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

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Abstract

Mycobacterium bovis is the main causative agent of bovine tuberculosis (BTB) in cattle and it is also responsible for a proportion of human TB cases. The annual cost of BTB worldwide is estimated at US$3 billion. Zebu cattle are considered to be more resistant to some infectious diseases than Holstein-Friesian (HF) cattle, including BTB. However, epidemiological studies do not necessarily take into account usage differences of the two types of cattle. It could be argued that HF cattle suffer greater metabolic stress due to their mainly dairy use, whereas Zebu cattle are mainly used for beef production. However, in experiments comparing Zebu and European cattle, the number of animals has been too small to draw statistically robust conclusions on the differences in the level of resistance between these breeds of cattle. Here, we used a recently developed vaccination-and-BCG challenge model to compare the ability of naïve and vaccinated Zebu and HF cattle to control/kill mycobacteria. Young male cattle of both breeds with similar ages were housed in the same accommodation for the duration of the experiment; after correcting for multiple comparison, we found that there was a trend for vaccinated HF cattle to have lower cfu numbers than non-vaccinated HF cattle ($\rho = 0.057$). No such trend was observed between vaccinated and non-vaccinated Zebu cattle ($\rho = 0.560$); similarly, no difference was observed between naïve HF and Zebu ($\rho = 0.862$) cattle. In contrast, evaluation of antigen-specific IFNγ secretion indicated that Zebu and HF cattle differed in their response to mycobacteria. Thus, under the conditions used in this work, the data indicate that there are no differences between Zebu and HF cattle. Further experiments, using larger numbers of animals may be required to determine whether Zebu and HF cattle differ in their susceptibility to infection with M. bovis.

Introduction

Bovine tuberculosis (BTB) is a zoonosis caused mainly by*Mycobacterium bovis* . BTB results in productivity loss, 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, Palmer, Thacker, Vordermeier, & Waters, 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 in many low- and middle-income countries (LMIC), in which this type of control is 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); widely used to vaccinate humans against tuberculosis. Like in humans, BCG has also shown variable efficacy in cattle, both at population and individual animal levels (H. M. Vordermeier, Jones, Buddle, Hewinson, & Villarreal-Ramos, 2016). The reasons for this variability are largely unknown, although pre-sensitisation with environmental mycobacteria interfering with BCG-induced immunity has been put forward as a possible explanation (Brandt et al., 2002) (Buddle, Wards, Aldwell, Collins, & de Lisle, 2002; Hope et al., 2005).

Humped cattle (Zebu, *Bos taurus* ssp. *indicus*) are considered more resistant to some infectious diseases, including BTB, than non-humped cattle (*B. taurus* ssp *taurus*) (Murray et al., 2013). Studies in Ethiopia have shown that Zebu cattle have a lower prevalence of skin test positivity under similar husbandry settings than 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 (M. Vordermeier et al., 2012). However, these preliminary data using small numbers of animals need to be confirmed. Previous experiments compared the protection conferred by BCG vaccination between Zebu and European cattle breeds; whilst Zebu cattle were better protected, the number of European and crossbreed cattle used was too small to allow to draw of statistically robust conclusions on whether BCG-vaccinated Zebu cattle were differentially protected compared to other breeds (Ellwood & Waddington, 1972).

Therefore, in this study we set out to compare 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 to control mycobacteria *in vivo* using age- and gender-matched animals housed at the same location for the duration of the experimental period.

Materials and Methods

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.

Cattle

Thirty-two Zebu cattle and 31 Holstein-Friesian (HF) cattle were sourced from farms free of TB. 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). Zebu cattle varied between 2 and 8 months of age with a median age of 3 months, whilst HF cattle varied between 4 and 7 months of age with a median age of 6 months.

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.

Vaccination and challenge experiments.

Sixteen Zebu cattle and 16 Holstein-Friesian cattle were vaccinated with*c* $1x10^6$ 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 $1x10^7$ cfu each. 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%.

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, U.K.) for 2 min with 7 ml of PBS. One hundred µl of LN macerate was spread in each of 2 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 the line indicating the limit of detection.

Evaluation of immune 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 from *Mycobacterium avium* (PPD-A) or *M. bovis* (PPD-B), or medium alone as negative control (Villarreal-Ramos et al., 2014). Levels of IFNγ were determined by using the Bovigam assay (Prionics). Data are shown as mean ± SEM.

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 Kruskall Wallis with Dunn's correction for multiple comparisons was carried out. For IFNγ secretion results were analysed using Mann-Whitney ANOVA.

Data availability

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

Results

Evaluation of bacterial load

Figure 1 shows the bacterial load in LN of control and vaccinated cattle recovered at three weeks after challenge. Statistical analysis correcting for multiple comparisons indicated that there was a strong trend, almost reaching statistical significance (P = 0.057), for a reduced recovery of mycobacteria from BCG vaccinated HF cattle compared to non-vaccinated HF cattle. No such trend was observed in the number of mycobacteria recovered from vaccinated or non-vaccinated Zebu cattle (P = 0.5606). Similarly, no difference in the number of recovered mycobacteria was detected between non-vaccinated HF and non-vaccinated Zebu cattle (P = 0.8622).

