Assessment of an Oral *Mycobacterium bovis* BCG Vaccine and an Inactivated *M. bovis* Preparation for Wild Boar in Terms of Adverse Reactions, Vaccine Strain Survival, and Uptake by Nontarget Species

Beatriz Beltrán-Beck,⁎ Beatriz Romero,⁎ Iker A. Sevilla, Jose A. Barasona, Joseba M. Garrido, David González-Barrio, Iratxe Díez-Delgado,⁎ Esmeralda Minguijón, Carmen Casal, Joaquín Vicente, Christian Gortázar,⁎ Alicia Aranaz

Sanidad y Biotecnología (SaBio), Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ciudad Real, Spain; Centro de Vigilancia Sanitaria Veterinaria (VISAVET)⁎ and Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain; NEIKER-Tecnalia, Animal Health Department, Derio, Spain

Wildlife vaccination is increasingly being considered as an option for tuberculosis control. We combined data from laboratory trials and an ongoing field trial to assess the risk of an oral *Mycobacterium bovis* 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 nontarget 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 postvaccination (*n* = 27)). No BCG excretion was detected in fecal samples (*n* = 162) or in urine or nasal, oral, or fecal swab samples at 258 days postvaccination (*n* = 29). In the field, we found no evidence of loss of BCG viability in baits collected after 36 h (temperature range, 11°C to 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 for all bird species. We found large proportions of chewed capsules (29%) (likely ingestion of the vaccine) and lost baits (39%) (presumably consumed), and the proportion of chewed capsules showed a positive correlation with the presence of wild boar. 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 strict timing of bait deployment.

**C**attle tuberculosis (TB), due mainly to *Mycobacterium bovis*, is a reemerging 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 Calmette-Guérin (BCG), an attenuated strain, has long been the only available vaccine (reviewed in reference 8). It has been evaluated for oral vaccination against tuberculosis in cattle, and it is increasingly being 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 (i) viability must be maintained until delivery and uptake and (ii) the consequent immune response must confer protection (10). In addition, key considerations in designing a vaccine bait delivery strategy are (i) adverse reactions and potential effects of high vaccine doses on the health of target animals, (ii) potential survival of *M. bovis* BCG in vaccinated individuals, (iii) potential excretion of *M. bovis* BCG by vaccinated animals, and (iv) vaccine-containing bait uptake by nontarget species (4).

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 the substrate (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).

BCG has been isolated at necropsy from tissues of vaccinated animals long after vaccination, with differences depending on the species, the route of administration, and the type of vaccine used (Table 1). In some cases, BCG has caused lesions in vaccinated but nonchallenged 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 environmental contamination. However, in a recent study of BCG-vaccinated white-tailed deer (*Odocoileus virginianus*) that shared alternatively the same pen as cattle, no transmission between the two species was evident (24). BCG could also present a risk of accidental exposure of nontarget scavengers through consumption of vaccinated prey.

Soil could present a risk of environmental contamination...
TABLE 1 *Mycobacterium bovis* BCG isolation at the time of necropsy in wild and domestic animals reported in the literature

