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Role of environmental mycobacteria in the efficacy of the BCG vaccine against cattle tuberculosis

Papel de las micobacterias ambientales sobre la eficacia de la vacuna contra la tuberculosis bovina

González-Ruiz, S.[1]* , Bárcenas-Reyes, I. [1] , Contreras-Magallanes, Y.G. [2] , Sosa-Gallegos, S.L. [1] , Santillán-Flores, M.A. [3] , Milián-Suazo, F. [1]

[1] *Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro. Av. De las Ciencias S/N, Juriquilla, Delegación Santa Rosa Jáuregui, C.P. 76230. Querétaro, Qro.*
[2] *Facultad de Medicina Veterinaria y Zootecnia Campus II, Universidad Autónoma de Chiapas.*
[3] *Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP).*

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ABSTRACT

Bovine tuberculosis is an infectious disease that causes severe direct and indirect economic losses, due to restrictions on the movement and marketing of live animals and animal products. In Mexico, disease control is based on “test and slaughter,” where animals reactive to the tuberculin tests are sent to slaughter. An alternative for controlling this disease is vaccination with the BCG strain. Under experimental conditions, this vaccine has shown efficacy to reduce infection and disease dissemination, with an efficacy of about 60 %; however, there are some concerns about environmental mycobacteria masking this efficacy. The objective of this study was to rule out the influence of these mycobacteria by detecting IFN-γ in response to the antigens PPD-*bovis* and PPD-*avium*. Using blood samples from animals vaccinated and animals unvaccinated with the BCG, and the challenged with a field strain of *M. bovis*, the overall average of IFN- response was significantly higher in the stimulation with PPD-*bovis*. We conclude that environmental mycobacteria do not affect the efficacy of BCG in reducing infection in vaccinated cattle.

KEY WORDS: Bovine tuberculosis, vaccination, goats, IFN-γ, mycobacteria.

***Corresponding Author:**

Sara González-Ruiz. Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro. Av. de las Ciencias s/n; Juriquilla, Delegación Santa Rosa Jáuregui, C.P. 76230, Querétaro, Qro., México. Teléfono: (442) 192 1200 ext. 5360.
E-mail: sara.gonzalez@uaq.mx

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RESUMEN

La tuberculosis bovina es una enfermedad infecto-contagiosa, que ocasiona severas pérdidas económicas, directas e indirectas, como limitaciones en la movilización y comercialización de animales y sus productos. En México, el control de la enfermedad se basa en la "prueba y sacrificio", donde los animales reactores son enviados a rastro. Una alternativa para el control es la vacunación con BCG, la cual ya se ha probado en condiciones experimentales, demostrando una eficacia mayor al 60 %; sin embargo, existe la duda de que micobacterias ambientales enmascaran dicha eficacia. El objetivo de este trabajo fue descartar la presencia de micobacterias ambientales a través de la detección de IFN-γ en respuesta a los antígenos PPD-*bovis* y PPD-*avium*. A partir de muestras sanguíneas de animales vacunados y no vacunados con BCG-Phipps, y posteriormente desafiados a una cepa de campo de *M. bovis*, se obtuvo un promedio general de respuesta de IFN-γ, el cual fue significativamente superior cuando el estímulo se hizo con PPD-*bovis*, en comparación a la estimulación con PPD-*avium*, por lo que se concluye que las micobacterias ambientales no tienen influencia sobre la respuesta a la vacunación.

PALABRAS CLAVE: Tuberculosis bovina, vacunación, cabras, IFN-γ, micobacterias.

Introduction

Bovine tuberculosis (BTb) is a chronic infectious disease found worldwide that affects several domestic and wild animal species. It is caused by *Mycobacterium bovis* (*M. bovis*), a member of the *Mycobacterium tuberculosis* complex (Taye *et al*., 2021). In addition to the severe economic losses it causes to the livestock industry, it poses a risk to public health; between 3 and 10 % of cases of human tuberculosis are caused by *M. bovis* (World Health Organization, 2020).

The cattle inventory in Mexico is approximately 30 million head, 91 % are for meat and dual-purpose production, and the remaining 9 % (2,700,000 head) are for milk production. Until December 2024, tuberculosis control in Mexico was based on a "test and slaughter" strategy, which had been successful in beef cattle, where the prevalence of the disease is <0.5 % (Rojas-Martínez *et al*., 2021), but with little success in dairy cattle, where the prevalence rate is greater than 0.5 % (range 0.1–14.2 %) (Perera-Ortíz *et al*., 2021; SENASICA, 2024). A possible explanation for this difference is that while beef cattle producers have the economic incentive to keep their herds clean to export live animals to the United States (US), dairy producers do not export live animals, and most of the milk they produce goes to pasteurization plants; in addition, the epidemiological performance of the intradermal caudal fold test is poor: sensitivity from 65.6 to 72 % and specificity of 68 to 98.6 % (Rivera *et al*., 2009; Klepp *et al*., 2019). However, for public health and economic

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reasons, the eradication of BTb in Mexico is an imperative task. Therefore, to control the disease, a legal agreement was recently established for the operation of the National Campaign against Bovine Tuberculosis (*Mycobacterium bovis*). In this agreement modern complementary diagnostic tests are included, such as the *in vitro* IFN-γ test, which distinguishes positive animals by means of the cellular release of IFN-γ, specific against *M. bovis* antigen in plasma, and PCR. Non-tuberculous mycobacteria (NTM) normally inhabit soil and water; there are more than 200 species, meaning that both animals and humans are frequently exposed to them from the moment we encounter the outside environment (Falkinham, 2022).

