Oral BCG Vaccine and an Inactivated *Mycobacterium bovis*

Preparation for Wild Boar (*Sus scrofa*): Adverse Reactions, Vaccine Strain Survival and Uptake by Non-Target Species

Running title: Safety of vaccines against tuberculosis for wild boar

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ABSTRACT

Wildlife vaccination is increasingly considered an option for tuberculosis control. We combined data from laboratory trials and an ongoing field trial to assess the risk of oral BCG vaccine and a prototype heat-inactivated Mycobacterium bovis preparation for Eurasian wild boar (Sus scrofa). We studied adverse reactions, BCG survival, BCG excretion, and bait uptake by non-target species. No adverse reactions were observed after administration of BCG (n = 27) or inactivated M. bovis (n = 21). BCG was not found at necropsy, 175 to 300 days post-vaccination (n = 27). No BCG excretion was detected in fecal samples (n = 162) or in urine and nasal, oral and fecal swabs at 258 days post-vaccination (n = 29). In the field we found no evidence of loss of BCG viability in baits collected after 36 h (temperature range: 11-41°C). Camera trapping showed that wild boar (39%) and birds (56%) were the most frequent visitors to bait stations (selective feeders). Wild boar activity patterns were nocturnal while diurnal activities were recorded in all bird species. We found a high proportion of chewed capsules (likely ingestion of the vaccine; 29%) and lost baits (presumably consumed; 39%), and the proportion of chewed capsules showed a positive correlation with wild boar presence. Both results suggest proper bait consumption (68%). These results indicate that BCG vaccination in wild boar is safe and that, while bait consumption by other species is possible, this can be minimized by using selective cages and a strict timing of bait deployment.

Keywords: Disease control, Tuberculosis, Oral baits, Safety, Sus scrofa, Wild boar
INTRODUCTION

Cattle tuberculosis (TB) due mainly to *Mycobacterium bovis* is a re-emerging global concern, and wildlife reservoirs are often implicated in its maintenance [1-3]. Wildlife vaccination is increasingly being considered among the different options available for TB control at the wildlife-livestock interface [4-7].

*Mycobacterium bovis* Bacillus of Calmette and Guérin (BCG), an attenuated strain, has been for a long time the only available vaccine (reviewed in [8]). It has been evaluated for oral vaccination against tuberculosis in cattle, and nowadays it is increasingly studied for use in wildlife [8]. However, new vaccine formulations have been developed to improve efficacy and biosafety [8-9].

Important points to take into account with BCG vaccination are that: (1) the viability must be maintained until delivery and uptake, and (2) the consequent immune response must confer protection [10]. In addition, key aspects when designing a vaccine-bait delivery strategy are: (1) adverse reactions and potential effects of high vaccine doses on health of target animals, (2) potential survival of *M. bovis* BCG in vaccinated individuals, (3) potential excretion of *M. bovis* BCG by vaccinated animals, and (4) vaccine-containing bait uptake by non-target species [4].

**Adverse reactions.** Although reports of adverse reactions arising from the use of BCG are relatively uncommon [11], there are many factors believed to cause side effects [12] such as substrain [12, 13] and route of administration [11, 13-16]. In wildlife and domestic animals, although several species have been vaccinated by different routes [11, 17], no adverse reactions other than local reactions in badgers and some systemic reactions in cattle have been reported [11, 18]. In the case of the badgers, differences in the persistence of the lesions were also dependent on the strain and the route of administration [11, 18]. In cattle, adverse effects have been attributed to high
doses used for vaccination (10⁹ CFU) and to contamination of the BCG preparation [11].

**Survival of *M. bovis* BCG.** BCG has been isolated at necropsy from tissues of vaccinated animals long after vaccination, with differences depending on the species, the route and the type of vaccine used (Table 1). In some cases, BCG has caused lesions in vaccinated but non-challenged animals [19-22]. Dissemination of the vaccine to multiple sites has been observed [20, 23]. The persistence of BCG in tissues could be related to the administration of high doses (10⁹ CFU) [3].

*M. bovis* BCG transmission from vaccinated animals has been demonstrated [20] likely due to the environmental contamination. However, in a recent study of BCG-vaccinated white-tailed deer (*Odocoileus virginianus*) that shared alternatively the same pen than cattle, no transmission between both species was evidenced [24]. BCG could also represent a risk of accidental exposure of non-target scavengers through consumption of vaccinated prey.

