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ORIGINAL ARTICLE

Evolution of IFN-γ response against mycobacterial antigens used for the diagnosis of bovine tuberculosis in BCG vaccinated cattle under a natural transmission setting in central Chile

María B. Benavides[a], Pedro E. Ábalos[a], Nicolás Valdivieso[b], Patricio Retamal[a*]

ABSTRACT. Bovine tuberculosis (bTB) is a chronic disease of animals mainly caused by *Mycobacterium bovis*, a zoonotic pathogen that generates economic losses in the milk and meat industry. In central Chile, the Metropolitan Region concentrates dairy herds with the highest bTB prevalence of the country and the official veterinary service has supported the evaluation of the *M. bovis Bacillus* Calmette-Guerin (BCG) vaccine in this area with the replacement of tuberculin purified protein derivative (PPDs) by the DIVA (Differentiating Infected from Vaccinated Animals) peptides for the bTB diagnosis in the herds. This study aimed to describe the IFN-γ response against PPDs (bovine and avian PPD) and DIVA antigenic cocktails (ESAT-6/CFP-10 and Rv3615c) in BCG vaccinated 11-month-old heifers under a natural transmission scenario. Sixty-two animals were vaccinated via subcutaneous route with a 2-8 x 10^5 colony forming units of BCG Russia strain and 60 control animals received sterile saline. Blood sampling was performed at time 0, previous to vaccination, and then at 3, 6, 9, 12, 15 and 18 months post-inoculation. The follow up of the IFN-γ response in animals determined that the BCG vaccination interferes with the diagnosis of bTB using the traditional bovine PPD between 9 and 12 months post-inoculation. Furthermore, the sensitization with non-tuberculous mycobacteria (NTM) was also interfering the diagnosis relying in PPDs, suggesting the need of using DIVA antigens under this epidemiological condition, whether or not the BCG vaccine is administered in cattle, in order to improve the accuracy of bTB diagnosis in central Chile.

Keywords: IFN-γ response, BCG, cattle, Chile.

INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic infectious disease mainly caused by *Mycobacterium bovis* (Olea-Popelka *et al*., 2017), a pathogen distributed across the world which causes significant economic losses to the dairy industry, especially in low- and middle-income countries. Due to the mandatory milk pasteurization programs in many countries, the zoonotic infection currently becomes more important in occupational settings where the frequent contact with diseased animals in closed enclosures is the highest risk activity (Torres-Gonzalez *et al*., 2013; Vayr *et al*., 2018).

The clinical disease in cattle generally appears along their productive cycle, although asymptomatic and infectious animals contribute significantly to bacterial transmission. Thus, an effective control scheme of bTB requires diagnostic tests to identify infected animals and their prompt removal from herds. However, without compensations for these animal losses, this sanitary management is generally not affordable by farmers in high prevalence areas (Buddle *et al*., 2018). This epidemiological and economic scenario encourages the study of the vaccination as an additional strategy for supporting the control of the disease.

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[a]Laboratorio de Enfermedades Infecciosas, Departamento de Medicina Preventiva Animal, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile.

[b]Servicio Agrícola y Ganadero, Coyhaique, Chile.

*Corresponding author: pretamal@uchile.cl

In animals, vaccination strategies have been proposed and tested in many studies, evaluating attenuated, subunit and recombinant products (Vordermeier *et al*., 2016). However, the *M. bovis* BCG strain constitutes a major vaccine candidate for use in cattle due to its approved use in humans, its availability and safety record (Buddle *et al*., 2018). What has prevented its official implementation is the interference with the standard tuberculin skin test, on which control and eradication programs rely for the *in vivo* diagnosis of animals. To overcome this limitation, novel DIVA (Differentiating Infected from Vaccinated Animals) antigens are now available among which ESAT-6, CFP-10 and Rv3615c have shown comparable sensitivity and specificity levels with the tuberculin test (Vordermeier *et al*., 2016; Srinivasan *et al*., 2019). Both ESAT-6 and CFP-10 are proteins encoded by genes located within the RD1 region of *M. bovis* and *M. tuberculosis* genome, which was deleted from the genome of *M. bovis* BCG vaccine strains (Vordermeier *et al*., 2016). Due to this, the IFN-γ response against these antigens discriminates between *M. bovis*-infected and BCG-vaccinated cattle (Vordermeier *et al*., 2016). The Rv3615c antigen is encoded in the BCG genome, but its secretion is dependent on a secretion system encoded in the RD1 region (Millington *et al*., 2011). In BCG-vaccinated populations, the use of Rv3615c peptide cocktail in combination with ESAT-6/CFP-10 peptide cocktail in DIVA tests has the potential to significantly increase diagnostic sensitivity without reducing specificity (Sidders *et al*., 2008; Vordermeier *et al*., 2016).