One strategy to reduce the prevalence of BTb in dairy cattle that has been interesting to producers is the incorporation of the BCG vaccine into the current control program. Bacillus Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* that has been used in humans for over 100 years, proving effective in protecting against severe forms of the disease (Okafor *et al*., 2023). After 230 subcultures, a live attenuated strain that did not cause disease when inoculated into animals was obtained, and a variant genetically distant from its pathogenic ancestor was generated. This attenuation is attributed to the loss of Differential Region 1 (RD1) (Brosch *et al*., 2007). BCG has been experimentally tested for potential use in different animal species, determining that low doses ($1x10^4$-$1x10^6$) are more effective than high doses ($1x10^8$) (Buddle *et al*., 2018), that the BCG strain used does not make much difference in efficacy (Srinivasan *et al*., 2021) and that vaccinating one-week-old animal provides better results than vaccinating 4-6-month-old calves or adults (Hope *et al*., 2005). Similarly, vaccine efficacy has been reported to be 67 % on average, based on lesion counts and lesion sizes in vaccinated versus unvaccinated animals, as well as the number of viable bacilli per lesion (Vordermeier *et al*., 2002). In addition, vaccination has been shown to be a valuable tool for controlling the disease in populations where the “test and slaughter” strategy is unfeasible due to the costs involved (Fromsa *et al*., 2024).

Our group has shown that primary vaccination with the BCG-Phipps strain, boosted with a purified protein extract (EPP) was 83 % effective in reducing the number of lesions, the size of the lesions, and the number of bacilli per lesion in vaccinated animals compared to unvaccinated animals (Canto-Alarcón *et al*., 2013). In addition, it was found to be equally effective in heifers and entirely harmless for pregnant heifers (Milian-Suazo *et al*., 2011). It has also been determined that the use of boosters with EPP improves protection (De Lisle *et al*., 1999; Young *et al*., 2007). Several vaccination studies in cattle have reported that sensitization of calves to environmental mycobacteria prior to vaccination negatively affects the protective efficacy of BCG, as it can cause cross-reactive immune responses, blocking the efficacy of the vaccine (Buddle *et al*., 2002, Skinner *et al*., 2003, Buddle, 2010, Parlane & Buddle, 2015). Furthermore, lacking the Differential Region 1 (RD1), the BCG vaccine does not induce an IFN-γ response to the CFP-10 and ESAT-6 antigens, allowing its use in differential diagnosis (Denis *et al*., 2007; Sopp *et al*., 2008).

The official *in vivo* test for diagnosing BTb is the double-comparative intradermal tuberculin test, which uses a purified protein derivative of *M. bovis* (PPD-*bovis*) and *M. avium* (PPD-*avium*). The use of these two antigens is necessary to rule out the possible interference of environmental mycobacteria, such as *M. avium*, *M. paratuberculosis*, or atypical mycobacteria,

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which could result in false-positive animals (OMSA, 2019). In general, an animal infected with *M. bovis* has lymphocytes in its blood that recognize its own antigens in PPD-*bovis*, resulting in an INF-γ response that can be detected by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (Sánchez-López *et al*., 2017). In experimental studies with vaccines for BTb, INF-γ is considered the best marker of immune response and vaccine efficacy; however, there is always doubt as to whether the release of INF-γ is due to exposure to the vaccine or exposure to environmental bacteria. Therefore, to rule out the latter, it is necessary to incorporate the antigen PPD-*avium* to the test (Díaz-Otero *et al*., 2008; Borham *et al*., 2022). Therefore, this study compares, through *in vitro* testing of INF-γ (Bovigam), the response of vaccinated animals to *M. bovis* (vaccine antigen) and *M. avium* (antigen representative of environmental mycobacteria) in goats immunized with the strain BCG-Phipps and challenged with a field strain of *M. bovis*.

