Assessment of safety and interferon gamma responses of *Mycobacterium bovis* BCG vaccine in goat kids and milking goats

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Abstract

Vaccination of domestic animals has emerged as an alternative long-term strategy for the control of tuberculosis (TB). A trial under field conditions was conducted in a TB-free goat herd to assess the safety of the *Mycobacterium bovis* BCG vaccine. Eleven kids and 10 milking goats were vaccinated with BCG. Bacterial shedding and interferon gamma (IFN- γ) responses were monitored throughout the study. Comprehensive pathological examination and mycobacterial culture of target tissues were performed.

BCG vaccine strain was only isolated from the draining lymph node of the injection site of a kid euthanized at week 8 post-vaccination. The remaining animals were euthanized at week 24. Six out of 20 showed small granulomas at the injection site. BCG shedding was not detected in either faeces or in milk throughout the study. All vaccinated kids showed BCG-induced IFN- γ responses at week 8 post-vaccination.

BCG vaccination of goats showed no lack of biological safety for the animals, environment and public health, and local adverse reactions were negligible.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) organisms, which affects a wide range of animals. Caprine TB, mainly caused by *M. bovis* and *M. caprae*, is responsible for economic losses in endemic areas due to trade limitations and to depopulation of positive herds, when the eradication strategies fail [1,2]. Furthermore, recent reports evidenced that infected goats may be a source of TB in cattle [3,4].

In high-prevalence areas, the eradication strategy of caprine TB based on test and slaughter may pose important economic costs for the producers and the public sector. Therefore, vaccination has been suggested as an option for reducing the prevalence prior to start eradication programs [5]. Nowadays, the *M. bovis* bacillus Calmette-Guerin (BCG), an attenuated strain of *M. bovis*, is the only available vaccine against TB. A large number of studies since the early 20th century have demonstrated very few adverse reactions either in humans or animals [6]. Even though the safety of BCG vaccine is already extensively guaranteed for both public health and the environment, comprehensive safety studies are required to allow acceptance by regulators in domestic species [5]. Vaccination of cattle against TB with BCG is currently banned in EU countries, mainly because it may compromise the specificity of standard tuberculin-based diagnostic tests. Nevertheless, it is not explicitly forbidden in other species and a BCG vaccine has been licensed for use in badgers in the UK.

Trials under field conditions provide sufficient data regarding the adverse reactions and the potential shedding or survival of BCG in tissues, in order to assess the risk of meat and dairy products from vaccinated animals [7]. As such, this is the first study under field conditions with the aim to assess the safety by evaluating the injection site as well as bacilli excretion via fecal and milk routes after subcutaneous BCG vaccination of kids and milking goats.

2. Materials and Methods

2.1. Herd and experimental animals

Experiments were conducted in a milking goat herd from the experimental farm of the Autonomous University of Barcelona (Catalonia, Spain). The entire goat herd was first confirmed as negative to TB by the interferon gamma (IFN- γ) release assay (BovigamTM, Thermo Fisher Scientific, Schlieren, Switzerland) prior to carry out the study. Twenty-two Murciano-Granadina goat kids, aging 2 month old (10 to 13 kg), and 10 adult milking goats, were selected for the study. The 22 kids were randomly divided into two experimental groups of 11 individuals each and were housed separated by a fence, which allowed the direct animal-to-animal contact. The adult milking goats were maintained together with the rest of the herd throughout the experiment.

All animal experimental procedures were undertaken in accordance with the European Union Laws for protection of experimental animals (86/609), with experimental procedures approved by the Animal Research Ethic Commission of the *Generalitat de Catalunya* and the Biosecurity Committee of the *Universitat Autònoma de Barcelona*.

2.2. Vaccination schedule

The BCG vaccine inoculum was prepared as previously described [8]. Briefly, *M. bovis* BCG Danish strain (ATCC, Ref. 35733TM) was sub-cultured in Middlebrook 7H9 media (BD Diagnostics, Sparks MD, USA) and subsequent growth culture was diluted to 10⁶ CFU/ml by suspension in sterile PBS (Sigma-Aldrich). A single dose of 0.5 ml of this suspension (5 × 10⁵ CFU) was administrated subcutaneously (neck, left side; 2.5 cm needle length) in the 10 adult milking-goats and a group of eleven kids, whereas the other 11 kids remained as unvaccinated controls. Experimental animals were observed for clinical signs after vaccination and throughout the experiment.

