Received: 25 May 2020 | Revised: 16 November 2020 | Accepted: 23 November 2020

DOI: 10.1111/tbed.13939

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Association of immune responses of Zebu and Holstein-Friesian cattle and resistance to mycobacteria in a BCG challenge model

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Funding information
British Council, Grant/Award Number: 276305277; Consejo Nacional de Ciencia y Tecnología, Grant/Award Number: 277863

Abstract

Mycobacterium bovis is the main cause of bovine tuberculosis (BTB) in cattle and can also infect humans. Zebu cattle are considered more resistant to some infectious diseases compared with Holstein-Friesian (HF) cattle, including BTB. However, epidemiological studies may not take into account usage differences of the two types of cattle. HF cattle may suffer greater metabolic stress due to their more or less exclusive dairy use, whereas Zebu cattle are mainly used for beef production. In experiments conducted so far, the number of animals has been too small to draw statistically robust conclusions on the resistance differences between these cattle breeds. Here, we used a BCG challenge model to compare the ability of naïve and vaccinated Zebu and HF cattle to control/kill mycobacteria. Young cattle of both breeds with similar ages were housed in the same accommodation for the duration of the experiment. After correcting for multiple comparisons, we found no difference between naïve HF and Zebu ($\rho = 0.862$) cattle. However, there was a trend for vaccinated HF cattle to have lower cfu numbers than non-vaccinated HF cattle ($\rho = 0.057$); no such trend was observed between vaccinated and non-vaccinated Zebu cattle ($\rho = 0.560$). Evaluation of antigen-specific IFNγ secretion by PBMC indicated that Zebu and HF cattle differed in their response to mycobacteria. Thus, whilst there may be difference in immune responses, our data indicate that with the number of animals included in the study and under the conditions used in this work, we were unable to measure any differences between Zebu and HF cattle in the overall control of mycobacteria. Whilst determination of different susceptibilities between Zebu and HF cattle using the BCG challenge model will require larger numbers of animals than the number of animals used in this experiment, these data should inform future experiments.

KEYWORDS

BCG, Holstein-Friesian cattle, mycobacteria, natural resistance, vaccine, Zebu cattle

1 | INTRODUCTION

Bovine tuberculosis (BTB) is a zoonotic disease caused mainly by *Mycobacterium bovis*. BTB results in productivity loss in cattle, imposition of trade barriers and risk of spread of infection to other domestic livestock, wildlife and humans. The current annual worldwide cost of BTB is estimated at US$3 billion (Maggioli et al., 2015). Conventionally, in developed countries, control of BTB is based on 'test-and-slaughter' policies, in which tuberculin skin test positive cattle are deemed to be infected with *M. bovis* and killed. However, this approach is not applied universally, particularly not in many low- and middle-income countries (LMIC), in which this type of control is either deemed unaffordable or societally unacceptable (https://www.oiebulletin.com/wp-content/uploads/bulletins/panorama-2019-1-en.pdf). In these countries, vaccination could be used as a sustainable supplementary tool to control policies based on test and slaughter. The lead candidate vaccine against BTB is the live attenuated *M. bovis* bacillus Calmette-Guerin (BCG), which is widely used to vaccinate humans against tuberculosis. As in humans, BCG has also shown variable efficacy in cattle, both at population and individual animal levels (Vordermeier et al., 2016). The reasons for this variability are largely unknown; however, presensitization of the adaptive immune response with environmental mycobacteria interfering with BCG-induced immunity is proposed as a plausible explanation (Brandt et al., 2002; Buddle et al., 2002; Hope et al., 2005).

Humped cattle (Zebu, *Bos taurus* ssp. *indicus*) are considered to be more genetically resistant to some infectious diseases, including BTB, than non-humped cattle (*B. taurus* ssp *taurus*) (Murray et al., 2013). Indeed, studies in Ethiopia have shown that Zebu cattle have a lower prevalence of BTB skin test positivity under similar husbandry settings compared with Holstein-Friesian (HF) cattle (Ameni et al., 2007). There are also preliminary results indicating that Zebu (Boran) cattle were more resistant to a low dose experimental *M. bovis* infection than Holstein cattle (Vordermeier et al., 2012). However, these preliminary data using relatively small numbers of animals, six Zebu and six Holstein, need to be confirmed.