Materials y Methods

Experimental animals

The experiment was conducted as described by Contreras-Magallanes *et al*. (2021). In summary, the size of the animal groups was determined by resource availability, included 35 goats aged three to five months, crossbreed between Alpine and Nubian, confirmed free of tuberculosis with the tuberculin and ELISA-IFN-γ (Bovigam) tests, and free of paratuberculosis with the ID Screen® diagnostic kit for serum and plasma samples (LABGENE Scientific SA, Châtel-Saint-Denis, Switzerland). The animals were randomly divided into five groups of seven animals each and placed in pens specifically prepared for the experiment, with sufficient space, shade, water, and food. The study was conducted in strict accordance with recommendations of the Guide for the Care and Use of Animals of the Autonomous University of Queretaro, Mexico. The Animal Experimentation Ethics Committee of the Autonomous University of Queretaro approved the protocol (Protocol Number: 20FCN2019).

Vaccination, booster, and challenge

The details are provided in Contreras-Magallanes *et al*. (2021). In summary, the experimental animals were randomly assigned to treatments in a completely randomized design. The vaccine was administered subcutaneously into the neck. The treatments were: 1) unvaccinated control group, 2) vaccination with$1x10^3$ CFU of BCG, 3) vaccination with$1x10^3$ CFU of BCG with EPP booster (720 µg/mL) one month after primary vaccination, 4) vaccination with $1x10^2$ CFU of BCG encapsulated in chitosan with EPP (720 µg/mL) booster one month after primary vaccination + PLGA (polylactic-co-glycolic acid), and 5) vaccination with $1x10^3$ CFU of BCG plus booster with EPP 720 (µg/mL) + chitosan. The adjuvant Montanide (MontanideTM) was added to all treatments. Two months after primary vaccination, all animals were challenged with a field strain of *M. bovis* and euthanized seven months after the start of the experiment.

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Blood sampling and antigen stimulation

As described by Contreras-Magallanes *et al*. (2021). In summary, blood samples for the determination of IFN-γ were collected every two to three weeks until week 30, with a total of 13 samples. Blood was collected from the jugular vein into heparinized vacutainer tubes. For the test, 750 µl of whole blood from each animal was used, which was incubated in microplates with 50 µl of PPD-*bovis* at a concentration of 0.9 to 1.1 mg/ml and 50 µl of PPD-*avium* at a concentration of 0.45 to 0.55 mg/ml. PBS was used as a negative control, and 50 µl of pokeweed mitogen at a concentration of 1 mg/mL (Sigma-Aldrich, Gillingham, UK) was used as a positive control. The microplates were incubated in a humidifier with 5 % CO_2 at 37°C for 20 hours. The optical densities (OD) of the PBS negative control were used to normalize the ELISA readings to calculate the optical densities of each antigen. Thus, the final OD values were obtained by subtracting the readings from the PBS samples. The concentration of IFN-γ in blood *in vitro* was determined over 30 weeks (with a total of 16 samples) using the commercial kit [Bovine IFN-γ microplate Enzyme-Linked Immunosorbent Assay kit (ELISA; Bovigam®; Prionics AG, USA)].

Statistical analysis

An analysis of variance (ANOVA) was used to compare the average concentration of IFN-γ in blood (OD in raw data) per experimental group for each sampling period. The IFN- concentrations in response to PPD-*bovis* and PPD-*avium* stimulation were compared using Student's t-test. A confidence level of 95 % was used in all analyses. All statistical analyses were performed using SPSS version 22.

Results and Discussion

IFN-γ response to PPD-*bovis* in blood. The concentration of IFN-γ in blood is considered one of the best indicators of BCG vaccine efficacy in animal models (Rothel *et al*., 1992; Vordermeier *et al*., 2002). In our study, the concentration of IFN-γ, measured by optical density (OD) at 450 nm in an ELISA with the PPD-*bovis* antigen, is shown in Table 1. No significant differences were observed between the experimental groups in samples one, three, and six ($p > 0.05$); however, the control group consistently showed a lower concentration of IFN-γ than the vaccinated groups (two to five). Similarly, at the end of the experiment (weeks 24, 28, and 30), no significant differences were detected between groups ($p > 0.05$).

In contrast, between weeks eight and 22, significant differences ($p < 0.05$) were observed between the vaccinated and control groups, consistent with previous reports highlighting that BCG vaccination induces an IFN-γ mediated by cellular immune response against *M. bovis* (Buddle *et al*., 2005; Buddle *et al*., 1995). The maximum peak concentration of IFN-γ was reached in week 20 (sampling nine) for most groups, except for group two, which reached its maximum in week 18, that is, one sampling earlier. It should be noted that five weeks after challenge (week 18), group two, vaccinated only with the BCG strain, showed the highest IFN-γ response, while the control

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group showed the lowest. These results are consistent with those reported by Vordermeier *et al*. (2002), who demonstrated that animals vaccinated with BCG had a more robust IFN-γ response than unvaccinated animals. Similarly, Hope *et al*. (2005) reported that IFN-γ peaks usually occur a few weeks after experimental challenge, which is consistent with the findings of this study. However, our results differ partially from those reported by Skinner *et al*. (2003), who observed that prior exposure to environmental mycobacteria could reduce the magnitude of the IFN-γ response; in our case, the involvement of environmental mycobacteria appears to have been minimal, as the vaccinated groups maintained significant differences from the control group throughout the experiment.