The tuberculin skin test consists in the intradermal inoculation of purified protein derivative (PPD) prepared from a culture of *M. bovis* (PPDB) or *M. avium* (PPDA)

(Vordermeier *et al*., 2016). In the last years, the interferon-γ (IFN-γ) release assay (IGRA) has been implemented as an ancillary test for the diagnosis of bTB, using PPD and DIVA antigens (Vordermeier *et al*., 2011; Buddle *et al*., 2018).

In Chile, the distribution of bTB is characterized by low prevalence areas in northern and southern regions and a high prevalence area in the central zone (Rivera & Vega, 2014) where farmers have been less receptive to cull reactor animals due to the lack of compensation (Max *et al*., 2011). On the other hand, the prevalence of non-tuberculous mycobacteria (NTM) in dairy cattle has been scarcely reported with some evidence in southern Chile about *M. avium* subsp. *paratuberculosis* infection in dairy herds, with a within-herd prevalence ranging between 3.2% and 38% (Verdugo *et al*., 2018) and a herd-level prevalence between 28% and 100%, depending on the herd size (Kruze *et al*., 2013).

In some dairy herds from the central zone, the Agricultural and Livestock Service of Chile (SAG) allowed a field trial for the study of BCG vaccination in cattle, obtaining a significant efficacy which encourages its implementation as an alternative strategy for the prevention of the disease in high prevalence farms (Retamal *et al*., 2022). The aim of this work is to describe the dynamics of IFN-γ responses against tuberculin PPD (PPDB and PPDA) and DIVA (ESAT-6/CFP-10 and Rv3615c) antigens in BCG vaccinated and unvaccinated cohorts of heifers raised under a natural transmission setting in central Chile.

MATERIALS AND METHODS

The dairy herd, with 294 Holstein-Friesian breeding cows in milk production, was located in the Metropolitan Region. It had a Brucellosis-free status and a bTB incidence of 24% at the beginning of the BCG vaccination in 2017. The presence of other infectious diseases was unknown. For controlling bTB, the owner was periodically using the single intradermal tuberculin test for detecting reactor animals, some of which were segregated and others culled. For the study in this farm, SAG allowed the use of the IGRA with traditional and DIVA antigens (described below) in replacement of the tuberculin test as an official diagnostic technique.

ANIMAL VACCINATION SCHEDULE

Between May 2017 and July 2018, 11-month-old Holstein-Friesian female heifers were included in a double-blinded cohort study, in which 62 animals were vaccinated by the subcutaneous route with 2-8 x 10^5 colony forming units (CFU) (0.1 mL) of the BCG Russia strain (Serum Institute of India, Pune, India). The control group was constituted by 60 animals that received 0.1 mL of NaCl 0.9% (vaccine diluent).

This study was originally designed to evaluate the BCG efficacy in heifers, in which a protection of 66.5% was determined (Retamal *et al*., 2022). Hence, vaccinated and unvaccinated animals that were reactors to DIVA IGRA were assumed as infected with *M. bovis* and were excluded from subsequent sampling activities. In addition, a group of animals gradually disappeared from the study due to herd management practices, which were locally prioritized by health or production issues. For these reasons, 41 and 27 animals were included in the last sampling activity in the vaccinated and control groups, respectively.

BLOOD SAMPLING

Animals were sampled the day of inoculation (time 0) and at 3, 6, 9, 12, 15 and 18-months post vaccination. For this, 5 mL of blood was collected in heparinized tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and maintained at environmental temperature until arriving at the laboratory.