**Excretion of *M. bovis* BCG by vaccinated animals.** Soil could represent a risk of environmental contamination through BCG excretion in feces from vaccinated animals. The persistence of the vaccine in feces from captive wild animals has been confirmed in both orally vaccinated possums and badgers (doses > 10⁸ CFU of lipid-formulated BCG-Pasteur) for up to 7 and 17 days post vaccination (pv), respectively [25, 26]. One of 12 possum fecal samples collected after BCG-ingestion and stored under similar conditions to those of a forest floor environment was culture-positive for up to 5 weeks [26].

**M. bovis** BCG viability and bait uptake by non-target species. BCG viability and stability are two important factors to consider in order to achieve a good immunization [27]. This is a severe constraint when vaccinating wildlife orally in the
field. Different studies have assessed the duration of BCG survival in laboratory and
field conditions (Table 2).

Many species compete for bait consumption; the species depend on the region
and the type of bait [28-30]. Some kinds of oral baits have been found highly palatable
to different non-target wild and domestic animals [28], thus strategies ensuring that only
target species gain access to bait are necessary [31, 32]. More studies concerning bait
deployment and BCG viability are in progress [8].

Wild boar (Sus scrofa) is the main wild reservoir of TB in Spain [1], therefore
recent research has focus on immune response in this species and the protection
conferred after oral immunization with BCG and a prototype based on heat-inactivated
M. bovis preparation [4, 33, 34]. Target animals for vaccination are 3-4 month-old
piglets [28, 32], age usually achieved by early summer. The aim of this study was to
assess through data compiled from both already published and non-published studies,
the potential risks of the field deployment of oral BCG and the prototype inactivated M.
bovis preparation for wild boar considering adverse reactions in the target host, risks
due to vaccine strain survival or excretion in vaccinated individuals, and bait uptake by
non-target species.

MATERIALS AND METHODS

All the experiments used handling procedures designed to reduce stress and health risks
for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D.
1021/2005), and were approved by the institutions Ethics Committees.

Bait-vaccine delivery system. The baits had a hemispherical shape
(Ø3.4 × 1.6 cm) and are made with piglet feed, wheat flour, paraffin, sucrose and
cinnamon-truffle powder attractant as described [35]. Vaccine formulations were
delivered into sterile airtight polypropylene or polyethylene 0.2 ml eppendorf tubes (capsules) which were dipped into the bait.

This research was performed using two formulations: BCG and a prototype killed *M. bovis* preparation. The BCG Danish (CCUG 27863) was cultured in the laboratory and the suspension turbidity was adjusted to 1.0 McFarland standard. The amount of BCG CFU were calculated by plating aliquotes of $10^{-3}$ to $10^{-6}$ dilutions onto Coletsos medium in duplicate as described [33, 34]. The baits contained 0.150 ml of this suspension which equals $5.2 \times 10^5 - 7.6 \times 10^5$ CFU. In the case of parenteral BCG, an intra-dermal dose of 0.1 ml containing 0.075 mg of BCG Danish strain (Statens Serum Institute, Copenhagen, Denmark) was administered [36]. The inactivated preparation was made with a *M. bovis* field isolate cultured in the laboratory and the suspension adjusted to 1.0 McFarland standard. Tenfold serial dilutions were plated onto OADC-enriched agar-solidified Middlebrook 7H9 to assess the CFU in the suspension. The inoculum was then heat-inactivated (34). Animals received the equivalent to $6 \times 10^6 - 10^7$ CFU. In the case of parenteral administration, the preparation used Montanide ISA 50 V (Seppic, Castres, France).

**Adverse reactions to *M. bovis* BCG and inactivated *M. bovis* preparation.** Data originated from several vaccination experiments. In total, data on possible adverse effects such as signs of fever, loss of appetite and body condition deterioration were available for 7 wild boar vaccinated with BCG-Danish by the parenteral route and 20 wild boar vaccinated by the oral route in four different experiments [33, 34, 36, unpublished data]. For the heat-inactivated vaccine, data were available for 9 parenterally vaccinated wild boar [34, unpublished data] and 12 orally vaccinated wild boar [34, unpublished data]. Vaccinated animals were subsequently challenged with a *M. bovis* field strain administered by the oropharyngeal route at doses ranging from $10^5$. 
to $10^6$ CFU, with the exception of two animals with a minimum dose of $10^2$ and two animals with a medium dose of $10^4$ [33, 34, unpublished data]. Feeding and behavior was also monitored throughout the entire experiments and body weight, head and body length, and kidney fat index (KFI) of the wild boar were recorded at necropsy.