Finally, all groups returned to baseline levels by sampling 13 (week 30), which is consistent with previous observations that the IFN- response tends to decline over time, even in the presence of vaccination (Buddle *et al*., 2002). This pattern underscores the need to consider booster schedules or combined strategies to prolong the protective response in bovine tuberculosis vaccination campaigns (Figure 1).

Table 1. IFN-γ concentration in experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*bovis* for each sampling week.

Sampling week	*Experimental group*	*Mean of IFN-γ (OD 450nm)*	*Standard deviation*	*95% IC*	*p-value*
	1	0.1866[a]	0.2419	-0.0672; 0.4405	
	2	0.0914[a]	0.0164	0.0762; 0.1066	
1	3	0.0970[a]	0.0366	0.0361; 0.1308	0.713
	4	0.1661[a]	0.2277	-0.0440; 0.3767	
	5	0.1101[a]	0.0751	0.0313; 0.1890	
	1	0.01453[a]	0.0350	0.1085; 0.1821	
	2	0.2711[a]	0.0350	-0.0340; 0.5763	
3	3	0.3282[a]	0.1801	0.1616; 0.4949	0.25
	4	0.2578[a]	0.1251	0.1421; 0.3736	
	5	0.1245[a]	0.0331	0.0897; 0.1592	
	1	0.1235[a]	0.0367	0.0849; 0.1620	
	2	0.1377[a]	0.0772	0.0662; 0.2091	
6	3	0.2185[a]	0.1406	0.0884; 0.3486	0.357
	4	0.2197[a]	0.2122	0.0233; 0.460	
	5	0.1158[a]	0.0275	0.0869; 0.1447	

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Continuation

Table 1. IFN-γ concentration in experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*bovis* for each sampling week.

Sampling week	*Experimental group*	*Mean of IFN-γ (OD 450nm)*	*Standard deviation*	*95% IC*	*p-value*
	1	0.2021[b]	0.0180	0.1831; 0.2211	
	2	0.0947[a]	0.0106	0.0849; 0.1044	
8	3	0.1114[a]	0.0352	0.0788; 0.1440	0.001
	4	0.0925[a]	0.0366	0.0586; 0.1264	
	5	0.1495[ab]	0.0663	0.0798; 0.2191	
	1	0.1755[a]	0.0436	0.1296; 0.2213	
	2	.3252[ab]	0.0759	0.2550; 0.3954	
11	3	.5140[b]	0.1366	0.3877; 0.6402	0.05
	4	.3640[ab]	0.2260	0.1750; 0.5529	
	5	.4252[ab]	0.3734	-0.0385; 0.8889	
	1	0.0988[a]	0.0204	0.0773; 0.1203	
	2	0.2364[a]	0.1450	0.1022; 0.3705	
13	3	0.2405[a]	0.0820	0.1647; 0.3164	0.49
	4	0.1848[a]	0.1010	0.0914; 0.2783	
	5	0.1475[a]	0.0401	0.1053; 0.1896	
	1	0.2556[a]	0.1852	0.0612; 0.4501	
	2	0.4712[ab]	0.2387	0.2504; 0.6921	
16	3	0.3667[ab]	0.2463	0.1389; 0.5945	0.002
	4	0.7475[b]	0.4321	0.3478; 1.1472	
	5	0.1013[a]	0.04951	0.0493; 0.1532	
	1	0.0933[a]	0.0205	0.0717; 0.1149	
	2	1.1730[b]	0.8178	0.4166; 1.9293	
18	3	0.6585[ab]	0.4943	0.2014; 1.1157	0.002
	4	0.4650[ab]	0.3746	0.1184; 0.8115	
	5	0.1053[a]	0.0307	0.0730; 0.1375	
	1	0.7015[ab]	0.2699	0.4181; 0.9848	
	2	1.022[ab]	0.6780	0.3949; 1.6490	
20	3	0.7787[ab]	0.5017	0.3146; 1.2427	0.50
	4	1.4491bc	0.8747	0.6401; 2.2581	
	5	0.4566[ab]	0.3581	0.0807; 0.8325	

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Continuation

Table 1. IFN-γ concentration in experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*bovis* for each sampling week.