2.3. Adverse reactions

All goats were daily observed for local and general adverse reactions to BCG vaccination. Clinical signs in the inoculation site (skin induration, fibrosis and nodulations) were recorded. Pain and/or inflammation of the main draining lymph node (LN), i.e. left pre-scapular LN, were also recorded.

2.4. *M. bovis* BCG excretion

(i) Milk: Fresh milk samples (20 to 30 ml) were collected from each milking goat at days 0, 3, 7, 14, 21, 28, and then every two weeks until week 24 post-vaccination for bacterial isolation. Samples were centrifuged at 1800 g for 15 min. The pellet was washed twice with PBS and then suspended in 1 ml of PBS. The suspension was cultured in Löwenstein-Jensen with pyruvate and Coletsos media (bioMérieux España, Madrid, Spain). Cultures were incubated at 37 °C for 90 days before being considered negatives.

(ii) Faeces: Faecal samples were directly collected from each goat kid at days 0, 7, 14, 21, 28, 56, 112 and 168 post-vaccination and were homogenized, decontaminated and cultured in Löwenstein-Jensen with pyruvate and Coletsos media following the procedure previously published [9].

Virulent *M. bovis* and BCG reference strains were used as positive controls in each culture session. Isolates were identified as BCG by multiplex PCR, using two pairs of oligonucleotide primers previously described [10]. These two pair of primers are specific for the DNA sequences of the RD1 region (absent in the BCG genome) and the MPB70 protein of the MTBC (which includes BCG), respectively.

2.5. Necropsy and tissue samples collection

Vaccinated animals were euthanized by intravenous sodium pentobarbital overdose at day 168 (week 24). At necropsy, a comprehensive examination for local (injection site and draining lymph node) and systemic presence of granulomatous lesions was conducted. A fragment of the injection site was processed for histopathology (Haematoxylin-Eosin and Ziehl-Neelsen stainings) when a granuloma was macroscopically observed. In kids, the injection site (when a lesion was detected), the regional draining LN (left pre-scapular LN), a respiratory LN (caudal mediastinal LN) and a fragment of the spleen were aseptically removed, weighted and processed for mycobacterial culture.

2.6. Bacterial culture of tissue samples

All tissue samples were homogenized, decontaminated and 10-fold dilutions were plated in 7H11 medium as previously described [11]. Plates were incubated at 37 °C for 28 days. Afterwards, CFUs were counted and bacterial load was measured as CFU per g of tissue.

2.7. Whole blood IFN- γ assays

All goats were bled prior to vaccination (week 0) and at the end of the experiment (week 24). Kids were additionally bled at weeks 8 and 16. Blood was collected from the jugular vein in 10 ml heparinized tubes.

The 22 goat kids were sampled at week 0, 8, 16 and 24. Whole blood samples from each animal were stimulated in 96-deepwell cell culture plates (Eppendorf Ibérica, Madrid, Spain) with *M. avium* and *M. bovis* tuberculins (PPD-A and PPD-B, respectively; CZV, Porriño, Spain), both used at a final concentration of 20 μ g/ml; peptide cocktails Prionics™ PC-HP (based on ESAT-6, CFP-10 and 4 additional mycobacterial antigens) and Prionics™ PC-EC (based on ESAT-6 and CFP-10), used at the manufacturer recommended concentration; and E/C, a mixture 1:1 of recombinant proteins ESAT-6 and CFP-10 (Lionex, Braunschweig, Germany; used at a final concentration of 10 μ g/ml). Blood samples stimulated with PBS or pokeweed mitogen (PWM;

Sigma-Aldrich, Steinheim, Germany; at 10 µg/ml) were used as negative and positive controls, respectively. The results interpretation for the standard IFN-γ assay (PPD-B and PPD-A) and the 3 additional IFN-γ assays (PC-HP, PC-EC and E/C) was conducted as previously described [12].