IFN-γ RELEASE ASSAY (IGRA)

The stimulation of whole blood was performed on the day of sampling with 250 IU/mL of the traditional avian (PPDA) and 300 IU/mL of bovine (PPDB) Purified Protein Derivatives (Applied Biosystems® Bovigam®) and DIVA antigens, represented by the ESAT-6 and CFP-10 combined peptides (21 peptides in total) and the Rv3615c peptides (11 peptides in total) (Pepceuticals Ltd., United Kingdom) (Sidders *et al*., 2008; Vordermeier *et al*., 2001). These DIVA peptides were diluted in RPMI 1640 medium with Glutamax® supplement (Gibco, Grand Island, NY, USA) at a final concentration of 55 µg/each peptide/mL. Then, 25 µL of this solution was used to stimulate 250 µL of blood. In addition, a PBS control solution and the Pokeweed mitogen (Applied Biosystems® Bovigam®) were also utilized for blood stimulation. After an incubation period at 37°C for 18 h, plasma supernatants were harvested and stored at -20°C. For the IFN-γ detection, plasmas were thawed and processed with the Bovigam 2G® Test Kit for cattle (Prionics AG, Tullamarine, Australia), according to manufacturer recommendations.

The cut-off value for both bovine and avian PPD was a ≥ 0.05 difference (ΔOD_{450}) between them (PPD B-A for PPDB reactors and PPD A-B for PPDA reactors) and a ≥ 0.05 difference with the phosphate-buffered saline (PBS) control solution, according to the national control and eradication program definition. The cut-off value for DIVA antigens was OD_{450} ≥0.1 difference with the PBS control in either cocktail. The criterion for diagnosing DIVA reactors was testing positive to at least one peptide cocktail.

ANALYSIS OF RESULTS

The IGRA results (ΔOD_{450}) were compared between vaccinated and unvaccinated groups of animals at each sampling time with the non-parametric Mann-Whitney

Table 1. Comparisons of interferon gamma release assay (IGRA) reactors using PPD and DIVA antigens for the diagnosis of bovine tuberculosis in BCG vaccinated and control groups of animals.

Month	BCG				Control			
	PPD N° (%)	DIVA N° (%)	p	k	PPD N° (%)	DIVA N° (%)	p	k
3	15 (24.2)	2 (3.2)	0.001	0.06	6 (10)	8 (13.3)	0.79	-0.13
6	16 (28.1)	2 (3.5)	<0.001	0.05	7 (14.6)	2 (4.2)	0.12	0.17
9	8 (15.7)	1 (2.0)	0.04	-0.04	2 (5.3)	0 (0)	<0.001	0
12	6 (12.5)	0 (0)	<0.001	0	3 (7.7)	3 (7.7)	0.63	0.28
15	5 (11.4)	2 (4.6)	0.45	-0.07	5 (14.3)	2 (5.7)	0.38	0.22
18	8 (19.5)	0 (0)	<0.001	0	2 (7.4)	2 (7.4)	0.5	0.46

PPD, purified protein derivative; DIVA, differentiating infected from vaccinated animals; BCG, *Mycobacterium bovis* BCG strain.
p, McNemar test; k, Kappa test.

test. The comparison within groups between sampling times was performed with the Friedman test. The bTB diagnosis attained with PPD and DIVA antigens was contrasted with the McNemar and Kappa tests, to identify changes in proportions and interrater reliability of results, respectively.

These analyses were performed using Infostat® software (Di Rienzo *et al*., 2016).

RESULTS

The IGRA results with PPD and DIVA antigens can be seen in Figure 1 and Table 1S. The unique stimulus in which IFN-γ responses were significantly different between vaccinated and control groups was observed with the bovine PPD at 3, 6 and 18 months post-vaccination ($p<0.05$, Figure 1B).

