Testing of a palatable bait and compatible vaccine carrier for the oral vaccination of European badgers (*Meles meles*) against tuberculosis

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Abbreviations: HPO, hydrogenated (hardened) peanut oil; QC, quality control; PT, paste-bait; EP, European pharmacopoeia; USP, United States pharmacopoeia; IPA, iophenoxic acid; TAMC, total aerobic microbial count; TYMC, total yeast and mould count.
Abstract

The oral vaccination of wild badgers (*Meles meles*) with live Bacillus Calmette–Guérin (BCG) is one of the tools being considered for the control of bovine tuberculosis (caused by *Mycobacterium bovis*) in the UK. The design of a product for oral vaccination requires that numerous, and often competing, conditions are met. These include the need for a highly palatable, but physically stable bait that will meet regulatory requirements, and one which is also compatible with the vaccine formulation; in this case live BCG. In collaboration with two commercial bait companies we have developed a highly attractive and palatable bait recipe designed specifically for European badgers (*Meles meles*) that meets these requirements. The palatability of different batches of bait was evaluated.
against a standardised palatable control bait using captive badgers. The physical
properties of the bait are described e.g. firmness and colour. The microbial load in the bait
was assessed against European and US Pharmacopoeias. The bait was combined with an
edible vaccine carrier made of hydrogenated peanut oil in which BCG vaccine was stable
during bait manufacture and cold storage, demonstrating <0.5 log_{10} reduction in titre after
117 weeks’ storage at 20 °C. BCG stability in bait was also evaluated at +4 °C and under
simulated environmental conditions (20 °C, 98% Relative Humidity; RH). Finally, iophenoxic acid biomarkers were utilised as a surrogate for the BCG vaccine, to test variants of the vaccine-bait design for their ability to deliver biomarker to the gastrointestinal tract of individual animals. These data provide the first detailed description of a bait vaccine delivery system developed specifically for the oral vaccination of badgers against *Mycobacterium bovis* using live BCG

**Key Words**

Badger; BCG; oral vaccination; bait; tuberculosis

**1. Introduction**

The package of control measures aimed at the eradication of bovine tuberculosis (TB) in England and Wales includes the development of an oral vaccine for badgers (*Meles meles*) against the causative agent, *Mycobacterium bovis* [1]. The first injectable vaccine for TB in badgers, BadgerBCG, was licensed in 2010 and has been used in specific areas of England and Wales since then [2,3]. The beneficial effects of the injectable vaccine have been demonstrated in terms of reducing disease severity and progression in captive badgers and reduced serological evidence of infection in wild badgers [4]. The major limitation of BadgerBCG is the need to trap badgers to inject the vaccine. A cost-effective BCG-based oral vaccine could achieve wider coverage, overcoming some of the financial and logistical issues associated with the widespread deployment of BadgerBCG [5]. Oral
vaccines against TB are in development for a number of wildlife species besides badgers [6–9]. In all cases, the development and delivery of a licensed oral vaccine product to the field faces many challenges, including effective delivery of the vaccine by consumption, vaccine stability, and environmental safety [10]. These are best exemplified by the comprehensive programme of research and development of the oral rabies vaccine for foxes (*Vulpes vulpes*) in Europe [11], the end product being a ‘tailor-made’ species-specific bait-vaccine product that can be manufactured and deployed at scale. Oral vaccine delivery mechanisms developed for one species are not necessarily appropriate for another, even if the product is palatable. For example, a bait-vaccine carrier developed for wild boar (*Sus scrofa*) was also attractive to badgers in Spain, but of 150 baits consumed by badgers, 87% had the vaccine carriers (plastic capsules) rejected and of these, 99% were separated from the bait intact with the payload of water still inside [12]. Here we present the first detailed description of a vaccine delivery system developed specifically for the oral vaccination of badgers with live BCG. Numerous baits and possible vaccine carriers were trialed with both captive and wild badgers. Captive animals were used to screen large numbers of different products of which some were selected for field-testing; only selected data are presented here. The selection of the best bait was dependent on many factors including the potential for ease of manufacture at relatively low cost, as well as the results of associated field studies. The data we present are crucial for the on-going development and eventual licensing of an oral vaccine product for badgers, including: (a) identification and description of important physical characteristics of the bait for potential future quality control (QC) purposes for large-scale manufacture; (b) design of a bait with a compatible and palatable vaccine carrier; (c) evidence that the bait-vaccine carrier design can deliver biomarker to the GIT of badgers as a surrogate for BCG; and (d) evidence that the BCG vaccine remains viable in the delivery system through laboratory production processes, cold-storage and simulated field conditions.
2. Materials & Methods