<table>
<thead>
<tr>
<th>Species</th>
<th>BCG strain</th>
<th>Route(^a)</th>
<th>Dose</th>
<th>Tissue(s) with confirmed BCG isolation</th>
<th>Time after BCG vaccination</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>White-tailed deer (Odocoileus virginianus)</td>
<td>Danish</td>
<td>SC</td>
<td>10(^6) CFU</td>
<td>Superficial cervical, tracheobronchial, mediastinal, and hepatic lymph nodes</td>
<td>8 mo</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>SC</td>
<td>10(^7) CFU</td>
<td>Superficial cervical lymph nodes and lung</td>
<td>8 mo</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Danish</td>
<td>O</td>
<td>10(^8) CFU</td>
<td>Tonsils, lymph nodes (retropharyngeal, mediastinal, hepatic, and ileocecal), jejunum, and cecum</td>
<td>3 mo</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Danish, lipid-encapsulated BCG bait</td>
<td>O</td>
<td>10(^7) CFU</td>
<td>Tracheobronchial, hepatic, and mesenteric lymph nodes</td>
<td>9 mo</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Danish, liquid suspension</td>
<td>O</td>
<td>10(^9) CFU</td>
<td>Lymph nodes (head, thoracic, and abdominal pool)</td>
<td>12 mo</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymph nodes (head and thoracic pool)</td>
<td>9 mo</td>
<td>3</td>
</tr>
<tr>
<td>Red deer (Cervus elaphus)</td>
<td>Pasteur and BCG Pasteur recombinant strain</td>
<td>SC</td>
<td>10(^7) CFU</td>
<td>Lymphoid tissues (site of injection and draining lymph nodes)</td>
<td>3 mo(^b)</td>
<td>42</td>
</tr>
<tr>
<td>Possums (Trichosurus vulpecula)</td>
<td>Pasture, lipid-formulated</td>
<td>O</td>
<td>10(^8) CFU</td>
<td>Mesenteric lymph nodes and Peyer’s patches</td>
<td>3 wk to 2 mo</td>
<td>26</td>
</tr>
<tr>
<td>Badgers (Meles meles)</td>
<td>Pasture, lipid-formulated</td>
<td>O</td>
<td>10(^8) CFU</td>
<td>Cervical lymph nodes(^c)</td>
<td>7 mo</td>
<td>25</td>
</tr>
<tr>
<td>Mice</td>
<td>Danish</td>
<td>SC</td>
<td>10(^8) CFU</td>
<td>Inguinal lymph nodes, spleen, and lungs</td>
<td>5 mo (spleen)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>SC</td>
<td>10(^8) CFU</td>
<td>Spleen</td>
<td>7–8 mo</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Pasture</td>
<td>O</td>
<td>10(^8) CFU</td>
<td>Mesenteric lymph nodes</td>
<td>3 mo</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Pasture, lipid-encapsulated</td>
<td>O</td>
<td>10(^8) CFU</td>
<td>Mesenteric lymph nodes</td>
<td>7 mo</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>SC</td>
<td>7,000, 60 CFU</td>
<td>Ears, local draining (auricular) lymph nodes, and spleen</td>
<td>1 mo (skin) or 3 mo (lymph nodes)</td>
<td>44</td>
</tr>
<tr>
<td>Guinea pigs</td>
<td>BCG</td>
<td>IP</td>
<td>50 mg</td>
<td>Abscess of epididymis caused by inoculation</td>
<td>9–10 mo</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>IP</td>
<td>1 mg</td>
<td>Mesenteric lymph nodes</td>
<td>19 mo</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>SC</td>
<td>10 mg (4 × 10(^7) ± 8 × 10(^6) CFU/mg)</td>
<td>Site of inoculation and distant lymph nodes, spleen, liver, and lungs</td>
<td>6 mo</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
<td>O</td>
<td></td>
<td>Cervical and bronchial lymph nodes</td>
<td>2–6 days</td>
<td>48</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Pasteur</td>
<td>IV</td>
<td>1 mg</td>
<td>Mesenteric and tracheobronchial lymph nodes and occasionally spleen and kidney</td>
<td>14 mo</td>
<td>19</td>
</tr>
<tr>
<td>Primates (Macacus rhesus)</td>
<td>Pasteur</td>
<td>SC</td>
<td>2 doses of 50 mg with a 1-mo interval</td>
<td>Site of inoculation and eight vertebral glands</td>
<td>7 mo(^d)</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>IT</td>
<td>10 mg</td>
<td>Bronchial lymph nodes</td>
<td>6 mo</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>O</td>
<td>1,030 mg over 10 wk</td>
<td>Submaxillary, mesenteric, ileocecal, and colic lymph nodes and spleen</td>
<td>4 mo</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>IV</td>
<td>10 mg</td>
<td>Lung, bronchial lymph nodes and spleen</td>
<td>1 mo</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pasteur</td>
<td>EI</td>
<td>100 mg in four doses over 12 days</td>
<td>Submaxillary, submaxillary, and mesenteric lymph nodes</td>
<td>3 mo</td>
<td>49</td>
</tr>
</tbody>
</table>

\(^a\) SC, subcutaneous; O, oral; IP, intraperitoneal; IV, intravenous; IT, intratracheal; EI, eye instillation.