2.8. Data analysis

Non-parametric Wilcoxon test was used to compare IFN-γ responses between different time points within the vaccinated group. Significance was set at *P* value < 0.05. Statistical analysis was performed with R package v2.15.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Local and systemic adverse reactions

No adverse reactions were detected in vivo in the studied animals by means of visual inspection or palpation in the injection site or pre-scapular LN throughout the study. A goat kid was euthanized at day 48 on welfare grounds (severe clinical enteritis), and was subsequently necropsied. Fatal dissemination of BCG was discarded. A plate-like lesion was found in the injection site. Histopathology revealed a granulomatous infiltrate with presence of multinucleated giant cells and acid-fast bacilli (Fig. 1A-B). The rest of experimental animals (10 young and 10 milking goats) were necropsied at the end of the experiment (week 24). A young goat and 5 adult milking goats showed small subcutaneous granulomas at the injection site (Fig. 1C-D). Acid-fast bacilli were detected in four of these lesions (Fig. 1E). All post-mortem results are shown in Table 1.

3.2. *M. bovis* BCG isolation

MTBC was not isolated from any milk or faecal sample throughout the experiment. A positive culture for *M. bovis* BCG was obtained from the left pre-scapular LN (2×10^2 UFC/g) of the kid

163 euthanized at week 8. No isolations were obtained from tissue samples of the rest of the
164 animals at the end of the experiment (week 24, Table 1).

165 3.3. *IFN- γ responses to vaccination*

166 All vaccinated kids were positive to the standard IFN- γ assay at week 8 (10 out of 10). Eight and
167 7 kids remained positive at weeks 16 and 24, respectively. One of them was also positive to the
168 PC-HP specific IFN- γ assay at week 24. The rest of the tests in vaccinated kids as well as all the
169 tests in unvaccinated kids were negative.

170 In quantitative terms, a peak-up of PPD-B specific IFN- γ responses was detected in vaccinated
171 kids at week 8 (Fig. 2), followed by a significant decrease at week 16 ($P = 0.043$). A mild, but
172 not statistically significant decrease, was found between weeks 16 and 24 ($P = 0.358$).

173 4. Discussion

174 In the last two decades, considerable efforts have been allocated to assess BCG safety and
175 efficacy for its potential application in cattle, as well as in wildlife species such as Eurasian
176 badgers, brushtail possums, white-tailed deer or Eurasian wild boar [13]. The efficacy of BCG
177 vaccination to reduce pulmonary bacterial burden and to avoid extra-pulmonary spread of the
178 infection in experimentally infected goats has also been previously demonstrated by our
179 research group [8,14]. Herein, we have addressed aspects concerning the safety and IFN- γ
180 responses of BCG Danish strain vaccination in the domestic goat by carrying out a trial under
181 field conditions in a goat flock.

182 BCG shedding in milk and to the environment should be addressed to allow risk assessment for
183 consumer products from vaccinated animals. The present study was conducted taking into
184 account the principles of the European regulation for veterinary medicinal products
185 (Commission Directive 2009/9/EC) and the recommendations of EFSA for cattle vaccination [7],

to confirm the previously published results obtained in laboratory conditions [8] and, in addition, to provide further safety data regarding the potential excretion of BCG via milk and feces after vaccination, as well as information concerning the local survival of the vaccine strain at the injection site and its potential systemic dissemination in the host.

Our results did not show evidences of BCG shedding or animal-to-animal transmission, although additional large scale studies are required to confirm the lack of BCG excretion to the environment. Adverse reactions were circumscribed to the injection site, similar to that found in analogous safety studies with inactivated mycobacterial vaccines, where goats showed focal lesions in the injection site and the draining LN [12,15]. In the present study, BCG was only isolated from the injection site draining LN in the kid euthanized at week 8. It was not isolated in any of the remaining animals euthanized at week 24. Additionally, the granulomas of the injection site, when present, were almost completely reabsorbed (less than 1 cm in diameter).

The persistence of BCG within the host tissues for a period might be necessary for development of an effective immune response but poses a safety concern in animals that may be consumed for food. Even though transient tissue colonization cannot be excluded, this study suggests a complete removal of viable bacilli occurs before 24 weeks after vaccination. According to this, other BCG safety studies conducted in wild boars confirmed the absence of BCG in key tissues at 6 month after vaccination [9]. On the other hand, in studies carried out in deer, BCG has also been primarily detected in the injection site and the draining LN after subcutaneous vaccination [16,17], although, in some animals, BCG was later isolated from hepatic, pulmonary or mesenteric LN as far as 9 months after vaccination, suggesting an hematic or lymphatic dissemination [17]. However, all data indicate a low potential risk for human exposure to BCG through meat consumption, especially if we consider that lymphoid organs are not generally consumed and that the food cooking should kill the mycobacteria.