2.1 Animals

Badgers were either brought in from the wild from TB-free areas under licence, or born in captivity. Wild-caught animals were demonstrated to be free of TB on the basis of IFN\(\gamma\) and clinical sample culture testing and housed in groups (two to five animals per pen) in open-air pens with artificial setts, as described elsewhere [13]. Animals were fed a mixture of commercial dog food, peanuts and occasionally fruit and specified pathogen-free eggs. Each pen was equipped with a motion-sensitive infra-red CCTV camera (Secom Security Systems PLC., Kenley, UK). Groups of two to five penned animals were used in palatability and bait design tests as animals could not be repeatedly housed individually for animal welfare reasons; animals were individually caged for a single night for the biomarker study only. The work was carried out under licences (PL 70/6864 and PL 70/7878) from the UK Home Office under the Animal (Scientific Procedures) Act 1986 and approved by the Animal and Plant Health Agency (APHA) Local Ethical Review Panel.

2.2 QC of the bait components

The bait (referred to as either ‘PT’ or ‘paste bait’), is based on a proprietary recipe and was developed with Pest-Tech Ltd. (Leeston, New Zealand) and Connovation Ltd. (Manukau, New Zealand). The paste is free of anti-microbial preservatives, genetically-modified organisms or animal-derived products. Two physical attributes, namely firmness and colour, were assessed for batches of paste bait post-production. Firmness measurements (kgf) were obtained using a calibrated fruit pressure tester (FT011 with 8 mm tip; ACE Supplies Industrial Ltd., Staplehurst, UK) applied to a minimum of three bait portions (~11 g) from each batch. Colour was visually assessed by comparison with a colour chart [14]
and the closest match recorded for each batch. Between one and three samples of paste bait from each batch were submitted for microbiological testing (Wickham Laboratories Ltd., Gosport, UK) as soon as possible after manufacture to assess microbial burden against the European pharmacopoeia (EP) and US pharmacopoeia (USP) specifications for ‘Non-aqueous preparations for oral use’: (a) total aerobic microbial count (TAMC) and total yeast and mould count (TYMC) with limits of $\leq 2 \times 10^3$ and $\leq 2 \times 10^2$ CFU g$^{-1}$ of bait, respectively; and (b) the absence of Escherichia coli in 1 g of material. Where more than one sample was tested per batch of bait, if any one sample exceeded any of the EP or USP specifications, it was considered to have failed QC. Three batches of the vaccine-carrier material, a solid, edible vegetable lipid (HPO, hardened [hydrogenated] peanut oil; Ph. Eur., Sigma-Aldrich Company Ltd., Gillingham, UK) were also submitted for microbiological testing against EP and USP criteria. Palatability testing of the paste bait was carried out between April and October, in both 2013 and 2014, in order to avoid the winter months when badgers exhibit reduced activity [15] and to correspond to when oral bait vaccine would most likely be deployed in the field. In each test (one test per batch of bait) between six and eight groups of animals were each presented with bait contained in five litre plastic tubs (two white and two grey), which were placed in a Latin square arrangement in each pen (tubs approx. 1 m from each other) in the late afternoon before the animals emerged from their setts. Two tubs contained 400 ± 1 g of a batch of paste bait each and two tubs contained 400 ± 1 g of control bait each, comprising a mix of peanuts and golden syrup (ratio 8:1), known to be highly palatable to badgers [16]. Tests were run overnight and normal feed was either withheld for the entire night or given to a group after they had consumed some, but not all, of either bait type; water was provided ad libitum. Limited consumption of bait by a group of animals (i.e. <20 g of one or both bait types, as recommended by the manufacturer) was not considered to be representative of a definitive preference and could result in incorrect palatability calculations. Therefore
groups which consumed <20 g of material were not included in the palatability calculations. Palatability (%) was calculated for each group of animals by dividing the weight of test bait consumed by the total quantity of bait (test and control) consumed by the group. The final palatability value for a batch of bait was calculated by taking an average across all groups. The peanut and syrup control provided a benchmark for palatability, as a minimum standard for a palatable bait. Therefore any test material was required to be at least as palatable as the control i.e. have a palatability of ≥50%.