\(^b\) At the time of necropsy, at 14 weeks, BCG was eliminated by 50% of the animals, and only low levels of residual organisms persisted in the hosts.

\(^c\) Some of these badgers had concurrent infections with BCG and the *M. bovis* challenge strain in the affected tissue.

\(^d\) Numerous acid-fast bacilli were observed in the gland, but cultures were negative (apparently dead).

through BCG excretion in feces from vaccinated animals. The persistence of the vaccine in feces from captive wild animals has been confirmed in orally vaccinated possums and badgers (doses of $\geq10^8$ CFU of lipid-formulated BCG Pasteur) for up to 7 and 17 days postvaccination (p.v.), respectively (25, 26). One of 12 possum fecal samples collected after BCG ingestion and stored under conditions similar to those of a forest floor environment was cultured positive for up to 5 weeks (26).

BCG viability and stability are two important factors to consider in order to achieve good immunization (27). These are severe constraints when vaccinating wildlife orally in the field. Different studies have assessed the duration of BCG survival under 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 bait have been found to be highly palatable to different non-target wild and domestic animals (28); thus, strategies ensuring that only target species gain access to the 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 focused on immune responses in this species and the protection conferred after oral immunization with BCG and a prototype based on a heat-inactivated *M. bovis* preparation (4, 33, 34). Target animals for vaccination are 3- to 4-month-old piglets (28, 32) (an age usually achieved by early summer). The aim of this study was to assess, through data compiled from both published and unpublished studies, the potential risks of field deployment of orally administered 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 nontarget species.

**MATERIALS AND METHODS**

**Animal handling.** All of the experiments used handling procedures designed to reduce stress and health risks for subjects, according to European (Council Directive 86/609) and Spanish (Royal Decrees 223/1988 and 1021/2005) laws, and were approved by the institutions’ ethics committees.

**Bait-vaccine delivery system.** The baits had a hemispherical shape (3.4 by 1.6 cm) and were made with piglet feed, wheat flour, paraffin, sucrose, and cinnamon-truffle powder attractant, as described previously (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, i.e., BCG and a prototype killed *M. bovis* preparation. BCG Danish (CCUG strain 27863) was cultured in the laboratory, and the suspension turbidity was adjusted to a 1.0 McFarland standard. The BCG CFU values were calculated by plating aliquots of 10⁻¹ to 10⁻⁶ dilutions onto Coletsos medium in duplicate, as described previously (33, 34). Bacterial suspensions were cultured in solid and liquid media using the Bactec MGIT system (Becton, Dickinson, Sparks, MD), as described by Garrido et al. (34). The isolates resulting from positive cultures were further characterized by spoligotyping (37), which allows identification of *Mycobacterium tuberculosis* complex strains based on the 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 bacilli in fecal samples. In both experiments, animals were housed in two rooms in class 3 biocontainment facilities. Samples from three different points in each room were collected at different postvaccination times (first experiment [n = 24], days 1, 3, 5, and 7 p.v.; second experiment [n = 54], days 0, 1, 2, 3, 4, 10, 20, 30, and 40 p.v.) and postinfection (p.i.) times (first experiment [n = 24], days 8, 21, 42, and 71 p.i.; second experiment [n = 18], days 60, 80, and 100 p.i.). Moreover, individual fecal samples from each animal were taken the day of the necropsy (first experiment [n = 20], day 189 p.v.; second experiment [n = 22], day 258 p.v.). These samples were cultured 24 to 48 h after collection. For decontamination, 2 g of each fecal sample was homogenized with 38 ml of 0.75% hexadecylpyridinium solution and left for 18 h. After collection of the upper part of the sediment (homemade; Neiker, Derio, Spain) were inoculated with 4 drops each. The tubes were incubated at 37°C 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 also collect urine samples from 5 of the 8 BCG-vaccinated wild boar at necropsy. The urine specimens were 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 and tissue kit, according to the manufacturer’s protocol (Qiagen, Germany). Detection of *M. tuberculosis* complex DNA was performed