Sampling week	*Experimental group*	*Mean of IFN-γ (OD 450nm)*	*Standard deviation*	*95% IC*	*p-value*
	1	0.2473[a]	0.1335	0.1072; 0.3874	
	2	0.7285[ab]	0.5109	0.2560; 1.2011	
22	3	0.4198[ab]	0.3974	0.0522; 0.7874	0.001
	4	1.2011[b]	0.7193	0.5357; 1.8665	
	5	0.1071[a]	0.0343	0.0711; 0.1432	
	1	0.4270[a]	0.2106	0.2059; 0.6480	
	2	0.3648[a]	0.2789	0.1068; 0.6228	
24	3	0.1421[a]	0.0583	0.0881; 0.1960	0.137
	4	0.4712[a]	0.4152	0.0872; 0.8553	
	5	0.2450[a]	0.1186	0.1204; 0.3695	
	1	0.3918[a]	0.3512	0.0232; 0.7604	
	2	0.1785[a]	0.0802	0.1043; 0.2528	
28	3	0.1441[a]	0.0780	0.0719; 0.2163	0.158
	4	0.2178[a]	0.1717	0.0590; 0.3676	
	5	0.2430[a]	0.0725	0.1668; 0.3191	
	1	0.0688[a]	0.0138	0.0542; 0.0833	
	2	0.1361[a]	0.0556	0.0847; 0.1875	
30	3	0.1174[a]	0.0529	0.0684; 0.1663	0.180
	4	0.1205[a]	0.0498	0.0744; 0.1667	
	5	0.0665[a]	0.0079	0.0581; 0.0748	

Source: Own elaboration

IFN-γ response to PPD-*avium* in blood.

Regarding the IFN-γ response in blood stimulated with the antigen PPD-*avium*, no significant differences were observed between the experimental groups in samples one, six, eight and 12 ($p > 0.05$); however, differences ($p<0.05$) were detected in samples two, three, four, five, seven, nine, 10, 11, and 13 (Table 2). In samples four and nine, group one (control group) had the

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highest response values, while in the other samples, greater variability was observed between the groups (Figure 2).

This behavior suggests that exposure to environmental mycobacteria can induce nonspecific IFN-γ responses, as previously reported in cattle and goats (Skinner *et al*., 2003; Buddle, 2010). Consistent with this, Hope *et al*. (2005) reported that PPD-*avium*-induced responses do not always reflect infection with *M. avium*, but may be due to frequent contact with environmental mycobacteria present in soil and water. Our results differ partially from those reported by Vordermeier *et al*. (2002), who observed that BCG vaccination did not elicit elevated responses to PPD-*avium*, thereby allowing differentiation between exposure to *M. bovis* and exposure to environmental mycobacteria. In our study, although in some samples the control group showed higher responses to PPD-*avium*, the vaccinated groups also showed variable reactivity, which is consistent with the proposal by Parlane & Buddle (2015), who argued that responses to PPD-*avium* may mask the true efficacy of BCG when there is high environmental exposure to non-tuberculous mycobacteria. Combined these findings reinforce the importance of considering the influence of environmental mycobacteria when interpreting tests based on IFN-γ production. Furthermore, they suggested that the complementary use of more specific antigens, such as ESAT-6 or CFP-10, could improve diagnostic capacity and reduce interference generated by PPD-*avium* (Vordermeier *et al*., 2001; Denis *et al*., 2007).

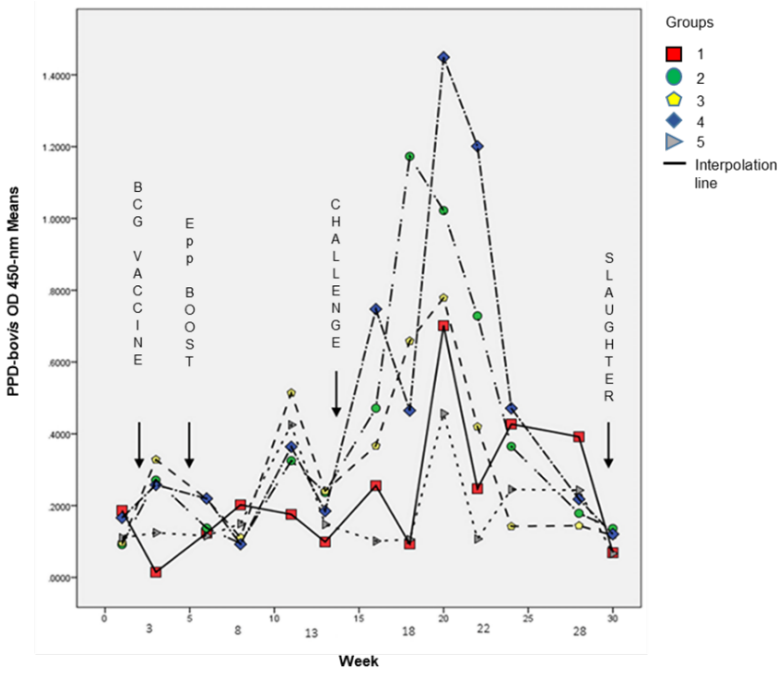

Figure 1. IFN-γ concentration in the blood of goats stimulated with the antigen PPD-*bovis* in the 13 sampling periods in the five experimental groups. Values are expressed as average optical densities (OD 450nm).