**Survival of** *M. bovis* **BCG.** Oropharyngeal tonsil, mandibular lymph nodes (LN), parotid and retropharyngeal LNs, lung, tracheobronchial LNs, mediastinal LN, spleen, ileocecal valve, mesenteric LNs and hepatic LN from 27 BCG-vaccinated wild boar of four different experiments were collected at necropsy (days: 175 pv, 189 pv, 258 pv and 300 pv) and cultured in solid media and in liquid media using the BACTEC™ MGIT™ system (Becton Dickinson, Sparks, MD 21152 USA) as described by Garrido et al. [34] The isolates resulting from positive cultures were further characterized by spoligotyping [37] which allows identification of MTC complex strains based on presence or absence of spacers in the Direct Repeat region. Specifically, BCG (SPB0120, www.Mbovis.org) is positive for spacers 21 and 26 to 29, whereas the *M. bovis* field strain used for challenge (SB0339) is negative.

**Excretion of** *M. bovis* **BCG by vaccinated animals.** Regarding the presence of BCG in feces, two experiments were carried out to detect the bacilli in fecal samples. In both experiments, animals were housed in two rooms in class 3 bio-containment facilities. Samples of three different points of each room were collected at different times post-vaccination [1st experiment ($n = 24$): days 1 pv, 3 pv, 5 pv and 7 pv; 2nd experiment ($n = 54$): days 0, 1 pv, 2 pv, 3 pv, 4 pv, 10 pv, 20 pv, 30 pv and 40 pv] and post-infection (pi) [1st experiment ($n = 24$): days 8 pi, 21 pi, 42 pi and 71 pi; 2nd experiment ($n = 18$): days 60 pi, 80 pi and 100 pi]. Moreover individual fecal samples of each animal were taken the day of the necropsy [1st experiment ($n = 20$) at day 189 pv; 2nd experiment ($n = 22$) at day 258 pv]. These samples were cultured between 24-48
After collection, for decontamination, 2 g of the fecal sample was homogenized with 38 ml of 0.75% hexadecylpiridinium solution and left for 18 h. After collecting the upper part of the sediment with a plastic disposable pipette, 2 tubes of Coletos medium (bio-Merieux SA, Marcy L’Etoile, France) and 2 tubes of Löwenstein-Jensen medium (homemade, Neiker, Derio, Spain) were inoculated with 4 drops each. The tubes were incubated at 37ºC and inspected monthly until the 16th week with a stereoscopic microscope for the presence of any growth. In the second experiment we were able to collect also urine from 5 out of the 8 BCG-vaccinated wild boar at necropsy. Urine was cultured as described above.

Additionally, in the second experiment, nasal, oral and rectal swabs from 8 BCG-vaccinated wild boar were analyzed after necropsy to detect the possible excretion of *M. bovis*. DNA was extracted using the DNeasy Blood & Tissue kit according to the manufacturer’s protocol (QIAGEN, Germany). Detection of MTC DNA was performed by an in-house Real-Time PCR detecting the MPB70 gene, including also an internal control (Castellanos et al., unpublished data). Moreover, these samples were cultured in solid media and in liquid media using the BACTEC™ MGIT™ system.

**M. bovis BCG viability and bait uptake by non-target species.** To evaluate the viability of the BCG vaccine once inserted into a bait, we tested baits containing $10^5$-$10^6$ CFU of BCG in the 0.2 ml plastic vial “capsules” (as described by Ballesteros et al. [35]) under both laboratory and field conditions. The vaccine preparation protocol was as described above. Time between preparation in the laboratory and deployment in the field was less than 24 h and temperature was kept at 4ºC. First, in order to know the effect of the temperature in south-central Spain on the BCG vaccine, we conducted two different field trials in summer 2012. A total of 95 BCG-baits were placed inside 12 bait stations (selective piglet feeders) (as used by Ballesteros et al. [31]) in two different
areas with similar environmental conditions. Baits were delivered at dusk and collected after different periods of time to evaluate the survival of viable BCG bacilli within the bait. In the first experiment, performed in late August, one pool of baits \((n = 20)\)

belonging to 8 selective piglet feeders was collected after being kept in the environment from 20.30 h to 8.30 h (12 h). Later, early in September, baits from 4 selective piglet feeders were also placed in the feeders from 20.30 h and collected after 12 h \((n = 35)\), 24 h \((n = 22)\) and 36 h \((n = 18)\). Baits were collected from each zone, capsules extracted and their content pooled. This mixture, together with 10-fold serial dilutions, was cultured on Löwenstein Jensen media (Difco FSM, Madrid, Spain) in duplicate. Colony forming units readings were taken after 8 weeks.