Comparisons performed with the Friedman test showed that the IFN-γ responses to the bovine PPD in vaccinated animals increased significantly at 3, 6, 9, 12 and 15 months post-vaccination ($p<0.0001$), in contrast to time 0. Within the unvaccinated group, differences were only observed between times 0 and 3 months ($p<0.05$). In the analysis of PPD B-A, the vaccinated group showed differences between times 0 and 6, 9, 15 and 18 months post-vaccination ($p<0.01$). The unvaccinated group did not fluctuate significantly alongside the study ($p>0.05$). With DIVA antigens, the unique difference was observed with ESAT-6/CFP-10 cocktail in the vaccinated group, between time 0 and 6 months ($p<0.05$).

The diagnosis of bTB through IGRA using cut-off values, showed variable proportions of reactor animals and interrater agreements when IFN-γ responses to PPD and DIVA antigens were compared, especially in the BCG vaccinated group (Table 1).

Within the group of DIVA positive animals, 58% of them (14/24) were also identified as *M. avium* reactors (PPD A-B ≥0.05), 29% (7/24) were PPDB reactors (PPD B-A ≥0.05) and 12.5% (3/24) were classified as negative by PPD antigens.

Table 2. Interferon gamma release assay (IGRA) reactors using PPD antigens (PPD A-B ≥0.05 and PPD A-PBS ≥0.05) in BCG vaccinated and control groups of animals.

Month	PPDA reactors N° (%)	
	BCG	Control
0	19 (30.6)	22 (36.6)
3	27 (43.5)	29 (49.2)
6	17 (29.8)	12 (25)
9	18 (35.3)	9 (23.7)
12	14 (29.2)	13 (33.3)
15	9 (20.5)	10 (28.6)
18	10 (24.4)	5 (18.5)

PPD, purified protein derivative; PPDA, PPD avium; PPDB, PPD bovis; PBS, phosphate-buffered saline; BCG, *Mycobacterium bovis* BCG strain.

The sensitization with non-tuberculous mycobacteria (NTM) was similar in both groups of animals during all sampling times ($p>0.05$), with the highest values of avium PPD reactors at 3 months post-vaccination (Table 2).

The strength of IFN-γ responses of DIVA positive animals was also compared between groups without significant differences ($p>0.05$) (Table 3).

DISCUSSION

The application of the *M. bovis* BCG vaccine in cattle has been evaluated in experimental and natural transmission settings, at different doses, strains, ages and breeds, suggesting an average efficacy around 25% of vaccinated animals (Srinivasan *et al*., 2021) and some potential non-specific benefits (Retamal *et al*., 2022). Despite of this level of protection, which would significantly contribute to bTB

Table 3. Interferon gamma release assay (IGRA) results of DIVA reactor animals using PPD (PPDB, PPDA) and DIVA (ESAT-6/CFP-10, Rv3615c) antigens.

Group	DIVA reactors N°(%)	IFN-γ responses (ΔOD_{450} nm) (mean ± SEM)			
		PPD B-A	PPDB	ESAT-6/CFP-10	Rv3615c
BCG	7 (11.3)	-0.01 ± 0.15	0.92 ± 0.44	0.21 ± 0.05	0.08 ± 0.06
Control	17 (28.3)	-0.03 ± 0.21	0.75 ± 0.20	0.40 ± 0.16	0.17 ± 0.07

PPD, purified protein derivative; PPDA, PPD avium; PPDB, PPD bovis; DIVA, differentiating infected from vaccinated animals; BCG, *Mycobacterium bovis* BCG strain.

control and prevention (Conlan *et al*., 2015), the official veterinary services have not allowed its implementation, due to the interference that the vaccine elicits with the World Organization for Animal Health (WOAH)-recommended tuberculin skin test (Bayissa *et al*., 2021). However, the required diagnostic assays that discriminate infected from vaccinated animals (DIVA) have already been developed, for both skin and IGRA procedures (Srinivasan *et al*., 2019; Vordermeier *et al*., 2016).

In this study, the BCG vaccination elicited a raise in the IFN-γ response against the bovine PPD (Figure 1), which determined significant differences ($p<0.05$) with the diagnosis based on DIVA cocktails, especially during the first 12 months post-vaccination (Table 1), as has been observed previously (Whelan *et al*., 2011). Accordingly, the agreement between these PPD and DIVA peptides in vaccinated animals was negligible in all sampling times, contrasting with the control group, in which this indicator was progressively improving along the study (Table 1). This result accounts for the interference that the *M. bovis* BCG strain generates with the tuberculin-based diagnosis and justifies its replacement by a DIVA assay when the vaccination is applied in cattle (Vordermeier *et al*., 2001).