Source: Own elaboration based on Contreras-Magallanes *et al*. (2021).

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When comparing the average concentration of IFN-γ in peripheral blood cells in response to stimulation with PPD-*bovis* and PPD-*avium* antigens in the vaccinated groups with the control group, no significant difference was observed in samples 1-4, 6, 12, and 13 ($p > 0.05$), but there was a difference in samples five and 7-11 (Figure 3). The peak concentration of IFN-γ for both cases was reached in week 20, for both PPD-*bovis* and PPD-*avium*, returning to its baseline level in week 30. These results indicate that there was no prior exposure to *M. avium* or other environmental mycobacteria that could have influenced the response to the BCG-Phipps vaccine reported in this study. Studies have described this and shown that exposure to environmental mycobacteria can skew the immune response to PPD-*avium* (Alcaraz-López *et al*., 2021).

In contrast to our findings, Buddle *et al*. (2002) and Parlane & Buddle (2015) reported that sensitization to environmental mycobacteria can decrease the protective efficacy of BCG and increase reactivity to PPD-*avium*, complicating the interpretation of immunodiagnostic tests. However, the absence of this effect in our study is consistent with that described by Vordermeier *et al*. (2002), who found that BCG vaccination induced more robust responses to PPD-*bovis*, with PPD-*avium* having no significant impact on the overall response.

Table 2. Average IFN-γ release for experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*avium* for each sampling period.

Sampling week	Experimental group	Mean of IFN-γ (OD 450nm)	Standard deviation	95% IC	p-value
1	1	0.0975[a]	0.0492	0.0458; 0.1491	0.364
	2	0.0945[a]	0.0159	0.0797; 0.1093	
	3	0.0844[a]	0.0158	0.0697; 0.0990	
	4	0.072[a]	0.0130	0.0600; 0.0841	
	5	0.0808[a]	0.0166	0.0633; 0.0983	
3	1	0.1120[a]	0.0186	0.0923; 0.1316	0.037
	2	0.1748[a]	0.1233	0.0607; 0.2889	
	3	0.2498[a]	0.1068	0.1510; 0.3487	
	4	0.2135[a]	0.0985	0.1223; 0.3047	
	5	0.1158[a]	0.0279	0.0865; 0.1451	
6	1	0.1296[a]	0.0288	0.0993; 0.1599	0.043
	2	0.1435[b]	0.0485	0.0987; 0.1884	
	3	0.1182[a]	0.0355	0.0854; 0.1511	
	4	0.1060[a]	0.0201	0.0873; 0.1246	
	5	0.0880[a]	0.0125	0.0748; 0.1011	

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Table 2. Average IFN-γ release for experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*avium* for each sampling period.

Sampling week	Experimental group	Mean of IFN-γ (OD 450nm)	Standard deviation	95% IC	p-value
8	1	0.1955^{c}	0.0134	0.1814; 0.2095	0.0001
	2	0.0967^{a}	0.0168	0.0811; 0.1123	
	3	0.0894^{a}	0.0159	0.0746; 0.1042	
	4	0.0744^{a}	0.0049	0.0698; 0.0790	
	5	0.1565^{b}	0.0295	0.1255; 0.1874	
11	1	0.0905^{a}	0.0108	0.0790; 0.1019	0.0001
	2	0.2651^{bc}	0.0448	0.2237; 0.3065	
	3	0.3362^{bc}	0.1124	0.2322; 0.4403	
	4	0.3262^{bc}	0.1403	0.1965; 0.4560	
	5	0.1563^{b}	0.0980	0.0534; 0.2592	
13	1	0.1345^{a}	0.1051	0.0241; 0.2448	0.627
	2	0.2000^{a}	0.1642	0.0480; 0.3519	
	3	0.1385^{a}	0.0594	0.0836; 0.1935	
	4	0.1260^{a}	0.0531	0.0768; 0.1751	
	5	0.1918^{a}	0.1335	0.0516; 0.3320	
16	1	0.1018^{a}	0.0512	0.0480; 0.1555	0.001
	2	0.2734^{ab}	0.1031	0.1780; 0.3688	
	3	0.2661^{ab}	0.1201	0.1549; 0.3772	
	4	0.3870^{b}	0.1358	0.2613; 0.5126	
	5	0.1380^{a}	0.0986	0.0345; 0.2414	
18	1	0.3460^{a}	0.2259	0.1088; 0.5831	0.713
	2	0.4572^{a}	0.2444	0.2312; 0.6833	
	3	0.3542^{a}	0.3409	0.0389; 0.6695	
	4	0.3592^{a}	0.1565	0.2145; 0.5040	
	5	0.2651^{a}	0.1669	0.0899; 0.4404	
20	1	0.5986^{b}	0.3491	0.2322; 0.9651	0.046
	2	0.4795^{ab}	0.1787	0.3142; 0.6449	
	3	0.1715^{a}	0.0505	0.1248; 0.2182	
	4	0.3542^{ab}	0.2693	0.1051; 0.6034	
	5	0.4540^{ab}	0.3026	0.1364; 0.7715	

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Table 2. Average IFN-γ release for experimental groups of goats vaccinated against BTb and challenged with a field strain of *M. bovis* with the antigen PPD-*avium* for each sampling period.