However, a more stringent palatability threshold of 65% was set for this work to allow for the greater variability introduced when using a small number of groups for testing; ideally palatability tests would utilise large numbers of individually caged animals (R. Henderson, Pest-Tech Ltd., personal communication).

2.3 Bait design tests: bait consumption

Variants of the PT-HPO bait design (Fig. 1) were evaluated in two tests designed to investigate whether altering the PT:HPO ratio and varying the surface area of the exposed PT (to enhance odour release) affected bait disappearance and/or preference. Groups of captive animals were presented with bait portions under inverted terracotta plant pots (with a drainage hole in the base) placed on saucers. Baits, with each type under a different pot, were placed in the pens in the late afternoon prior to the animals emerging from their setts. The delivery of normal feed was delayed until 23:00 each night, to give the animals time to interact with the baits with no other food present. Infra-red CCTV footage of the night was then reviewed to determine (a) the numbers of baits taken - whether or not the bait was consumed in view of the cameras, and (b) preference as measured by the order in which different baits were taken (mean order baits taken across all groups). The peanut and syrup mix was prepared as described previously and presented in portions equal in weight to the baits presented in each pen. In test 1, three different designs of the PT-HPO bait (‘SH’, ‘M’ and ‘C’, Fig. 1), ranging in weight from 13 g to 16 g each, were presented to ten
groups of animals (between three and five animals per group) over two nights in three-
choice tests. The different baits (one bait of each type per group) were rotated through
each pen and each position, left to right, in view of the camera. Test 2 included the bait
designs from test 1 and an additional two designs ('T' and 'D', Fig. 1) in a four-choice test.
Bait designs were rotated through each group over three nights such that each potential
bait combination was presented to each group.

2.4 Bait design tests: biomarker delivery

Two different iophenoxic acid (IPA) biomarkers (PR euroCHEM Ltd., Cork, Ireland) were
incorporated into each of two bait designs (selected from those tested in tests 1 and 2
above) in order to determine whether they could, irrespective of packaging type, deliver
IPA to the GIT as a surrogate for the BCG vaccine. Each bait contained $80 \pm 4 \text{ mg (±5\%)}$
of Propyl-IPA powder (P-IPA; a-propyl,b-(3-hydroxy-2,4,6-triiodo)phenyl-propionic acid)
weighed directly into the centre of the bait within the HPO core in place of the BCG.
Isobutyl-IPA (I-IPA; a-isobutyl,b-(3-hydroxy-2,4,6-triiodo)phenyl-propionic acid) was
incorporated by evenly dissolving 80 mg throughout the HPO component to provide
internal calibration for the amount of actual vaccine carrier consumed. A total of 20
animals were presented with a single bait each and bait allocation was randomised. Ten
were given the PT-HPO ‘M’ in a greaseproof paper bag (approx. 140 x 170 mm,
Connovation Ltd.) and 10 were given the ‘SH’ bait packaged in a low density polyethylene
(LDPE)-lined paper bag (approx. 90 x 170 mm). Different types of packaging were being
evaluated in order to inform packaging field tests [17]. Materials such as plastic-lined
paper may offer increased protection to the bait against moisture, relative to a paper bag.
Individual animals were trapped overnight in cages with a single bait for one night only;
water was provided in a bowl. Animals were released the following morning and any bait
fragments found in the cages collected and weighed. Only animals that consumed some or
all of the bait were anaesthetised by an intramuscular injection of ketamine and
medetomidine (10 mg/kg; Vetalar® and 100 µg/kg; Domitor®, respectively; Pfizer Animal Health, New York, USA) and bled by jugular venepuncture into Serum Separation Tubes (SST Vacutainer tubes; BD, Plymouth, UK) 13–15 days after bait consumption, to coincide with peak IPA serum levels (based on unpublished findings). Serum samples were stored frozen (80 °C) and submitted for duplicate analysis by liquid chromatography-mass spectrometry (LC-MSMS; LGC, Teddington, UK), to quantify and differentiate between P-IPA and I-IPA.