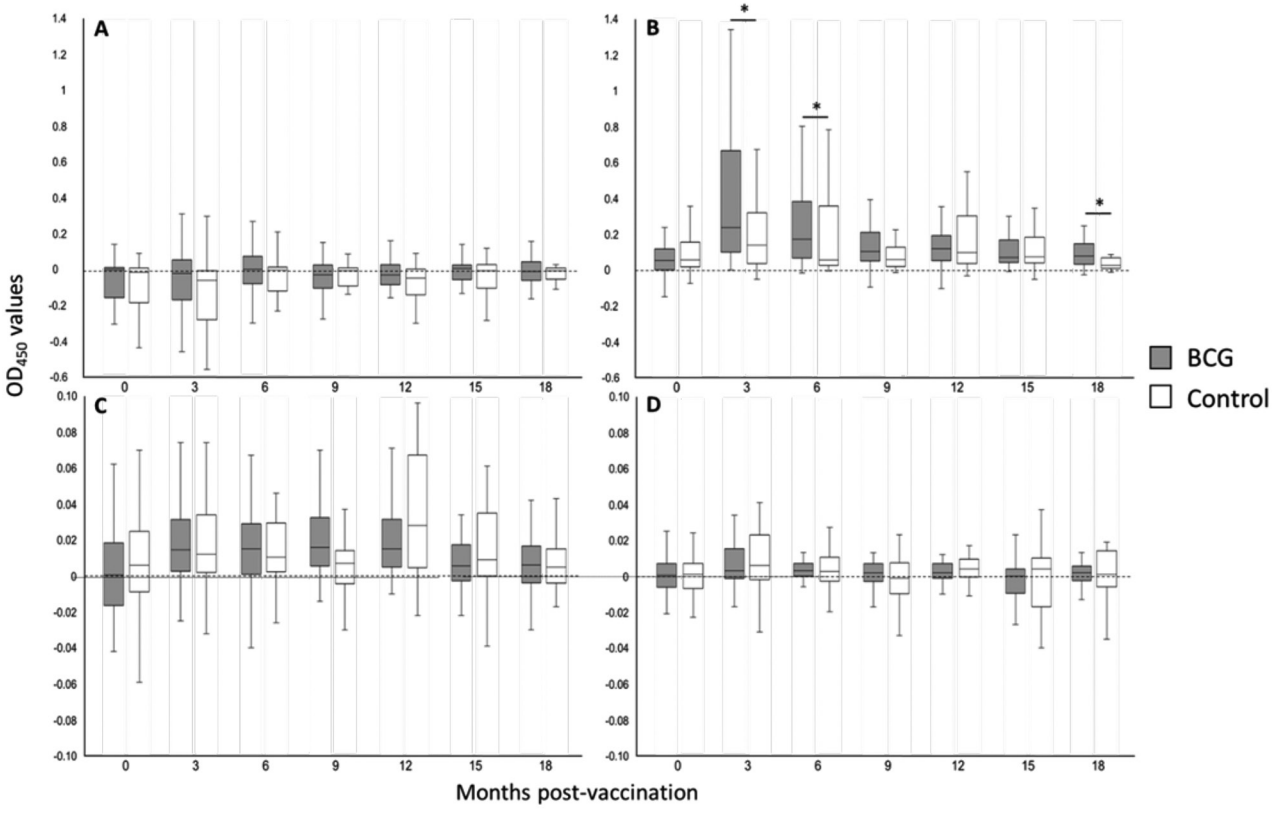

Figure 1. Box-plot diagrams showing IFN-γ responses at 0, 3, 6, 9, 12, 15 and 18 months post-vaccination in BCG and control groups. The figure shows the IFN-γ release assay (IGRA) results obtained with **A)** PPD B-A (bovis minus avium), **B)** PPDB minus saline, **C)** CFP-10/ESAT-6 and **D)** Rv3615c. Within box-plot diagrams, the median is represented with a line, the interquartile range with a box and the minimum and maximum with the whiskers. Outliers were not depicted (* $p<0.05$).