### Table 2: Stability studies of lipid- and non-lipid-formulated *Mycobacterium bovis* BCG under laboratory and field conditions at different temperatures

<table>
<thead>
<tr>
<th>Condition</th>
<th>Temperature (°C)</th>
<th>Formulation</th>
<th>Vaccine</th>
<th>Length of stability</th>
<th>Viability/potency</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient room</td>
<td>18–24</td>
<td>Lipid</td>
<td>BCG Danish</td>
<td>7 wk</td>
<td>Few viable <em>M. bovis</em> BCG isolates detected</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>10–25</td>
<td>Lipid</td>
<td>BCG Pasteur</td>
<td>8 wk</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Refrigerated</td>
<td>5</td>
<td>Nonlipid</td>
<td>BCG Pasteur</td>
<td>8 wk</td>
<td>Fell to level of 10–20%</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Lipid</td>
<td>BCG Pasteur</td>
<td>8 wk</td>
<td>Minimal loss of viability</td>
<td>51</td>
</tr>
<tr>
<td>Frozen</td>
<td>−20</td>
<td>Lipid</td>
<td>BCG Danish</td>
<td>8 mo</td>
<td>Predicted time to 1-log&lt;sub&gt;10&lt;/sub&gt; decline in bacterial viability of 17.3 mo</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>−20</td>
<td>Nonlipid</td>
<td>BCG Danish</td>
<td>8 wk</td>
<td>Fell to level of 10–20%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27</td>
</tr>
<tr>
<td>Field studies</td>
<td>Variable</td>
<td>Lipid</td>
<td>BCG Danish</td>
<td>3–5 wk in forest/pasture margin habitat</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> A higher potency of BCG vaccine is maintained at lower temperatures.
with an in-house real-time PCR assay detecting the MPB70 gene, including also an internal control (E. Castellanos-Rizaldos, S. Gómez, A. Aranaz, B. Romero, L. de Juan, L. Domínguez, and I. G. Fernández-de-Mera, unpublished data). Moreover, these samples were cultured in solid and liquid media using the Bactec MGIT system.

*M. bovis* BCG viability and bait uptake by nontarget species. To evaluate the viability of the BCG vaccine inserted into baits, we tested baits containing 10⁸ to 10⁹ 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. The time between preparation in the laboratory and deployment in the field was less than 24 h, and the temperature was kept at 4°C. First, in order to determine the effects of the temperatures 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 baits. In the first experiment, which was performed in late August 2012, one pool of baits (n = 20) from 8 selective piglet feeders was collected after being kept in the environment from 8:30 p.m. to 8:30 a.m. (12 h). Later, early in September 2012, baits from 4 selective piglet feeders were placed in the feeders at 8:30 p.m. and were collected after 12 h (n = 35), 24 h (n = 22), or 36 h (n = 18). Baits were collected from each zone, capsules were extracted, and their contents were pooled. This mixture, together with 10-fold serial dilutions, was cultured on Lowenstein-Jensen medium (Difco FSM, Madrid, Spain) in duplicate. CFU readings were taken after 8 weeks.

Environmental temperatures were monitored from 6 July 2012 to 6 September 2012 with 4 Microlite data loggers (Dostmann Electronic, Germany) set to record data every 30 minutes. Data loggers were placed at the bases of trees or shrubs located less than 2 meters from a selective piglet feeder, two at the presumably coolest points of the study sites and the other two at the presumably warmest (more sun-exposed) points, in order to record the broadest possible temperature range.

Moreover, BCG viability within the baits was tested under laboratory conditions exposing groups of four vaccine baits each to four different temperatures (4°C, 25°C, 37°C, and 42°C) for 24 h, 48 h, or 72 h. Bait contents (*M. bovis* BCG) and 10-fold serial dilutions were cultured in duplicate as described above. Data from both field and laboratory trials were analyzed by nonparametric Mann-Whitney U tests, using the SPSS 19.0 statistical package (IBM Corp., Somers, NY).