2.5 Stability of BCG in PT-HPO baits

Following successful additional field testing as a carrier (K. Palphramand et al., unpublished results), and on the basis of ease of manufacture, the ‘SH’ and ‘M’ PT-HPO baits (Fig. 1) were used to test vaccine stability. A 200 µl volume of BCG vaccine suspension (Danish strain 1331; ‘Concentrated Bulk BCG’ preparation, Statens Serum Institute, Copenhagen, Denmark), was encapsulated within the centre of the HPO (Fig. 1b). BCG stability (survival) within baits was evaluated at the storage temperatures of 20 °C and 4°C without humidity control, and at 20 °C at 98% relative humidity (RH) maintained using an environmental test chamber (THC Slimline 600/800/-40/ME; Sharetree Ltd., Stonehouse, UK). The conditions in the environmental chamber were based on RH measurements taken from badger setts by LogTag® recorders (HAXO-8; LogTag Recorders Ltd., Auckland, New Zealand) during a Spring-Summer bait deployment study (unpublished results) and from UK MET Office soil temperatures, as indicative of the conditions a product would experience if deployed down a sett entrance. BCG was extracted from baits at regular intervals by cutting each bait into small fragments to release the BCG from the centre. The fragments were submerged in Sauton liquid medium supplemented with 0.05% (v/v) Tween 80 and agitated to macerate the PT and wash the BCG from the fragments in order to form a suspension. Aliquots of this
suspension and dilutions thereof were plated on Modified 7H11 agar plates and BCG colonies enumerated after four weeks’ incubation at 37 °C. Log change values were calculated as $\log_{10} (\text{CFU per bait})$ at each sampling point minus the value at the first time point for the storage conditions under evaluation.

2.6 Statistical analyses

Chi-squared tests were used to test whether badgers exhibited evidence of preference for particular bait types (measured as the order in which baits were consumed), or whether they simply selected different bait types at random. Correlations between mean P-IP and mean I-IPA values of individual badgers were tested using Pearson correlation tests. Chi-square and correlation analyses were carried out using R 3.0.2 (cran.org). The numbers of badgers consuming ‘M’ and ‘SH’ bait types were compared using Fisher’s Exact Test (two-tailed) performed in GraphPad Prism® 6 software (GraphPad Software, Inc., USA).

3. Results

3.1 QC of the bait components

The firmness of the paste bait ranged between 1.8 and 3.2 kgf with a mean of 2.3 kgf (SEM 0.04 kgf; n = 87 tests for 16 batches of bait). Of these 16 batches, two had TAMC in excess of $2 \times 10^3 \text{ CFU g}^{-1}$ but none had TYMC above the limit of $2 \times 10^2 \text{ CFU g}^{-1}$; *E. coli* was not detected in any of the samples. The three batches of HPO were all free of *E. coli* and all had TAMC of $<1 \times 10^1 \text{ CFU g}^{-1}$ and TYMC of $\leq 5.0 \times 10^0 \text{ CFU g}^{-1}$. The colour of the paste bait was consistent between batches. Thirteen batches of paste bait were palatability tested and each batch was presented to 6–8 groups of animals. Overall mean palatability was 77% (67–87%) with none of the batches having a mean palatability of less than the 65% target threshold (Fig. 2).

