Cattle Husbandry in Ethiopia Is a Predominant Factor Affecting the Pathology of Bovine Tuberculosis and Gamma Interferon Responses to Mycobacterial Antigens

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Bovine tuberculosis is a major economic problem and a potential public health risk. Improved diagnostics like the gamma interferon (IFN-γ) test with ESAT6 and/or CFP10 could contribute to the control program. We assessed IFN-γ responses in zebu (Ethiopian Arsi breed) and Holstein cattle kept indoors or in a pasture to tuberculin purified protein derivative (PPD) and an ESAT6- CFP10 protein cocktail. Furthermore, the intensity and distribution of pathology of bovine tuberculosis were compared between the two breeds. Our data demonstrated significantly (all \( P < 0.02 \)) higher IFN-γ responses to avian PPD, bovine PPD, and the ESAT6-CFP10 protein cocktail in Holstein than in zebu cattle, while lesion severities in infected animals and tuberculin skin test responses did not differ significantly (\( P > 0.05 \)) between the two breeds. Holstein cattle that were kept indoors produced significantly (all \( P < 0.01 \)) higher IFN-γ levels in response to avian PPD, bovine PPD, and the ESAT6-CFP10 protein cocktail than did Holstein cattle kept in a pasture. Moreover, lesion severity was significantly higher in Holstein cattle kept indoors (\( P = 0.001 \)) than in those kept in the pasture. Lesions were localized predominantly in the digestive tract in cattle kept in a pasture, while they were localized in the respiratory tract in cattle kept indoors. In conclusion, in Holstein cattle, husbandry was a dominant factor influencing the severity of tuberculosis lesions and IFN-γ responses to mycobacterial antigens compared to breed. A difference in the cellular immune response between zebu and Holstein cattle was observed, while tuberculosis lesion severities were identical in the two breeds, when both were kept in a pasture.

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Human tuberculosis (TB) of animal origin, particularly that caused by *Mycobacterium bovis*, is becoming increasingly important in developing countries (24, 35). In sub-Saharan Africa, humans and animals share the same microenvironment and water holes, especially during droughts and the dry season, thereby potentially promoting the transmission of *M. bovis* from animals to humans. According to Cosivi et al. (13), 60% of the African, 47% of the Asian, and 38% of the Latin American and Caribbean countries have reported the occurrence of bovine TB from sporadic to enzootic levels. Approximately 85% of the cattle and 82% of the human populations of Africa live in areas where bovine TB is either partly controlled or not controlled at all (13). In such countries, where bovine TB is still common and pasteurization of milk is not practiced, an estimated 10 to 15% of human TB cases are caused by *M. bovis*. In cattle, BCG has been used in a series of experiments with various degrees of protection against *M. bovis* challenge (7, 10) where a significant level of protection in BCG-vaccinated cattle against *M. bovis* infection has been demonstrated.

However, a major constraint on the use of attenuated mycobacterial vaccines, such as BCG, is that vaccination of cattle interferes with the detection of TB by means of the tuberculin skin test. On the other hand, antigens such as early secretory target antigen 6kD (ESAT6) and culture filtrate protein 10kD (CFP10) have been identified and found to be effective as differential diagnostic reagents since they are able to differentiate *M. bovis* infection from BCG vaccination (8, 12, 16, 25, 30). The in vitro gamma interferon (IFN-γ) assay, which was developed in 1990 (32), has been evaluated with purified protein derivates (PPDs) in different geographic locations (2, 3, 28, 33, 34), and an ESAT6- and/or CFP10-based IFN-γ assay was found to be more specific than a PPD-based IFN-γ assay (8, 29, 30). However, most of the studies were performed with cattle of European origin, such as Holstein cattle, which belong to the *Bos taurus taurus* group of breeds. In contrast, the predominant breeds in Africa and Asia are humped cattle of the *B. taurus indicus* breed groups. As it is conceivable that...
different breeds display different strengths of cellular immune response, the present study was designed to evaluate IFN-γ responses in Holstein and Arsi (B. taurus indicus) cattle with confirmed bovine TB in Ethiopia. In addition, the husbandry system by which cattle are kept could influence the IFN-γ response to these antigens. To address some of these questions, we investigated lesion distribution and disease severity in cattle of different breeds kept under different husbandry conditions.