We used infrared radiation-triggered cameras (NightTrakker NT50 IR; Uway, Lethbridge, Canada) to assess bait uptake by target and nontarget species. Camera traps were set up to record three capture shots for 1 min and were fixed to posts or tree trunks focusing on the center of the selective piglet feeders (n = 46) in two different study sites (23 feeders in each site). We delivered a total of 8,280 vaccine baits; every night for 9 nights, 20 baits containing BCG and 20 baits containing the heat-inactivated *M. bovis* preparation were deployed in 23 feeders each (920 baits per night). At each feeder, baits were deployed at dusk and the unconsumed baits were collected the next morning, for destruction. The outcomes of the baits and vaccine capsules were classified as intact baits (untouched baits), consumed baits but untouched capsules with vaccine left in the feeder, consumed baits with chewed capsules (with likely ingestion of the vaccine), and “lost” baits (missing baits and capsules, presumably ingested) (Fig. 1). Cameras were kept over the entire length of the study (9 days). Picture details were processed by two independent researchers and were converted into Excel files (Microsoft Excel version 2007; Microsoft Corp.), recording the following variables regarding each feeder per day (from 8:00 p.m. to 8:00 p.m. 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 nonparametric 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, or body condition deterioration, were observed after BCG administration (n = 27). Also, no adverse reactions to the inactivated *M. bovis* prototype were recorded (n = 21), and the animals that received the inactivated vaccine via the parenteral route (n = 9) did not show swelling at the site of injection.

**Survival of M. bovis BCG.** Although specimens from at least 7 different tissues per animal were cultured in all of 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 tissue specimens 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.

**FIG 1** Examples of the outcomes of the baits and vaccine capsules in the field, i.e., consumed baits but untouched capsules with vaccine left (left), intact baits (center), and consumed baits with chewed capsules, indicating likely ingestion of vaccine (right).
Excretion of *M. bovis* BCG by vaccinated animals. After 16 weeks of incubation of fecal samples, no growth was observed in the inoculated culture medium from the antemortem and postmortem samples (*n* = 162) collected in the two experiments. The urine samples from 5 BCG-vaccinated wild boar and the nasal and fecal swabs from the 8 animals were negative at 258 days postvaccination. Oral swabs from 2 of the 8 animals were positive for *M. bovis* at 258 days p.v., but negative for BCG.

*M. bovis* BCG viability and bait uptake by nontarget species. Temperature data collected by the data loggers over 2 months revealed that the average temperature in the field sites from 6 July 2012 to 6 September 2012 was 21.97°C (mean ± standard deviation). Figure 2 shows the global average values and hourly maximum and minimum values. The maximum temperature exceeded 37°C from 11:00 a.m. to 7:00 p.m.

In August 2012, the temperature achieved an average of 24.51°C (range, 11.02°C to 41.32°C), with a minimum at 7:00 a.m. and a maximum at 1:00 p.m. In September 2012 (mean temperature, 22.18°C; range, 11.44°C to 40.06°C), baits were exposed to the minimum and maximum temperatures at 8:00 a.m. and 6:00 p.m., respectively. The BCG number was around 10⁵ CFU in both trials (mean, 5.6 × 10⁵ ± 1.7 × 10⁵ CFU [*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).

Under laboratory conditions, we recorded the BCG CFU that remained viable after being subjected to different temperatures. The initial bacterial counts (at room temperature) ranged from 5.1 × 10⁴ to 4.1 × 10⁵ CFU. The number of CFU remained quite stable at temperatures of 4°C and 25°C for 72 h. At 37°C and 42°C, however, the concentration began to decrease significantly after 24 h, to 5.3 × 10³ and 3.3 × 10³ CFU (*U* test, *z* = −2.309, *P* = 0.029, and *z* = −2.323, *P* = 0.029, respectively), until reaching final counts of 3.1 × 10⁰ and 3 × 10⁰ CFU, respectively, at 72 h.