3.2 Bait design tests
The percentage of baits taken in tests 1 and 2 was high for all designs (80–100%) and the order of bait selection was not significantly different from that expected if animals were selecting at random (Chi-squared tests, test 1: $\chi^2 = 6.67, p = 0.67$, test 2: $\chi^2 = 8.58, p = 0.37$). Of the bait designs, ‘SH’ and ‘M’ (Fig. 1), were selected for the biomarker study based on size, handling by the animals and ease of manufacture. The number of individual badgers consuming bait was 8/10 and 5/10 for the ‘M’ and ‘SH’ baits respectively (NS difference, Fisher’s Exact Test). Very few fragments were recovered post-consumption with their weight constituting 2.1% (n = 3) and 2.4% (n = 3) of the ‘M’ and ‘SH’ baits respectively. Every animal that consumed a bait had positive serum-IPA levels and mean serum values for P-IPA and I-IPA were closely correlated for the bait designs under evaluation (Pearson correlation, $t_{25} = 6.98, p < 0.001, r = 0.81$).

3.3 BCG stability in PT-HPO baits

The oral BCG dose required to successfully vaccinate badgers in the field is not known. In addition, storage, distribution and deployment of a commercial oral badger vaccine is likely to expose BCG-containing baits to a range of temperatures and for varying durations. For these reasons, the stability of BCG in bait was broadly characterised using a range of different starting concentrations, temperatures and durations: $2.90 \times 10^6$–$1.32 \times 10^8$ CFU/bait at $20 \degree C$, $1.24 \times 10^7$–$9.20 \times 10^7$ CFU/bait at $4 \degree C$ and $5.40 \times 10^7$–$6.20 \times 10^7$ CFU/bait at $20 \degree C$ and 98% RH. BCG-containing baits evaluated at $4 \degree C$ and $20 \degree C$ 98% RH had previously been stored at $20 \degree C$ for between 4 and 207 days, and 25 days, respectively. BCG retained viability in bait best when stored at $20 \degree C$ (Fig. 3a) with $<0.5$ log$_{10}$ decrease in CFU/bait up to 117 weeks storage. BCG was less stable in bait when stored at $4 \degree C$ or at $20 \degree C$, 98% RH, with notable and more rapid decreases in CFU/bait becoming apparent after approximately 7 days’ storage (Fig. 3b and c). The overall physical structure of the vaccine-baits remained intact over a 30-day period (Fig. 4).
4. Discussion

The data presented here fulfil the first important steps in the development and testing of a tailor-made oral vaccine product for UK badgers against TB, namely palatability, vaccine stability during bait manufacture and storage, determination of objective and descriptive criteria for QC, and demonstration of its ability to deliver biomarker after ingestion as a surrogate for the vaccine.

The data generated from the QC of the paste bait not only allowed for a better definition of the product but also demonstrated, for the majority of the batches tested, a consistent standard of manufacture. This is important for future bait production but also reassuring given the nature of some of the key bait ingredients which are plant-derived and preservative-free and therefore more variable in nature than, for example, more highly processed products such as the HPO component that will carry the vaccine. Slight variations in paste-bait consistency and colour did not affect its palatability to captive animals as the material was consistently more palatable (on average, more paste bait was consumed than control bait in all batch tests; Fig. 2) to captive animals than the peanut-syrup mix, which is known to be highly attractive to badgers. There are unavoidable limitations associated with the use of captive animals for bait tests, namely the limited sample size and the possible effects of an artificial diet on bait preference and consumption. However, promising materials were always field-tested and these tests reflected the high attractiveness and palatability of the PT-HPO baits observed with captive animals [17].

The paste bait component fulfils several important roles as an effective lure (release of olfactory cues), a palatable material and as an adaptable and malleable material compatible with a vaccine carrier (in this case HPO). Smell is a very important sense for badgers [18] and so the release of odour by the paste bait is likely to be important for
attracting individuals to the bait which is why the bait designs (Fig. 1) varied in the surface area of exposed PT (that not covered by the HPO). The PT component may also help to mask the taste of the vaccine payload; PT-HPO baits containing 200 µl of BCG have proven palatable (88% and 92% of baits taken) with individually caged badgers in two vaccine efficacy trials (S. Lesellier, personal communication). In the current study, variations in bait design did not appear to affect attractiveness (percentage of baits taken) allowing bait design to be driven by other criteria including ease of manufacture, cost (as determined by the quantities of the ingredients) and physical stability; bait size did not affect preference as baits of different sizes were also readily accepted by wild badgers [17] (K. Palphramand et al., Unpublished results).