Environmental temperature was monitored from July 6th to September 6th through 4 Microlite data loggers (Dostmann electronic, Germany) set up to record data every half hour. Data loggers were placed at the base of trees or shrubs located less than two meters from a selective piglet feeder: (1) two of them in the presumably coolest two points of the study sites, and (2) the other two in presumably warmest (more sun-exposed) points in order to record the possible broadest temperature range.

Moreover, BCG viability within the baits was tested in laboratory conditions exposing five groups of four vaccine baits each to four different temperatures \((4, 25, 37 \text{ and } 42 ^\circ \text{C})\) respectively for 24 h, 48 h and 72 h. Bait contents \((M. \text{ bovis} \text{ BCG})\) and 10-fold serial dilutions were cultured in duplicates as described above. Data from both field and laboratory trials were analyzed by non-parametric Mann-Whitney U tests using the SPSS statistical package (IBM 19.0 software, IBM Corporation, Somers, NY, USA).

We used infrared-triggered cameras (Uway NightTrakker NT50 IR, Lethbridge, Canada) to assess bait uptake by target and non-target species. Camera traps were set up to record three capture shots for one minute and fixed to posts or tree trunks focusing
the center of the selective piglet feeders ($n = 46$) in two different study sites (23 feeders in each site). We delivered a total of 8280 vaccine baits: every night, 20 baits containing BCG or 20 baits containing the heat-inactivated $M. bovis$ preparation were deployed in 23 feeders each vaccine during nine nights (920 baits per night). At each feeder, baits were deployed at dusk and the not consumed ones were collected in the next morning for destruction. The outcome of the baits and vaccine capsules was classified as intact baits (untouched baits), consumed baits but untouched capsules with vaccine left in the feeder, consumed baits with chewed capsules meaning likely ingestion of the vaccine, and "lost" baits (missing baits and capsules presumably ingested) (Figure 1). Cameras were kept over the entire length of the study (9 days). Picture details were processed by two independent researchers and converted into Excel files (Microsoft Excel, version 2007; Microsoft Corporation) recording the following variables regarding each feeder per day (from 20:00 h to 20:00 h of the following day): feeder location, date and time of capture, presence of each species, and presence inside/outside the feeders. Our findings are described in terms of positive minutes in relation to the presence (PMP) of each species. Data were analyzed by descriptive statistics and non-parametric Spearman’s correlations using the SPSS statistical package (IBM).

RESULTS

Adverse reactions. No signs of fever such as reduced activity or frequent drinking, loss of appetite and body condition deterioration were observed after BCG administration ($n = 27$). Also, no adverse reactions against the inactivated $M. bovis$ prototype were recorded ($n = 21$); and the animals that received the inactivated vaccine via parenteral ($n = 9$) did not show swelling at the site of injection.

Survival of $M. bovis$ BCG. Despite the fact that at least 7 different tissues per
animal were cultured in all the experiments, BCG was not found at the time of necropsy. The field *M. bovis* strain used for the challenge was isolated from 14 of the 27 wild boar. Of the 257 tissues analyzed, virulent *M. bovis* was isolated in 48 cases; all isolates had the same spoligotyping pattern as the challenge strain (SB0339), and none of them was BCG.

**Excretion of *M. bovis* BCG by vaccinated animals.** After sixteen weeks of incubation of fecal samples, no growth was observed in the inoculated culture media from the ante-mortem and post-mortem samples (*n* = 162) collected in the two experiments. The urine of 5 BCG vaccinated wild boar as well as the nasal and fecal swabs of the 8 animals were negative at 258 days post-vaccination. Oral swabs from 2 of the 8 animals were positive to *M. bovis* at 258 days pv, but negative to BCG.

**M. bovis** BCG viability and bait uptake by non-target species. Temperature data collected by the data loggers over two months revealed that the average temperature in the field sites from July 6th to September 6th was 21.97°C ± 8.09 (mean ± ı). Figure 2 shows the global average values and hourly maximum and minimum values. Maximum temperature exceeded 37°C from 11:00 to 19:00 h.

In August, temperature achieved an average of 24.51°C (11.02 - 41.32) with a minimum at 7:00 h and a maximum at 13:00 h. In September (mean 22.18°C; 11.44 - 40.06), baits were exposed to the minimum and maximum temperatures at 8:00 h and 18:00 h, respectively. The number of BCG was around 10^5 in both trials (mean CFU: 5.6 x10^5 ± 1.7x10^5; *n* = 95). Despite exposure of the vaccine to this huge environmental temperature variability, there was no significant evidence of loss of viability in the baits collected after 12, 24, or 36 h (Mann-Whitney U test; z=-1.481, p=0.178).