Camera trapping data recorded a total of 13,504 PMP from all 46 feeders in the 9 days of the experiment. The proportions according to 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* = 3,103 PMP), of which 82.92% were piglets, 5.31% juveniles, and 11.78% adults that put their heads between the feeder bars. The bird presence is detailed in Table S1 in the supplemental material. The PMP proportions of the different carnivore species in relation to the total presence of carnivores inside the feeders (1.65%) were as follows: red fox (*Vulpes vulpes*), 57.02%; stone marten (*Martes foina*), 40.53%; badger, 1.75%; common genet (*Genetta genetta*), 0.88%. Red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and fallow deer (*Dama dama*) were observed only outside the feeders (1.65%), in proportions of 69%, 20%, and 11%, respectively.

Wild boar and birds entered the feeders at different times. Wild boar activity patterns were nocturnal, while diurnal activities were recorded for all bird species. Hourly averages of the total PMP from the 46 feeders showed that wild boar activity began about 7:00 p.m. and the peak was observed at 11:00 p.m. (Fig. 3).

Regarding bait consumption, we found large proportions of “lost” baits (39.3% ± 31.2%) and chewed capsules (29.2% ± 27.7%) in relation to total delivered baits, with 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 were confirmed unconsumed bait capsules (31.5% ± 37%). Intact baits and intact capsules were mostly found inside the feeders (baits, 98.64%; capsules, 78.13%).

Considering only the time in which the baits were in the field, we combined the data on the presence of the species detected by the cameras with the data on the types of baits found in the morning. The presence of wild boar, carnivores, and other species (lagomorphs and small rodents) was negatively correlated with the 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 to support eradication programs and to promote the health of wildlife populations (10). However, several issues related to protection against infection but also 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 were 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, as well as for ethical reasons, we 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 were *M. bovis* BCG Danish and a prototype killed mycobacterial preparation that is being tested for potential use as a vaccine (34; Beltrán-Beck and Gortázar, unpublished), 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 on the safety of wild boar vaccination against TB. The main results belong to two groups of risks, one regarding the consequences of the use of BCG in wild boar and one regarding bait deployment. First, there were no adverse reactions to *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. Second, although rates of BCG survival inside baits in the environment were higher than expected, rates of bait uptake by nontarget species were low and could 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 ongoing field trials will allow increases in the sample size and, we hope, confirmation of the available results. In the field, data on hunter-harvested orally vaccinated wild boar will 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 the results of previous bait deployment experiments (32). Selective feeders allow targeted delivery of oral baits to wild boar piglets, at the preferred age for vaccination (31). Furthermore, the use of this type of feeders could avoid the possibility of bait consumption by cattle, since their heads are unable to enter through the bars to reach the baits.

No adverse reactions to BCG or the heat-inactivated *M. bovis* preparation were observed in the wild boar used in the different experiments. This suggests that both preparations are safe for wild boar and most likely also for its domestic relative, the pig.

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**TABLE 3** Correlations between bait outcomes recorded from each feeder and positive minutes in relation to the presence of the species detected by the cameras during the time baits were in the field

<table>
<thead>
<tr>
<th>Species</th>
<th>Intact baits</th>
<th>Intact capsules</th>
<th>Chewed capsules</th>
<th>Lost capsules</th>
<th>Lost baits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild boar</td>
<td>-0.361&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.018</td>
<td>0.429&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.103&lt;sup&gt;&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Wild boar, inside</td>
<td>-0.496&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.071</td>
<td>0.565&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.132&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Birds</td>
<td>0.139&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.015</td>
<td>-0.208&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.052&lt;sup&gt;&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Other ungulates</td>
<td>0.027&lt;sup&gt;&lt;/sup&gt;</td>
<td>-0.048</td>
<td>0.042&lt;sup&gt;&lt;/sup&gt;</td>
<td>-0.071&lt;sup&gt;&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Carnivores</td>
<td>-0.206&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.067</td>
<td>-0.146&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.364&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>-0.152&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.061</td>
<td>0.101&lt;sup&gt;&lt;/sup&gt;</td>
<td>0.133&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Shown are both positive and negative (inverse relationship) correlations between the presence of the different species and the numbers of intact baits (not touched), intact capsules (bait eaten but the vaccine within the capsule not ingested), chewed capsules (bait eaten, capsule chewed, and the vaccine likely ingested), and lost baits (bait and capsules presumably eaten).