Immune responses

Figure 2 shows immune responses, detected as secretion of IFNγ by peripheral blood cells stimulated with 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. 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 to responses at week 0 in any of the animal groups regardless of their vaccination status. In non-vaccinated HF cattle, responses to PPD-A were greater than responses to PPD-B at week 8; in non-vaccinated Zebu cattle, responses to PPD-A were greater than responses to PPD-B at week 4; responses to PPD-A in HF vaccinated cattle were greater than the responses to PPD-B at weeks 4 and 8; and in vaccinated Zebu cattle responses

to PPD-A were greater than responses to PPD-B at week 8. Interestingly, responses to PPDA or PPDB 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 $c\ 10^7$ 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 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 not vaccinated 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, i.e. no difference in responses at weeks 9 and 10 compared to responses observed in week 8. In contrast, in non-vaccinated Zebu cattle, intranodal challenge induced a statistically significant increase in the responses to PPD-A at week 9 ($\rho < 0.001$) but not at week 10; intranodal inoculation in the same group of animals induced a statistically significant increase in responses to PPD-B at weeks 9 ($\rho < 0.001$) and 10 ($\rho < 0.05$).

Discussion

In this work, we have compared the relative ability of Zebu and HF cattle to control BCG *in vivo* in an intranodal BCG challenge model (Villarreal-Ramos et al., 2014). We have also evaluated the immune responses of Zebu and HF cattle following vaccination and then intranodal challenge with BCG.

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 not vaccinated groups. It is possible that environmental conditions, such as prior exposure to environmental mycobacteria (see discussion of IFNγ data below), may have conferred a degree of protection to all animals prior to BCG vaccination which 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. It is pertinent to state that under these conditions, increasing the number of experimental animals may have provided the necessary numbers with which to obtain statistically significant data. Given that this is the first time that the model has been carried out under these conditions, this is important data to bear in mind for future trials.

The data also indicated that there was no statistically significant difference in the number of mycobacteria recovered from vaccinated Zebu cattle compared to 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. The current data would appear to suggest that HF cattle can be more effectively protected by BCG vaccination than Zebu cattle.

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 would indicate that cattle had been and/or were concomitantly being exposed to environmental mycobacteria. 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, Kwong, Villarreal-Ramos, Sopp, & Hope, 2002) (Coad, Clifford, Vordermeier, & Whelan, 2013; Jones, Whelan, Clifford, Coad, & Vordermeier, 2012),; it is possible that BCG vaccination boosted or synergistically increased immune responses to antigens shared with environmental mycobacteria.

In non-vaccinated animals, particularly non-vaccinated HF calves, responses to PPD-A increased at weeks 4 and 8 compared to week 0, which could be taken as confirmation that animals were responding to an on-going infection with environmental mycobacteria; however, whilst column medians were different, no statistically significant difference was detected between responses observed at week 0 with those observed at weeks 4

or 8 once corrections for multiple comparison were performed; nevertheless, the data indicated that these animals may also have been exposed to environmental mycobacteria and had therefore mounted an immune response to PPD-A. In the case of non-vaccinated Zebu cattle, as it would be expected with control animals, no significant responses to PPD-A or PPD-B were detected at weeks 4 and 8 compared to those observed at week 0.

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 (supplementary figure 1); it has been shown that the magnitude of the response to mycobacteria is positively correlated to antigen load, which could be an indicator of mycobacterial load, in this case *M. avium* . 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. A study aimed at determining the prevalence of *M. avium* infection in Uganda cattle, reported that*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, Reinacher, Loukopoulos, & Ojok, 2013). These data suggest that indeed, HF cattle may be more susceptible to *M. avium* than Zebu cattle.

In conclusion, the observed immune responses appear to indicate that prior to, or after BCG vaccination, cattle had been exposed to environmental mycobacteria. Prior exposure to environmental mycobacteria may have conferred a degree of protection against subsequent mycobacterial exposure, which may have reduced the power of differentiating between vaccinated and non-vaccinated animals by the BCG-challenge model. Nevertheless, the data indicate that under conditions under which *M. avium* exposure 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. To our knowledge, this is the first study that shows, in a statistically significant manner, that Zebu and HF cattle mount different responses to mycobacterial antigens.

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Conflict of interests

The authors declare no conflicts of interests.

References

Ameni, G., Aseffa, A., Engers, H., Young, D., Gordon, S., Hewinson, G., & Vordermeier, M. (2007). High prevalence and increased severity of pathology of bovine tuberculosis in Holsteins compared to zebu breeds under field cattle husbandry in central Ethiopia. *Clin Vaccine Immunol, 14* (10), 1356-1361. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17761523. doi:10.1128/CVI.00205-07

Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R., & Andersen, P. (2002). Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect Immun, 70* (2), 672-678. Retrieved from

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11796598

Buddle, B. M., Wards, B. J., Aldwell, F. E., Collins, D. M., & de Lisle, G. W. (2002). Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine, 20* (7-8), 1126-1133. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11803073.