The use of the IPA biomarkers demonstrated that irrespective of bait design, a core of biomarker could be ingested yielding a positive serum sample in every individual that consumed bait, thus complementing IPA-data from a vaccine carrier field study (K. Palphramand et al., unpublished results). In addition, very little of each bait was left behind in the cages of badgers which had consumed baits, which mirrors observations in the field that animals generally eat any bait fragments which fall from the mouth during consumption (K. Palphramand, personal communication); removal of bait fragments by badgers would also limit consumption by non-target species.

HPO fulfils many requirements of a vaccine carrier: (1) it is palatable, whether presented alone (S. Gowtage, unpublished results) or with the paste bait; (2) it provides good adherence and structural compatibility with the paste bait; (3) it provides an environment for long-term stability of BCG at 20°C; and (4) it is solid over the range of temperatures likely to be encountered in the field (HPO Ph. Eur. drop point specified range 32–43°C).

5. Conclusions
We have developed an oral bait-vaccine delivery system suitable for further evaluation with captive and wild badgers. Many of these tests are already underway, including evaluation of the efficacy of oral BCG fed to captive badgers in the context of PT-HPO and devising a pragmatic protocol for deployment of PT-HPO to wild badgers to maximise bait uptake. Future work will also comprise the necessary technology transfer for larger-scale manufacture. BCG was stable in the bait during prolonged periods of storage frozen. BCG viability declined at 4°C and 20°C but not appreciably until seven days at either temperature, indicating that BCG may retain sufficient viability during cold storage and field deployment to make it a viable oral vaccine. However, further studies will be required once the minimum efficacious dose of the vaccine is determined.

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Conflict of interest statement

Conflicts of interest: none
References


http://wales.gov.uk/topics/environmentcountryside/ahw/disease/bovinetuberculosis/intensive-action-area/badger-vaccination-iaa/?lang=en [accessed 03.02.16].


**Fig. 1.** Different designs of the PT-HPO bait. As referred to in the text – ‘SH’, ‘M’, ‘C’, ‘T’ and ‘D’; b) Vertical (top) and horizontal (bottom) sections of a frozen ‘M’ bait showing the location of the 200µl vaccine payload (arrows). Bar = 1 cm. All baits comprised the same basic structure in which the vaccine payload was held centrally in the HPO (white lipid); the IPA was located in the same position for the biomarker study. The HPO containing the vaccine was surrounded by a PT cylinder (brown paste bait) which in the case of the ‘SH’, ‘M’ and ‘C’ baits was then in turn surrounded by an external layer of HPO.

Abbreviations: PT, paste bait; HPO, hardened peanut oil; IPA, iophenoxic acid.
Fig. 2. Mean palatability (%) for each batch of paste bait (n=13). Each bar represents the mean palatability for a batch of bait and error bars indicate the range in palatability for each batch. Between 4-6 groups of animals (mean 5.2) consumed bait (>20 g) in each batch test. The dashed line is the cut-off of 65% below which a batch of bait was considered to be insufficiently attractive and palatable by the manufacturer.
Figure 3: Stability profiles of BCG in vaccine-baits stored under different conditions: (a) - 20 °C, (b) 4 °C and (c) 20 °C 98% relative humidity. Data points are the calculated mean log$_{10}$ change in CFU/vaccine-bait relative to the mean dose determined before incubation under the conditions stated. The numbers of vaccine-baits sampled at each point are indicated above the range error bars (where visible). Vaccine-baits evaluated at 4 °C and 20 °C 98% RH (relative humidity) had previously been stored at -20 °C for between 4 and 207 days, and 25 days, respectively.
Fig. 4. Photographs of packaged BCG-PT-HPO vaccine-baits stored at 20 °C and 98% RH for (a) 2 days and (b) 30 days. There was only limited absorption of water by the exposed PT component on the surface of the bait (area indicated by the scalpel in ‘b’).

Abbreviations: PT, paste bait; HPO, hardened peanut oil; RH relative humidity.