen or liver (two control animals had splenic lesions, one had liver lesions).

* Significantly different compared to nonvaccinated group ($P = 0.05$).

wildlife vaccine delivery programs for either mesocarnivores or porcines (Rosatte and Lawson, 2001; Blackwell et al., 2004; Campbell et al., 2006); however, it must be appreciated that possums reach much higher population densities in New Zealand bush because of their utilization of a tree canopy habitat and their monopolization of the browsing niche, thus requiring a higher baiting density. There are estimated to be over 70 million possums in New Zealand, with population densities over 2,000 animals per km^2 reported in favorable broadleaf forest habitats (Efford, 2000). The impact of a high possum density on potential wildlife vaccination became apparent in this study, since the recording of an 85% acceptance rate when the lipid baits were applied at 35 baits/ha suggested that a proportion of the animals could remain unvaccinated. It is possible that the unmarked 15% of animals were juveniles or subordinates, which typically feed in deference to dominant and established possums (Henderson and Hickling, 1997) and that a degree of bait monopolization was occurring because of the latter. This was evident by the achievement of a 100% acceptance rate when the baiting density was increased. Although in practical field operation terms it is unlikely that a baiting density of >70 baits/ha could ever be economically viable for a possum field vaccine operation, in reality such a high baiting density would not be required: mathematical modeling studies have indicated that a minimum effective vaccination coverage of 40–50% of animals would be sufficient to control Tb, if the vaccine was sufficiently efficacious and delivery was repeated annually (Barlow, 1991; Roberts, 1995).

Previous studies on captive possums have indicated that lipid-based oral BCG vaccines can induce cell-mediated immune responses and can limit the incidence and/or severity of Tb due to *M. bovis* infection (Aldwell et al., 2003a; Wedlock et al., 2005; Buddle et al., 2006;

Collins et al., 2007). Among the pathophysiologic impacts of Tb (and considering those parameters that may be ameliorated by vaccination), postinfection weight loss, lung weight (as an index of pulmonary pathology), lesion incidence and distribution, and tissue pathogen burdens have been reported as the most informative indices (McMurray, 2001). In the present study, chocolate-flavored Lipid-PK samples containing live BCG were able to induce cell-mediated immune reactivity in possums that had been fed the vaccine, indicating that immune responsiveness could be invoked with this formulation. Furthermore, following aerosol challenge with a virulent *M. bovis* isolate, vaccinated animals exhibited significantly lower weight loss than nonvaccinated animals, and correspondingly, vaccinated animals retained a lower lung:-body weight ratio, had numerically fewer extrapulmonary lesions, and had lower lung and spleen pathogen burdens compared to nonvaccinated controls, although statistical significance was not achieved in these latter cases. Prior studies of *M. bovis* challenge in vaccinated possums have reported numerical reductions in lung and spleen *M. bovis* burdens following oral BCG vaccination, although as here, these may not always achieve statistical significance (Buddle et al., 2006). Nevertheless, the beneficial effect of vaccination in reducing postchallenge weight loss indicates that the severe pathophysiologic impacts of clinical Tb could be ameliorated to an extent by BCG vaccination. The experimental challenge of possums with *M. bovis* is likely to be more severe than a natural exposure to the pathogen, as a recent field trial where lipid-formulated BCG was administered orally to wild possums demonstrated a significant reduction in prevalence of naturally acquired *M. bovis* infection (Tompkins et al., 2007). This follows results from a previous field trial that demonstrated that vaccination of wild possums with BCG administered by other mucosal routes (i.e., intranasally and intraconjunctivally administered vaccine) could provide a significant level of protection against natural exposure to *M. bovis* (Corner et al., 2002). Field delivery of a BCG-based vaccine to possums has the potential to control Tb in wild vector populations, and results here suggest that a lipid matrix vaccine may be sufficiently stable and palatable to achieve that aim in New Zealand. Further research will be necessary to confirm that a vaccine-mediated reduction in Tb in wild possums corresponds to a reduction in Tb reactor rate in adjacent livestock, as has been shown previously by extensive culling of possums to reduce the disease transmission risk via vector depopulation (Caley et al., 1999).

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