In laboratory conditions we recorded the CFU of BCG that remained viable after being subjected to different temperatures. The initial count of bacteria (at room
temperature) ranged from $5.1 \times 10^4$ to $4.1 \times 10^5$ CFU. The number of CFU remained quite stable at temperatures of 4 and 25°C for 72 h. However, at 37 and 42°C the concentration began to decrease significantly after 24 h, with $5.3 \times 10^4$ and $3.3 \times 10^3$ CFU (U test; $z=-2.309$, $p=0.029$; $z=-2.323$, $p=0.029$, respectively) until reaching a final count of $3.1 \times 10^2$ and $3 \times 10^2$ at 72 h.

Camera trapping data recorded a total of 13504 PMP from all the 46 feeders in the 9 days of the experiment. The frequency percentages by species groups were 39.26% wild boar, 56.37% birds, 1.65% carnivores, 1.65% other ungulates and 1.07% other species (lagomorphs and rodent species). Inside the selective feeders we observed a wild boar presence of 48.35% ($n = 3103$ PMP), of which 82.92% were piglets, 5.31% juveniles and 11.78% adults that put their head between the feeder bars. Bird presence is detailed in Table S1 (see supplementary files). The number of PMP of the different carnivore species in relation to the total presence of carnivores inside the feeders (1.65%) were the following: red fox (*Vulpes vulpes*) (57.02%), stone marten (*Martes foina*) (40.53%), badger (1.75%) and common genet (*Genetta genetta*) (0.88%). Red deer, roe deer (*Capreolus capreolus*), and fallow deer (*Dama dama*) were observed only outside the feeders (1.65%) and in a ratio of 69%, 20%, and 11%, respectively.

Wild boar and birds entered in the feeders at different times. Wild boar activity patterns were nocturnal while diurnal activities were recorded in all bird species. An hourly average of the total PMP from the 46 feeders, showed that wild boar activity began at about 19:00 h and the peak was observed at 23:00 h (Figure 3).

Regarding bait consumption, we found a high proportion of “lost” baits (39.3%±31.2) and chewed capsules (29.2%±27.7, 95% CI) in relation to the total delivered baits, both suggesting proper bait consumption (68.5%±37.07). Collected intact baits and capsules reached 25.3% (±38.2) and 6.2% (±11.9), respectively. These
are confirmed not consumed bait capsules (31.5%±37). Intact baits and intact capsules were mostly found inside the feeders (98.64% baits, 78.13% capsules).

Considering only the time in which baits were in the field, we combined the data of the presence of the species detected by the cameras with the data of the type of bait found in the morning. Presence of wild boar, carnivores and other species (lagomorphs and small rodents) was negatively correlated with number of intact baits, suggesting consumption by these species. The proportion of chewed capsules showed a significant positive correlation with wild boar while correlations with birds and carnivores were negative (Table 3).

**DISCUSSION**

Vaccination of wild species has been proposed as a tool for the support of eradication programs and to promote health of wildlife populations [10]. However, several issues related to protection against the infection but also with animal and environmental biosafety need to be addressed before this becomes a feasible option [1, 4, 8]. In this work we report on relevant safety issues related to administration of the vaccine to wild boar and to bait deployment. These experiments have been performed in the laboratory and specific biocontainment facilities and, for the first time, also in the field (under controlled conditions). Because of the complexity of the task and the difficulty of animal handling and ethical reasons we have focused the study on basic aspects mimicking the natural situations that are expected to be met in the field (Mediterranean habitat). The preparations studied here are *M. bovis* BCG Danish and a prototype killed mycobacterium preparation that it is being trialed for its potential use as a vaccine [34, unpublished data], which would have fewer cold-chain constraints and enhanced biosafety.
Combining the results of several laboratory experiments and one ongoing field study we obtained encouraging preliminary results of the safety of wild boar vaccination against TB. The main results belong to two groups of risks, one regarding consequences of use of BCG in wild boar and one regarding bait deployment. First, there were no adverse reactions to \textit{M. bovis} BCG; BCG was not detected in tissues of vaccinated wild boar after 175 days; and no BCG excretion by vaccinated wild boar was recorded. Secondly, although BCG survival inside baits in the environment was higher than expected, bait uptake by non-target species was low and can easily be minimized through management. This information is necessary to implement field vaccination with safety.