Therefore, in the present study we compared the protective efficacy of BCG in Zebu and HF cattle in an experimental setting. We used an established BCG challenge model (Villarreal-Ramos et al., 2014) to compare the relative innate (in naïve animals) and adaptive (in vaccinated animals) immune response capabilities of Zebu and HF cattle with control mycobacteria in vivo using age- and gender-matched animals housed at the same location for the duration of the experimental period.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The experiment was approved by UNAM's Facultad de Medicina Veterinaria y Zootecnia ethical review panel as # Protocolo 53; and APHA's AWERB committee under ASUF303 336/2017/002.

2.2 | Cattle

Thirty-two Zebu cattle and 31 Holstein-Friesian (HF) cattle were sourced from farms free of BTB. Due to their nature, Zebu cattle were sourced from the southern subtropical regions of Mexico (State of Veracruz) and Holstein-Friesian cattle were sourced from the central region of Mexico (State of Mexico). The age of Zebu cattle varied between 2 and 8 months, with a median age of 3 months, whilst the age of HF cattle varied between 4 and 7 months, with a median age of 6 months.

2.3 | Mycobacteria

The live attenuated strains *M. bovis* BCG SSI1331 and *M. bovis* BCG Tokyo were used for vaccination and challenge, respectively. Mycobacteria were grown to mid-log phase in 7H9 medium containing 0.05% Tween 80 and OADC; bacteria were aliquoted and frozen at −70°C until further use. Titre of the frozen aliquots was determined by thawing an aliquot and plating serial dilutions on 7H11 agar plates.

2.4 | Vaccination and challenge experiments

Sixteen Zebu cattle and 16 Holstein-Friesian cattle were vaccinated subcutaneously with 1×10^6 BCG SSI cfu/animal at week 0. Eight weeks after vaccination, 16 control and 16 vaccinated Zebu cattle, as well as 15 control and 16 vaccinated Holstein-Friesian cattle were challenged intranodally with BCG Tokyo, as indicated previously (Villarreal-Ramos et al., 2014), with 1×10^7 cfu each. Three weeks after challenge, all animals euthanized to recover prescapular lymph nodes for evaluation of protection conferred by vaccination with BCG, in vaccinated animals, and potential differences in the innate response to mycobacteria in the two breeds in non-vaccinated animals. The number of animals per group was determined based on previously published (Villarreal-Ramos et al., 2014) and non-published experimental data, which indicated that a comparisons between vaccinated and non-vaccinated HF cattle required 12 animals to reach a statistical power of 70.7%, whilst another experiment, using 17 animals, reached a statistical power of 96.7%.

2.5 | Determination of bacterial load in lymph nodes

Left and right prescapular lymph nodes (LN) were dissected from each animal at post-mortem. One of these LNs was used for evaluating bacterial load as previously described (Villarreal-Ramos et al., 2014). Briefly, LNs were trimmed and submerged briefly in 70% ethanol prior to weighing and slicing for processing in a stomacher (Seward) for 2 min with 7 ml of PBS. One hundred μl of LN macerate was spread in each of 2 7H11 plates, as well as preparing serial dilutions for plating on 7H11 agar plates (Gallagher & Horwill, 1977). Results are presented as counts per organ. The limit of detection of this assay in each individual plate is 70 cfu/organ; since we plated

two plates, the limit of detection for this assay could be considered to be 35 cfu/organ; the discontinuous line in the graph in Figure 1 indicates the limit of detection. Animals for which no cfu were detected in any of the two plates were placed below this line.