<sup>b</sup> The correlation was significant at the 0.01 level (bilateral).

<sup>c</sup> The correlation was significant at the 0.05 level (bilateral).
ing BCG, however, the absence of adverse reactions could be due in part to the medium-sized doses of vaccine used in our experiments (between 10³ and 10⁶ CFU). We used low doses in the experiments in the biocontainment facilities, to imitate those used in the field trials (dose in the field trials, 10⁵ CFU). Thus, even if an individual ingests several baits, it would be unlikely to consume doses higher than 10⁶ to 10⁷ CFU. At worst, to achieve a dose of 10⁸ CFU, the same individual would need to consume all of the baits (20 baits per feeder) in 6 different feeders for 9 nights, which is unlikely. Nevertheless, as mentioned in the introduction, even higher doses of BCG (10⁹ 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 175 to 300 days p.v.), and BCG was not detected in feces and swab samples obtained after 258 days p.v. Although we cannot exclude previous 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 under experimental conditions have shown that BCG shedding occurs only in low to moderate numbers and only for short periods of time (25, 26, 41). In wild boar, the lack of antemortem and postmortem 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); even if this were the case, it is still unknown what dose would actually infect nontarget animals after ingestion. For instance, it is believed that oral doses of BCG that could sensitize cattle would be near 10⁷ CFU (41). Such high doses are very unlikely to be excreted by wild boar vaccinated with doses below 10⁶ CFU.

Temperature stability was studied because bait deployment coincides with early summer, which is characterized by high temperatures in central-south Spain. In the laboratory, exposure to temperatures of 37°C and 42°C strongly reduced BCG viability by about 2 log units within 24 h. However, field viability was higher than expected, at least for 36 h after bait deployment, probably due to temperature fluctuations or effects of the soil temperature. This greater-than-expected stability in the field implies a logistic advantage for field vaccination, but it is a disadvantage regarding the possible access of nontarget 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 was correlated with the number of intact baits found (not consumed). This method of distributing the vaccine would also avoid exposure to temperatures above 37°C. Among the nocturnal nontarget species, the ones to be considered specifically are the carnivores. Foxes and stone martens were related to the bait losses, but their presence represented a much smaller percentage than that of wild boar. Although lagomorphs and rodents represent the lowest percentage of the presence of total species (1.07%), they were implicated in the bait losses and also the appearance of fewer intact baits in the feeders. In the case of the presence of other ungulates, the system of the selective piglet feeders prevents their access to the baits almost completely.

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 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.

ACKNOWLEDGMENTS

This is a contribution to Plan Nacional I+D+i AGL2011-30041 from the Ministerio de Economía y Competitividad (MINECO), Spain, and FEDER and to EU FP7 grant WildTBvac. B.B.-B. and I.D.-D. were supported by MINECO grants BES-2009-017401 and BES-2012-052490, respectively. I.A.B. was supported by MICINN grant FPU12/00980.

We are grateful to the mycobacterial unit staff for their technical support. We thank authorities in the Spanish Ministry of Agriculture, Food, and Environment and Junta de Comunidades de Castilla La Mancha for their continuous encouragement.

B.B.-B., B.R., A.A., J.M.G., J.A.B., and C.G. conceived, designed, and coordinated the study. B.R., I.A.S., J.M.G., E.M., and C.C. performed microbiological analyses and prepared the vaccines. B.B.-B., D.G.-B., I.D.-D., J.A.B., and J.V. carried out the field trials. All authors contributed in necropsies of the animals. B.B.-B. wrote the first draft, and all authors revised the drafts and approved the final version for submission.

We have no potential conflicts of interest to report.

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