Sample sizes, despite being reasonable for the level 3 biocontainment trials, were small. Future experiments and on-going field trials will allow increasing the sample size and hopefully confirming the available results. In the field, data on hunter-harvested oral-vaccinated wild boar will only become available in coming years. Meanwhile, the bait uptake results from this study make us confident that most vaccine capsules were actually consumed by the target wild boar piglets. This confirms results of previous bait deployment experiments [32]. Selective feeders allow a targeted delivery of oral baits to wild boar piglets, which is the preferred age for vaccination [31]. Furthermore, the use of this type of feeders could avoid the possibility of bait consumption by cattle, since its head is unable to enter through the bars to reach the baits.

No adverse reactions to BCG or the heat-inactivated \textit{M. bovis} preparation were observed in the wild boar used in the different experiments. This suggests that both are safe for wild boar, and most likely also for its domestic relative, the pig. Regarding BCG however, the absence of adverse reactions could in part be due to the medium
doses of the vaccine used in our experiments (between $10^5$ and $10^6$ CFU). We used low
doses in the experiments in biocontainment facilities to imitate those of the field trials
(dose in the field trial: $10^5$ CFU). Thus, even if an individual ingests several baits, it
would be unlikely to consume doses higher than $10^{6.5}$ CFU. At worst, to achieve a dose
of $10^8$ CFU, the same individual should consume all the baits (20 baits per feeder) of 6
different feeders during 9 nights, but this is unlikely. Nevertheless, as mentioned in the
introduction, even higher doses of BCG ($10^8$ CFU) have often been used in different
host species without secondary effects [12, 25, 26, 38-40]

At necropsy, BCG was not found in the key tissues of the experimentally
vaccinated wild boar in any of the 4 experiments (ranging from day 175 to 300 pv), and
BCG was not detected in feces and swab samples after 258 days pv. Although we
cannot discard previous and transient tissue colonization (further research is needed),
this study shows that after 175 days, BCG is not present in wild boar tissues. This fact
could be important to take into account prior to introducing this meat into the food chain
for human consumption. Moreover, although previous studies have occasionally
detected BCG in tissues, BCG has not been isolated from meat [21]. To date, wildlife
vaccination studies in experimental conditions have shown that BCG shedding occurs
only in low to moderate numbers and only for a short period of time [25, 26, 41]. In
wild boar the lack of ante- and post-mortem detection of BCG in feces of vaccinates
suggests that contamination of the environment by this route is unlikely. Furthermore,
for the moment, BCG has not been isolated from feces under field conditions [25], and
even if this was the case, it is still unknown which dose would actually infect non-target
animals after ingestion. For instance, it is believed that oral doses of BCG that could
sensitize cattle would be near to $10^7$ CFU [41]. Such a high dose is very unlikely to be
excreted by wild boar vaccinated with doses below $10^6$ CFU.
Temperature stability was studied because bait deployment coincided with early summer, characterized with high temperature in central-south Spain. In the laboratory, exposure to temperatures of 37°C and 42°C strongly reduced BCG viability by about two logarithms within 24 h. However, field viability was higher than expected, at least for 36 h after bait deployment, probably due to temperature fluctuations or effect of the soil temperature. This greater than expected stability in the field implies on one hand a logistic advantage for field vaccination, but on the other hand it is a disadvantage regarding the possible access of non-target species to viable BCG-baits. Ideally, baits should be distributed after sunset and collected at sunrise to avoid diurnal species, mainly birds. Nevertheless, field data suggest that birds are not involved in the consumption of baits, since their presence at feeders correlated with the number of intact baits found (not consumed). This way of distributing the vaccine would also avoid exposure to temperatures above 37°C. Among the nocturnal non-target species, the ones to be considered specifically are the carnivores. Foxes and stone martens were related to the bait losses, but their presence was a much smaller percentage than wild boar. Although lagomorphs and rodents represent the lowest percentage of the presence of the total of the species (1.07%), they were implicated in the bait losses and also with the appearance of less intact baits in the feeders. In the case of the presence of other ungulates, the system of the selective piglet feeders prevents almost completely their access to the baits.

In summary, the results indicate that BCG and heat-inactivated *M. bovis* vaccination in wild boar is safe and that, while consumption by other species is possible, this can be minimized by using specific management measures such as selective feeders and a strict timing of bait deployment and collection. The use of an inactivated vaccine would avoid most of the risks and logistic constraints of using BCG.
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Authors’ contribution: BBB, BR, AA, JMG, JAB and CG conceived, designed and coordinated the study. BR, IS, JMG and EM performed microbiology analyses and prepared the vaccines. BBB, DGB, IDD and JAB carried out the field trials. All authors contributed in the necropsy of the animals. BBB wrote the first draft and all authors revised the drafts and approved the final version for submission.