2.6 | Evaluation of secretion of IFNγ responses

Immune responses were evaluated as production of interferon gamma (IFNγ) in supernatants of peripheral blood cells incubated overnight at 37°C in a 5% CO_2 and 95% humidity atmosphere with purified protein derivative (PPD) from *Mycobacterium avium* (PPD-A, control antigen) or *M. bovis* (PPD-B, BTB-specific antigen), or medium alone as negative control (Villarreal-Ramos et al., 2014). Levels of IFNγ were determined using the Bovigam™ assay (Prionics) according manufacturer's recommendation. Data are expressed as mean $O.D._{450nm} \pm SEM$.

2.7 | Statistical analysis

Graph drawing and statistical analysis were carried out using GraphPad Prism v 5.02 (GraphPad Software, San Diego, CA) and GraphPad Instat v 3.06; for analysis of bacterial counts, a Kruskal–Wallis with Dunn's correction for multiple comparisons was carried out. For IFNγ secretion, results were analysed using a Mann–Whitney ANOVA.

3 | RESULTS

3.1 | Bacterial load in lymph nodes is not impacted on by breed

Figure 1 shows the bacterial load in LN of control and vaccinated cattle recovered at 3 weeks after challenge. Statistical analysis correcting for multiple comparisons indicated that there was a strong trend, almost reaching statistical significance ($p = .057$), for a reduced recovery of mycobacteria from BCG-vaccinated HF cattle compared with naive HF cattle. However, no such trend was observed in the number of mycobacteria recovered from vaccinated or naive Zebu cattle ($p = .5606$). Furthermore, no significant difference was seen in the number of recovered mycobacteria isolated from naive HF and naive Zebu cattle ($p = .8622$).

3.2 | Secretion of IFNγ

Figure 2 shows the antigen-specific IFNγ by peripheral blood cells stimulated with either PPD-A or PPD-B. Blood samples were taken prior to and after vaccination at weeks 0, 4 and 8, as well as after intranodal BCG Tokyo challenge (weeks 9 and 10). No statistically significant difference was observed between the responses to PPD-A and responses to PPD-B in any of the animal groups at week 0. Similarly, there was no statistically significant difference in the response to PPD-B detected at weeks 4 and 8 compared with responses at week 0 in any of the animal groups regardless of their vaccination status. In HF cattle, vaccinated or not, responses to PPD-A were higher than responses to PPD-B all through the experimental period, and the response to PPD-A and PPD-B, even though there was no significant difference, was slightly higher in vaccinated HF cattle compared with naïve HF cattle.

In Zebu cattle, the response to PPD-A and PPD-B was in general higher in vaccinated animals compared with naïve animals. In Zebu cattle, there was also a trend for responses to PPD-A to be higher than responses to PPD-B at weeks 4 and 8 after vaccination; however, this trend was less striking when compared to the trend in HF cattle. After challenge, responses to PPD-B and to PPD-A in Zebu cattle were very similar. Interestingly, responses to PPD-A or PPD-B observed in vaccinated HF were indistinguishable from responses observed in unvaccinated HF calves.

Eight weeks after subcutaneous vaccination with BCG, all cattle were inoculated intranodally with 10^7 cfu of BCG Tokyo in the challenge phase of the experiment. Responses to intranodal inoculation were also evaluated by measuring the production of IFNγ responses by peripheral blood cells stimulated with PPD-A and PPD-B at 1 and