Coad, M., Clifford, D. J., Vordermeier, H. M., & Whelan, A. O. (2013). The consequences of vaccination with the Johne's disease vaccine, Gudair, on diagnosis of bovine tuberculosis. *Vet Rec, 172* (10), 266. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23475045. doi:10.1136/vr.101201

Ellwood, D. C., & Waddington, F. G. (1972). A second experiment to challenge the resistance to tuberculosis in B.C.G. vaccinated cattle in Malawi. *Br Vet J, 128* (12), 619-626. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4567264.

Gallagher, J., & Horwill, D. M. (1977). A selective oleic acid albumin agar medium for the cultivation of Mycobacterium bovis. *J Hyg (Lond), 79* (1), 155-160. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/328766.

Hope, J. C., Thom, M. L., Villarreal-Ramos, B., Vordermeier, H. M., Hewinson, R. G., & Howard, C. J. (2005). Exposure to Mycobacterium avium induces low-level protection from Mycobacterium bovis infection but compromises diagnosis of disease in cattle. *Clin Exp Immunol, 141* (3), 432-439. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16045732. doi:CEI2882 [pii]

10.1111/j.1365-2249.2005.02882.x

Howard, C. J., Kwong, L. S., Villarreal-Ramos, B., Sopp, P., & Hope, J. C. (2002). Exposure to Mycobacterium avium primes the immune system of calves for vaccination with M. bovis BCG. *Clinical and Experimental Immunology., 130* (2), 190-195.

Jones, G. J., Whelan, A., Clifford, D., Coad, M., & Vordermeier, H. M. (2012). Improved skin test for differential diagnosis of bovine tuberculosis by the addition of Rv3020c-derived peptides. *Clin Vaccine Immunol, 19* (4), 620-622. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/22301696. doi:10.1128/CVI.00024-12

Maggioli, M. F., Palmer, M. V., Thacker, T. C., Vordermeier, H. M., & Waters, W. R. (2015). Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle.*PLoS One, 10* (4), e0122571. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25879774. doi:10.1371/journal.pone.0122571

Murray, G. G., Woolhouse, M. E., Tapio, M., Mbole-Kariuki, M. N., Sonstegard, T. S., Thumbi, S. M., . . . Hanotte, O. (2013). Genetic susceptibility to infectious disease in East African Shorthorn Zebu: a genome-wide analysis of the effect of heterozygosity and exotic introgression. *BMC Evol Biol, 13* , 246. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/24209611. doi:10.1186/1471-2148-13-246

Okuni, J. B., Reinacher, M., Loukopoulos, P., & Ojok, L. (2013). Prevalence and spectrum of Johne's disease lesions in cattle slaughtered at two abattoirs in Kampala, Uganda. *Trop Anim Health Prod, 45* (5), 1197-1202. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23274696. doi:10.1007/s11250-012-0346-3

Villarreal-Ramos, B., Berg, S., Chamberlain, L., McShane, H., Hewinson, R. G., Clifford, D., & Vordermeier, M. (2014). Development of a BCG challenge model for the testing of vaccine candidates against tuberculosis in cattle. *Vaccine, 32* (43), 5645-5649. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25138291. doi:10.1016/j.vaccine.2014.08.009

Vordermeier, H. M., Jones, G. J., Buddle, B. M., Hewinson, R. G., & Villarreal-Ramos, B. (2016). Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. *Annu Rev Anim Biosci, 4* , 87-109. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/26884103. doi:10.1146/annurev-animal-021815-111311

Vordermeier, M., Ameni, G., Berg, S., Bishop, R., Robertson, B. D., Aseffa, A., . . . Young, D. B. (2012). The influence of cattle breed on susceptibility to bovine tuberculosis in Ethiopia. *Comp Immunol Microbiol Infect Dis, 35* (3), 227-232. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/22304898. doi:10.1016/j.cimid.2012.01.003

Figure legends.

Figure 1. Evaluation of bacterial load in the prescapular LN of HF and Zebu cattle.

HF (and) and Zebu (and) cattle were vaccinated (and) or not (and) as described in figure 1 and 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 Zebu and HF cattle to mycobacterial antigens. Immune responses were evaluated as the secretion of IFNγ by peripheral blood cells from HF (A) and Zebu (B) cattle that had been vaccinated (white and horizontally hashed bars) with $c10^6$ BCG SSI cfu subcutaneously or not (black and diagonally hashed bars). Cells were stimulated with PPD-A or PPD-B. 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.

Supplementary figure 1.

Longitudinal immune responses in Zebu and HF cattle to PPD-A. Immune responses were evaluated as the secretion of IFNγ by peripheral blood cells stimulated with PPD-A from HF (black and horizontally hashed bars) and Zebu (white and diagonally hashed bars) cattle that had been vaccinated (black and white bars) with c 10^6BCG SSI cfu subcutaneously or not (horizontally and diagonally hashed bars). 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.

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