Furthermore, differences between PPD and DIVA peptides were also observed in the group of non-vaccinated animals, represented with low agreement rates between them during the first 9 months of the study (Table 1). In this regard, the higher IFN-γ response of animals to NTM during the first three months, assumed by the PPDA reactors (Table 2), determined that 9 out of 10 DIVA reactors were also identified as reactors to the avium PPD (Table 1S). This implies that when PPD antigens are occupied for bTB diagnosis in the context of a high NTM exposition, a proportion of truly *M. bovis* infected animals will be identified as PPDA reactors, decreasing the sensitivity of the test and the effectiveness of diagnosis for controlling the disease. In the whole study, a higher proportion of DIVA and PPDA reactors were observed in the control group (65%, 11/17) than the vaccinated group (43%, 3/7), probably due to BCG increased the IFN-γ response against bovine PPD, partially compensating the mentioned NTM effect in vaccinated animals. Therefore, the highest disagreement for *M. bovis* diagnosis between PPD and DIVA antigens were observed at 3 months in the control group (Table 1).

This NTM exposition was not explained by any known risk or management factor in animals. The NTM corresponds to a diverse group of microorganisms, widely distributed in nature, some of which can produce disease in animals (Pereira *et al*., 2020). In Chile, a high prevalence of *M. avium* subsp *paratuberculosis* has been reported in dairy cattle (Kruze *et al*., 2013), affecting the accuracy of PPD based tests for the diagnosis of bTB (Raffo *et al.*, 2017, 2020). Under this epidemiological context of high exposition to NTM, the use of DIVA reagents would also support diagnostic efforts of bTB.

Previous reports have shown that BCG vaccination also increases IFN-γ responses against avian PPD (Buddle *et al*., 2013; Vordermeier *et al*., 2001). However, in this work we did not observe differences between vaccinated and unvaccinated animals (data not shown), suggesting that the BCG strain, the age of inoculated animals or their previous exposition to NTM might be influencing the cross reaction between BCG and the avian PPD.

In *M. bovis* infected animals, the amount of secreted IFN-γ in response to DIVA peptides has been documented variable between vaccinated and unvaccinated animals, suggesting a negative correlation with protection and a positive correlation with bTB-associated pathology (Parlane *et al*., 2014; Vordermeier *et al*., 2002; Bayissa *et al*., 2021). Despite of this work determined a lower mean IFN-γ release in BCG vaccinated than control DIVA-reactors, such differences were not significant ($p>0.05$) (Table 3). To clarify if such prognostic association can also be observed in the Chilean field conditions, a higher number of reactor animals and the inclusion of *post-mortem* analyses are needed.

A major drawback to be addressed with BCG vaccination in cattle is the additional cost associated with the application of the DIVA test. Because of this, better cost-effective scenarios need to be explored, such as its application to confirm reactor animals to the traditional tuberculin skin test (Parlane & Buddle, 2015; Buddle *et al*., 2013; Jones *et al*., 2017).

It was determined that the implementation of DIVA-IGRA for the diagnosis of bTB was essential in BCG vaccinated dairy cattle, under a natural transmission setting in central Chile. In addition, this assay appeared useful in a context of high exposition of NTM, avoiding the interference that these mycobacteria could have with classical diagnosis based on bovine and avium PPD antigens. These results and the significant protection conferred by the vaccine (Retamal *et al*., 2022) suggest the need for a more committed effort of official veterinary services around the globe for the inclusion of BCG vaccine and DIVA diagnosis in bTB control programs, especially in those more prevalent areas.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS STATEMENT

The study was performed after an informed consent was signed by the farm owner. The University of Chile committee on the care and use of animals' guidelines were followed (Permit N°11021 VET-UCH).

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and analysis were performed by MBB. The organization and coordination of the field work was performed by PA and NV. Analyses of results was performed by MBB and PR. The manuscript was written by PR and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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