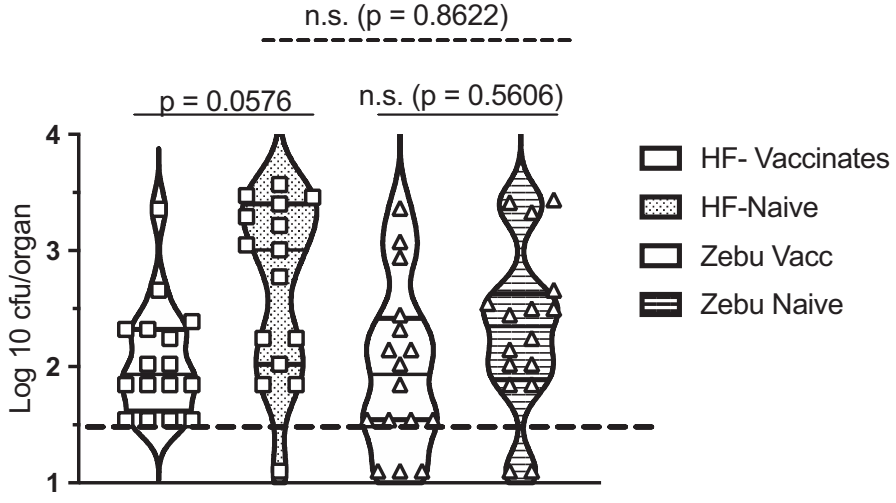

FIGURE 1 Evaluation of bacterial load in the prescapular LN of HF (▭ and ▩) and Zebu (▭ and ▤) cattle. HF (▭) and Zebu (▭) cattle were vaccinated or not (▩ and ▤) as described in materials and methods. Prescapular lymph nodes were harvested at post-mortem, three weeks post-intranodal challenge. Organs were macerated and an aliquot of the tissue macerate was plated in 7H11 plates. Counts are presented as cfu/organ; the limit of detection for this assay is 50 cfu