With regard to the immunological tests assessment, our results show that BCG vaccination of goats compromise the specificity of standard IFN- γ assay. Even after 24 weeks, 70% of vaccinated kids still responded positively to the test. This is not surprising, as most antigens of PPD-B are also present in *M. bovis* BCG [18]. In accordance to this, positive IFN- γ responses were consistently detected in cattle for more than 20 weeks post-vaccination [19]. The compromise of the specificity of the standard tuberculin-based diagnostic tests is the main reason to maintain the prohibition of BCG and mycobacterial vaccines in cattle in the EU. Similarly, a compromise of the IFN- γ assay specificity has also been reported in paratuberculosis vaccinated goat flocks [20]. Therefore, the use of reagents with higher specificity has been addressed in this study. A 100% diagnostic specificity of the IFN- γ assay was found in vaccinated animals when using either the peptide cocktail PC-EC or the whole antigen mixture rESAT-6/rCFP-10 (both reagents based on the proteins ESAT-6 and CFP-10, that are absent in the BCG strains), whereas 29 out of 30 tests (97%) were also negative when using the peptide cocktail PC-HP. Thus, these reagents may be optimal candidates for use in BCG vaccination campaigns. Notwithstanding, their sensitivity must be addressed in additional field trials.

The fact that none of the unvaccinated kids showed detectable antigen-specific IFN- γ responses, points to the absence of animal-to-animal transmission of BCG. This finding is in consistency with the non-detection of BCG neither in fecal samples nor in respiratory tissues.

5. Conclusions

The results indicate that BCG vaccination of goats is safe for both public health and the environment, and poses minimal adverse reactions. Nevertheless, as kids for meat consumption are slaughtered at 30-40 days of life, a vaccination strategy restricted to kids destined for breeding could be implemented to minimize the risk of BCG survival in meat. In

addition, the use of novel specific antigens, able to distinguish infected from vaccinated goats, improves the specificity of diagnostic tests. These new diagnostic reagents will be required for vaccination campaigns and should be further studied in field trials.

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TABLES

Table 1. Post-mortem findings after BCG vaccination

Animals	N	Time after BCG vaccination	No. of animals with granulomatous lesions ^a				BCG isolation
			Injection site	Left pre-scapular LN ^b	Mediastinal LN	Spleen	
Kid	1	8 weeks	1/1 (1) ^c	0/1	0/1	0/1	1/1 ^d
Kids	10	24 weeks	1/10 (1)	0/10	0/10	0/10	0/10
Adult goats	10	24 weeks	5/10 (3)	0/10	ND	ND	ND

^a Confirmed by histopathology (Haematoxylin and eosin staining).

^b Injection site draining LN.

^c No. of Ziehl-Neelsen staining positive lesions are shown in brackets.

^d Isolated from both the left pre-scapular LN.

FIGURE CAPTIONS

Fig. 1. Adverse reactions found at the vaccine injection site. (A) A plaque-like lesion measuring 4 x 2.5 x 0.5 cm was found between the cervical superficial muscles of the kid euthanased 8 weeks after vaccination. (B) Histologically, multiple coalescing foci of encapsulated granulomatous infiltrates were observed surrounded by a mononuclear inflammatory infiltrate. Minimal necrosis and mineralization areas were observed. Ziehl Neelsen stain evidenced acid-fast bacilli within the lesions (inset). (C) Much smaller subcutaneous lesion, less than 1 cm in diameter, found in a young goat euthanased 24 weeks after vaccination. (D) Microscopically well encapsulated, mineralized, necrotic lesions were observed. (E) Surrounded by a layer of macrophages, multinucleated giant cells and lymphocytes. Ziehl Neelsen stain evidenced acid-fast bacilli within the lesions (inset).

Fig. 2. Antigen-specific IFN- γ responses after BCG vaccination of kids. The IFN- γ release was measured by ELISA after the stimulation of whole blood of each animal with 4 antigenic regents prior and after 8, 16 and 24 weeks of the BCG vaccination. (A) *M. bovis* PPD (PPD-B). (B) Peptide cocktail PC-HP. (C) Peptide cocktail PC-EC. (D) Antigenic mixture of recombinant proteins rESAT-6/rCFP-10. The results are expressed as individual Δ OD at 450nm (OD450nm of antigen-stimulated wells minus OD450nm of non-stimulated wells). Dashed horizontal line indicates the cut-off point for positivity (Δ OD450nm $\geq$ 0.05).

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Figures