Sampling week	Experimental group	Mean of IFN-γ (OD 450nm)	Standard deviation	95% IC	p-value
22	1	0.1671[b]	0.0881	0.0746; 0.2597	0.003
	2	0.4264[bc]	0.2908	0.1574; 0.6954	
	3	0.0751[a]	0.0428	0.0354; 0.1147	
	4	0.4662[c]	0.3359	0.1556; 0.7769	
	5	0.0940[ab]	0.0730	0.0173; 0.1706	
24	1	0.1738[ab]	0.0910	0.0782; 0.2693	0.010
	2	0.0765[ab]	0.0160	0.0617; 0.0913	
	3	0.1465[ab]	0.0573	0.0935; 0.1995	
	4	0.0708[a]	0.0407	0.0332; 0.1085	
	5	0.1818[b]	0.1031	0.0735; 0.2900	
28	1	0.1838[a]	0.0914	0.0879; 0.2797	0.765
	2	0.2385[a]	0.1733	0.0782; 0.3988	
	3	0.1557[a]	0.0859	0.0762; 0.2352	
	4	0.2058[a]	0.1489	0.0681; 0.3435	
	5	0.2476[a]	0.1934	0.0446; 0.4506	
30	1	0.0725[ab]	0.0226	0.0487; 0.0962	0.001
	2	0.0418[a]	0.0213	0.0221; 0.0616	
	3	0.0535[a]	0.0177	0.0371; 0.0700	
	4	0.0521[a]	0.0118	0.0411; 0.0630	
	5	0.0928[b]	0.0270	0.0644; 0.0112	

Fuente: Elaboración propia

Overall, our results reinforce the hypothesis that, under controlled conditions, interference from environmental mycobacteria in the immune response induced by BCG may be minimal or non-existent. This finding is relevant from a practical standpoint, as it supports the potential use of BCG-Phipps in vaccination campaigns against bovine tuberculosis in regions with frequent exposure to environmental mycobacteria.

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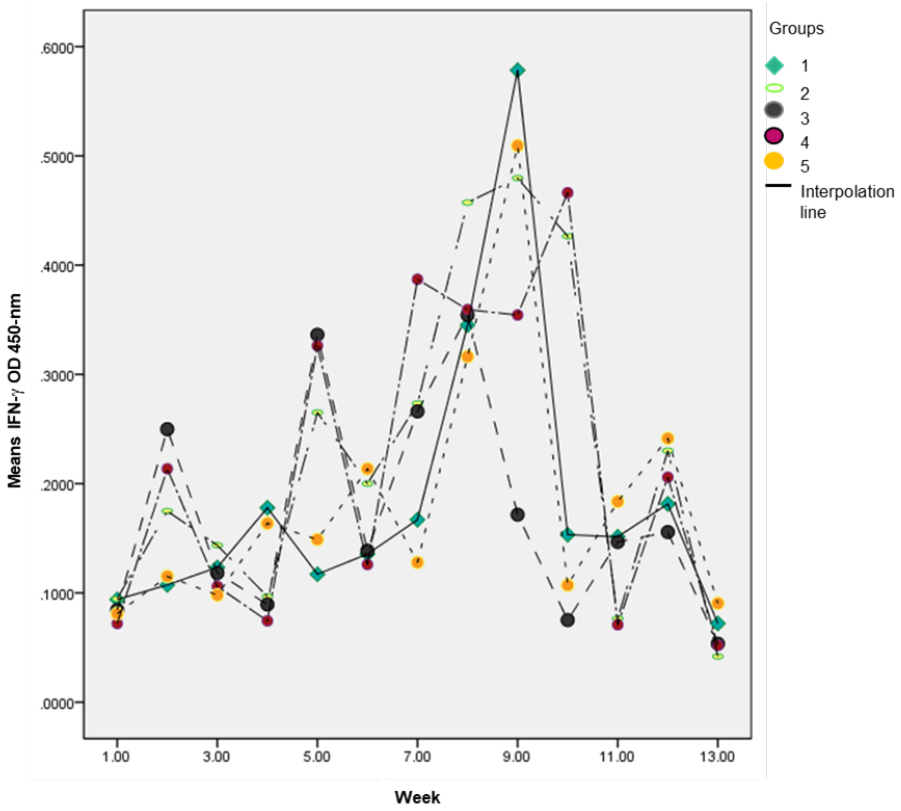

Figure 2. Concentration of IFN-γ in goats blood stimulated with the antigen PPD-*avium* for the 13 samples from the five experimental groups.