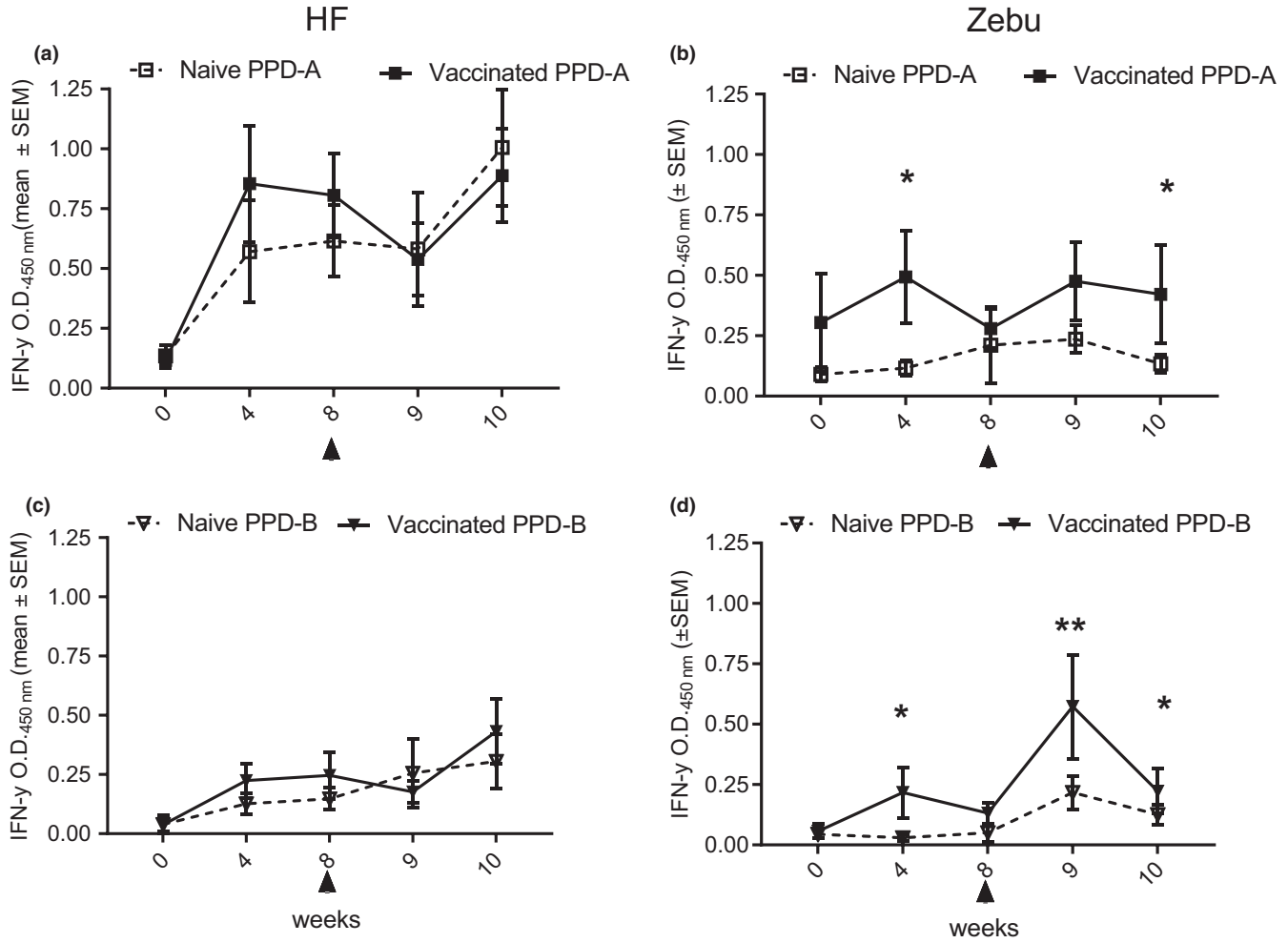

FIGURE 2 Longitudinal immune responses in HF and Zebu cattle to mycobacterial antigens. Immune responses were evaluated as the secretion of IFNγ by peripheral blood cells from HF (a and c) and Zebu (b and d) cattle that had been vaccinated (filled symbols) with *c* 10^6 BCG SSI cfu subcutaneously or not (empty symbols). Cells were stimulated with PPD-A (a and b) or PPD-B (c and d). Vaccination occurred at week 0 and all animals were challenged in both prescapular lymph nodes with *c* 10^7 BCG Tokyo cfu each lymph node at week 8 (arrow). For a closer description of statistics, please see text in results and discussion

2 weeks post-intranodal challenge (weeks 9 and 10 after the initial BCG vaccination, respectively) (Figure 2). The data indicated that, following challenge with BCG, the responses to PPD-A or PPD-B detected at weeks 9 and 10 in vaccinated or naive HF cattle were not different from the responses detected at week 8, prior to challenge; this was similar to what was observed in vaccinated Zebu cattle, that is no difference in responses at weeks 9 and 10 compared with responses observed in week 8. In non-vaccinated Zebu cattle, intranodal challenge induced a statistically significant increase in responses to PPD-B at weeks 9 ($\rho < 0.001$) and 10 ($\rho < 0.05$).

4 | DISCUSSION

The present study was designed to determine the potential differences in resistance to mycobacterial infection between Zebus and HF cattle, and to evaluate the potential differences in the immune response between HF and Zebu cattle using the recently in HF cattle developed BCG challenge model (Villarreal-Ramos et al., 2014). The premise of the model is that cattle which have been successfully vaccinated against, or which are naturally resistant to *M. bovis*, should be able to control the live attenuated *M. bovis* BCG strain better compared with cattle that have not been vaccinated or which are naturally susceptible to infection with *M. bovis*. However, being an attenuated mutant, it would also be expected that an immunocompetent host that has not been vaccinated or which would be susceptible to *M. bovis* would, with time, also be able to control BCG. Thus, in this model, any potential differences in the ability to control BCG between resistant and susceptible cattle, or between vaccinated or not vaccinated cattle would occur in the first 2 or 3 weeks after challenge, which shortens the possibility of performing long-term immunological post-challenge studies. Therefore, we evaluated bacterial recovery as well as the immune responses of Zebu and HF cattle following vaccination with BCG and 2 weeks after intranodal challenge.

In terms of bacterial recovery, the data indicated that there was a strong trend, which almost reached statistical significance, for lower

numbers of mycobacteria recovered from BCG-vaccinated HF cattle than from non-vaccinated HF cattle. The data indicated that the BCG challenge model could, to a large extent, differentiate between HF-vaccinated and non-vaccinated groups. The data also indicated that there was no statistically significant difference in the number of mycobacteria recovered from vaccinated Zebu cattle compared with non-vaccinated Zebu cattle. No difference was observed between the number of mycobacteria recovered from non-vaccinated HF and non-vaccinated Zebu cattle or between vaccinated HF and vaccinated Zebu cattle.