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FIG 1 Examples of the outcome of the baits and vaccine capsules in the field: consumed baits but untouched capsules with vaccine left (left), intact baits (center), and consumed baits with chewed capsules meaning likely ingestion of the vaccine (right).

FIG 2 Maximum and minimum hourly temperature and average temperature collected by the data loggers from July 6th to September 6th. Temperature reached a maximum of 48°C at 18:00 and a minimum of 4.52°C at 8:00. The average for the two months achieved 21.97 °C.

FIG 3 The presence of each species at the feeders was evaluated as total number of positive minutes in relation to the presence (PMP) in which the species was detected by the infrared-triggered cameras. Results show total PMP obtained every hour at the 46 feeders during the 9 days Wild boar (WB) activity began almost at the same time that birds activity ended up.
**TABLE 1** Mycobacterium bovis BCG-isolation at the time of necropsy in wild and domestic animals reported in the literature. Species, BCG strain, administration route, dose and time after vaccination are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>BCG-Strain</th>
<th>Route</th>
<th>Dose</th>
<th>Tissues with confirmed BCG isolation</th>
<th>Time since BCG vaccination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>White-tailed deer</td>
<td>BCG-Danish SCa</td>
<td>$10^7$ CFU</td>
<td>Superficial cervical, tracheobronchial, mediastinal, hepatic lymph nodes</td>
<td>8 months</td>
<td>[20]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Pasteur SC</td>
<td>$10^7$ CFU</td>
<td>Superficial cervical lymph node, lung</td>
<td>8 months</td>
<td>[22]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Danish O</td>
<td>$10^7$ CFU</td>
<td>Tonsil, lymph nodes (retropharyngeal, mediastinal, hepatic, ileocaecal), jejunum, caecum</td>
<td>3 months</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Danish SC</td>
<td>$10^6\cdot10^7$ CFU</td>
<td>Tracheobronchial, hepatic and mesenteric lymph nodes</td>
<td>9 months</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Danish lipid encapsulated BCG bait O</td>
<td>$10^8$ CFU</td>
<td>Lymph nodes (head, thoracic and abdominal pool)</td>
<td>12 months</td>
<td>[3]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Danish liquid suspension O</td>
<td>$10^8$ CFU</td>
<td>Lymph nodes (head and thoracic pool)</td>
<td>9 months</td>
<td>[3]</td>
<td></td>
</tr>
<tr>
<td>Red Deer (Cervus elaphus)</td>
<td>BCG-Pasteur and BCG-Pasteur recombinant strain SC</td>
<td>$10^6$ CFU</td>
<td>Lymphoid tissues (site of injection, draining lymph nodes)</td>
<td>3 months</td>
<td>[42]</td>
<td></td>
</tr>
<tr>
<td>Possums (Trichosurus vulpecula)</td>
<td>Lipid-formulated BCG-Pasteur O</td>
<td>$10^8$ CFU</td>
<td>Mesenteric lymph node, Peyer’s patches</td>
<td>3 weeks-2 months</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>Animals</td>
<td>Strain</td>
<td>CFU</td>
<td>Location</td>
<td>Time After Inoculation</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------</td>
<td>-------</td>
<td>---------------------------------------</td>
<td>------------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Badgers</td>
<td>BCG-Pasteur</td>
<td>O</td>
<td>Cervical lymph node</td>
<td>7 months</td>
<td>[25]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipid-formulated</td>
<td>O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG-Danish</td>
<td>SC</td>
<td>Inguinal lymph nodes, spleen, lungs</td>
<td>5 months (spleen)</td>
<td>[23]</td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>BCG-Pasteur</td>
<td>O</td>
<td>Mesenteric lymph node</td>
<td>3 months</td>
<td>[43]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipid-encapsulated</td>
<td>O</td>
<td>Mesenteric lymph node</td>
<td>7 months</td>
<td>[43]</td>
<td></td>
</tr>
<tr>
<td>Guinea pigs</td>
<td>BCG</td>
<td>IP</td>
<td>Abscess of the epididymis caused by</td>
<td>9-10 months</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>IP</td>
<td>Mesenteric lymph nodes</td>
<td>19 months</td>
<td>[46]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>Site of inoculation and distant lymph nodes, spleen, liver and lungs</td>
<td>6 months</td>
<td>[47]</td>
<td></td>
</tr>
<tr>
<td>Rabbits</td>
<td>BCG-Pasteur</td>
<td>IV</td>
<td>Mesenteric, tracheobronchial lymph nodes, and occasionally spleen and kidney</td>
<td>14 months</td>
<td>[19]</td>
<td></td>
</tr>
</tbody>
</table>
SC, subcutaneous; O, oral; IP, intraperitoneal; IV, intravenous; IT, intratracheal; EI, eye instilation.