Values are expressed as average optical densities (OD 450nm).

Source: Own elaboration based on Contreras-Magallanes *et al*. (2021).

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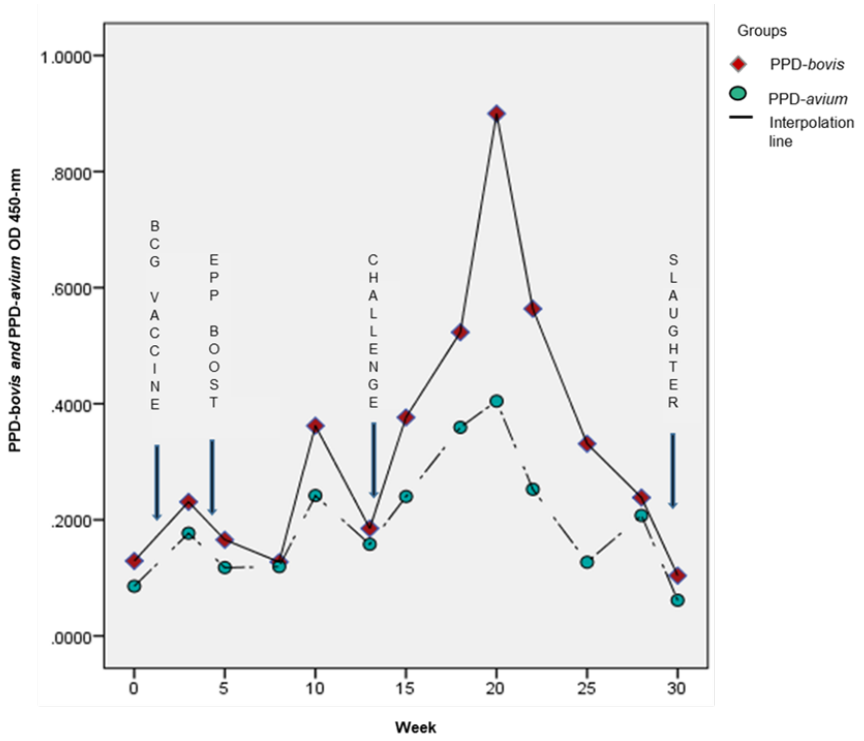

Figure 3. Antigen-specific IFN-γ response in goats postvaccination, boostering and challenging with a field strain of *M. bovis*.

PPD-*bovis* and PPD-*avium* were used for *in vitro* whole blood stimulation. IFN-γ response values are expressed as optical densities (OD 450nm).

Source: Own elaboration based on Contreras-Magallanes *et al*. (2021).

Conclusions

Our results show that the IFN-γ response to peripheral blood cell stimulation with the specific antigen PPD-*bovis* was consistently higher than that obtained with PPD-*avium* in vaccinated animals, compared to the control group, after being challenged with a field strain of *M. bovis*. Likewise, the overall average response in all vaccinated groups was significantly higher against PPD-*bovis* than against PPD-*avium*, confirming the specificity of the vaccine-induced immune response.

These findings suggest that the involvement of environmental mycobacteria in modulating the response to the BCG vaccine (Phipps strain) was minimal or nonexistent in this experiment and did not influence its protective efficacy. In practical terms, the results support the feasibility of incorporating BCG vaccination into bovine tuberculosis control and eradication programs, even

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in regions with high exposure to environmental mycobacteria, as these would not significantly compromise the vaccine-induced immune response. This indicates that environmental mycobacteria were not involved in the vaccine response in this experiment and did not influence the vaccine's efficacy.

Understanding in detail the impact of exposure to MNTs on the efficacy of the BCG vaccine is critical for the continued development of new vaccines against TBb, as it could be a decisive factor in their success in animal vaccination.

Authors' contributions

Conceptualization of work, González-Ruiz, Contreras-Magallanes, Milián-Suazo.; development of the methodology, González-Ruiz, Contreras-Magallanes, Milián-Suazo, Bárcenas-Reyes.; experimental validation, González-Ruiz, Contreras-Magallanes, Milián-Suazo, Sosa-Gallegos; analysis of results, González-Ruiz, Contreras-Magallanes, Milián-Suazo, Bárcenas-Reyes.; data management, González-Ruiz, Contreras-Magallanes, Milián-Suazo.; writing and preparation of the manuscript, González-Ruiz, Contreras-Magallanes, Milián-Suazo, Bárcenas-Reyes, Santillán-Flores.; project administrator, González-Ruiz, Contreras-Magallanes, Milián-Suazo.; acquisition of funds, González-Ruiz, Contreras-Magallanes, Milián-Suazo.

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Ethical statements

The authors declare prior authorization for the project by the Bioethics Committee of the Faculty of Natural Sciences of the Autonomous University of Queretaro with file number 20FCN2019.

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Conflict of interest

The authors declare that they have no conflict of interest.

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