With regard to the immunological response, we used PPD-A and PPD-B as antigens that would permit detection of BCG-specific induced responses. PPD-A, derived from *M. avium*, was used as an antigen representative of environmental mycobacteria. Although responses to PPD-B and PPD-A were low in all groups at week 0, peripheral blood responses to mycobacterial antigens after vaccination with BCG exhibited a bias towards PPD-A, rather than towards PPD-B in BCG-vaccinated HF and Zebu cattle; these results could indicate that cattle had been and/or were concomitantly being exposed to either *M. avium* or cross-reacting environmental mycobacteria. Whilst it was not possible to observe differences in the responses to PPD-B induced by BCG vaccination or challenge, it is clear that the two breeds of cattle responded differently to PPD-A following inoculation with BCG. HF cattle showed higher IFNγ responses to PPD-A than Zebu cattle (Figure 2). Thus, differences in the immune response to PPD-A could be an indication that Zebu cattle handle mycobacteria in different ways to the way HF cattle handle mycobacteria. Indeed, in a study aimed at determining the prevalence of *M. avium* infection in Uganda cattle, *M. avium* could be found in both breeds; however, despite examining almost twice as many Zebu cattle as HF cattle, the number of HF cattle determined as positive for *M. avium* was greater than the number of Zebu cattle (Okuni et al., 2013). These data suggest that indeed, HF cattle may be more susceptible to *M. avium* than Zebu cattle.

It is possible that a prior exposure to environmental mycobacteria may have conferred a degree of protection to BCG vaccination, similar as described before (Hope et al., 2005; Howard et al., 2002). This protection may have reduced the expected difference in bacillary recovery between the vaccinated and unvaccinated HF calves and, thus, the power of the BCG challenge model to differentiate between vaccinated and non-vaccinated HF cattle. Similar to our results, studies have shown that exposure of cattle to environmental mycobacteria prior to vaccination with BCG or infection with *M. bovis* has the effect of biasing the ensuing immune response towards PPD-A (Hope et al., 2005; Howard et al., 2002) (Coad et al., 2013; Jones et al., 2012). It is pertinent to state that under the conditions in this study, increasing the number of experimental animals may have provided the necessary numbers with which to obtain statistically significant data, compared with the number of animals used in the present experiment, which was based on results obtained in European cattle. Given that this is the first time that the model has been carried out under field conditions in Mexico, this is an important datum to bear in mind for future trials under similar conditions.

Nevertheless, our data indicate that under conditions in which exposure to environmental mycobacteria is suspected, the BCG challenge model could be a useful tool provided the number of animals per group being tested is increased. We have also established that the responses to mycobacteria induced by BCG vaccination are different, at least in terms of secretion of IFNγ by mycobacteria-stimulated peripheral blood cell between Zebu and HF cattle.

ACKNOWLEDGEMENTS

The authors wish to dedicate this manuscript to the memory of our friend and colleague Omar Antonio Alcaraz-López, whose life has been sadly claimed by the currently ongoing COVID-19 pandemic. This work was funded by the Consejo Nacional de Ciencia y Tecnología, México, through an Institutional Links grant: FONCICYT 277863; and the Birtish Council, through Institutional Links Newton Grant 276305277. The authors wish to thank colleagues at INIFAP, Queretaro for the excellent care and upkeep of the animals used in this experiment and all their help with the collection and processing of samples. The authors also wish to thank Mr Alejandro Nava and Ms Itzel Jiménez for their help in the handling of the animals at the time of inoculation. BVR and MV are Sêr Cymru II Professors at IBERS, AU, funded by the European Research Development Fund and Welsh Government.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

DATA AVAILABILITY STATEMENT

All data generated in this work are supplied in the figures.

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How to cite this article: Alcaraz-López OA, Flores-Villalva S, Cortéz-Hernández O, et al. Association of immune responses of Zebu and Holstein-Friesian cattle and resistance to mycobacteria in a BCG challenge model. *Transbound Emerg Dis*. 2020;00:1–6. https://doi.org/10.1111/tbed.13939