4 Time of necropsy, at 14 weeks BCG was eliminated by the 50% of the animals and only low levels of residual organisms persisted in the hosts.

5 Some of these badgers had concurrent infection with BCG and the M. bovis challenge strain in the affected tissue.

6 Numerous acid-fast bacilli were observed in the gland, but cultures negative (apparently dead).
TABLE 2 Stability studies of lipid and non-lipid formulated *Mycobacterium bovis* BCG in laboratory and field conditions at different temperatures

<table>
<thead>
<tr>
<th>Temperatures</th>
<th>Temperatures</th>
<th>Formulation</th>
<th>Vaccine</th>
<th>Stability</th>
<th>Viability/Potency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient room</td>
<td>18-24°C</td>
<td>Lipid formulation</td>
<td>BCG-Danish</td>
<td>7 weeks</td>
<td>-</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td>10-25°C</td>
<td>Lipid formulation</td>
<td>BCG-Pasteur</td>
<td>8 weeks</td>
<td>Few viable <em>M. bovis</em> BCG detected</td>
<td>[51]</td>
</tr>
<tr>
<td>Refrigerated</td>
<td>5°C</td>
<td>Non-lipid formulation</td>
<td>-</td>
<td>8 weeks</td>
<td>Fell to the level of 10-20%</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>Lipid formulation</td>
<td>BCG-Pasteur</td>
<td>8 weeks</td>
<td>Minimal loss of viability</td>
<td>[51]</td>
</tr>
<tr>
<td>Frozen</td>
<td>-20°C</td>
<td>Lipid formulation</td>
<td>BCG-Danish</td>
<td>8 months</td>
<td>Predicted time to 1 log10 decline in bacterial viability of 17.3 mo</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>Non-lipid formulation</td>
<td>-</td>
<td>8 weeks</td>
<td>Fell to the level of 10-20%†</td>
<td>[27]</td>
</tr>
<tr>
<td>Field studies</td>
<td>Variable temperature</td>
<td>Lipid formulation</td>
<td>BCG-Danish</td>
<td>3-5 weeks in a forest/pasture margin habitat</td>
<td>-</td>
<td>[50]</td>
</tr>
</tbody>
</table>

† A higher potency of BCG vaccine is maintained under lower temperature.
TABLE 3 Correlations between bait outcome recorded from each feeder and positive minutes in relation to the presence of the species detected by the cameras during the time in which baits were in the field. The table represents both positive and negative (inverse relationship) correlations between the presence of the different species and the number of intact baits (not touched), intact capsules (eaten bait but the vaccine within the capsule not ingested), chewed capsules (eaten bait but the capsule chewed and vaccine likely ingested) and lost baits (baits and capsules presumably eaten).

<table>
<thead>
<tr>
<th></th>
<th>Intact baits</th>
<th>Intact capsules</th>
<th>Chewed capsules</th>
<th>Lost baits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild boar</td>
<td>-0.361**</td>
<td>-0.018</td>
<td>0.429**</td>
<td>0.103</td>
</tr>
<tr>
<td>Wild boar inside</td>
<td>-0.496**</td>
<td>0.071</td>
<td>0.565**</td>
<td>0.132*</td>
</tr>
<tr>
<td>Birds</td>
<td>0.139*</td>
<td>-0.015</td>
<td>-0.208**</td>
<td>0.052</td>
</tr>
<tr>
<td>Other ungulates</td>
<td>0.027</td>
<td>-0.048</td>
<td>0.042</td>
<td>-0.071</td>
</tr>
<tr>
<td>Carnivores</td>
<td>-0.200**</td>
<td>0.067</td>
<td>-0.146*</td>
<td>0.364**</td>
</tr>
<tr>
<td>Others</td>
<td>-0.152*</td>
<td>0.061</td>
<td>0.101</td>
<td>0.133*</td>
</tr>
</tbody>
</table>

** The correlation is significant at 0.01 level (bilateral), * the correlation is significant at 0.05 level (bilateral).