MATERIALS AND METHODS

Study animals. This study was conducted with 91 head (54 Holstein and 37 zebu) of skin test-positive cattle. The study animals were obtained from two districts of Ethiopia, namely, Holeta and Selalle. Cattle husbandry consisted of zero grazing (intensive farming) in Holeta, where 29 Holstein cattle were recruited, and free grazing in a pasture in Selalle, where 25 Holstein cattle and 37 zebus were obtained in Selalle. In Selalle, both the Holstein cattle and zebus were obtained from the same villages and had been grazing on communal pastureland. Further, in most cases, the two breeds and their crossbred hybrids were kept together in a single herd. The 29 Holstein cattle obtained from a government farm in Holeta were kept under intensive farming conditions. This farm has a known history of bovine TB, and at the time of this study, 47.8% of its animals were positive reactors in the comparative intradermal tuberculosis test.

Comparative intradermal tuberculosis test. Two sites on the skin on the right side of the mid-neck of the animal, 12 cm apart, were shaved, and skin thickness was measured with a caliper. One site was injected with an aliquot of 0.1 ml of 2,500 IU/ml bovine PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom), into the dermis, and the other was similarly injected with 0.1 ml of 2,500 IU/ml avian PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom). After 72 h, the skin thickness at the injection sites was measured. Results were interpreted according to the recommendations of the Office International des Epizooties (23). Briefly, when the change in skin thickness was greater at the avian PPD injection site, the animal was considered positive for mycobacterial species other than the mammalian type (M. tuberculosis and M. bovis). When increases were observed at both injection sites, the difference between the two reaction sizes was considered. Thus, if the increase in skin thickness at the injection site for bovine PPD (B) was greater than the increase in skin thickness at the injection site for avian PPD (A) and B minus A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, suspect, or positive for bovine TB, respectively.

Whole-blood culture and Bovigam IFN-γ assay. Blood samples were collected from the jugular vein into heparinized Vacutainers and transported to the laboratory within 8 h of collection. Whole blood was dispensed at a 250-μl volume into the wells of 96-well flat-bottom culture plates. Antigens were added in 25-μl aliquots to give the following final assay concentrations: avian PPD, 10 μg/ml; bovine PPD, 10 μg/ml (both tuberculins were obtained from the Veterinary Laboratories Agency, Weybridge, United Kingdom); ESAT6-CFP10 protein cocktail, each protein at 5 μg/ml (kindly provided by M. Singh, Braunschweig, Germany). Phytohemagglutinin (PHA; 5 μg/ml) and saline (25 μl) were used as positive and negative controls, respectively. Cultures were incubated at 37°C in a humid 5% CO2 atmosphere for 48 h, and supernatants were harvested and frozen. Levels of IFN-γ in the supernatants were measured by an enzyme-linked immunosorbent assay with the bovine IFN-γ (Bovigam) test kit (Commonwealth Serum Laboratories, Victoria, Australia) in accordance with the manufacturer's instructions.

Postmortem examination and pathology scoring. Skin test reactor cattle were bought and sacrificed, and detailed postmortem examinations were performed. The lungs and lymph nodes were removed for the investigation of tuberculous lesions. The seven lobes of the two lungs, including the (i) left apical, (ii) left cardiac, (iii) left diaphragmatic, (iv) right apical, (v) right cardiac, (vi) right bronchial, (vii) hepatic, and (viii) mesenteric lymph nodes, were sliced into thin sections (circum 2 mm thick) and inspected for the presence of visible lesions. When gross lesions suggestive of bovine TB were found in any of the tissues examined, the animal was classified as lesioned. Animals in which lesions were not found were classified as nonlesioned.

The severity of the gross lesions was scored by applying the semiquantitative procedure developed by Vordermeier et al. (29), with minor modifications to facilitate performance under field conditions. Briefly, lesions in the lobes of the lungs were scored separately as follows: 0, no visible lesions; 1, no gross lesions but lesions apparent on slicing of the lobe; 2, fewer than five gross lesions; 3, more than five gross lesions; 4, gross coalescing lesions. The scores of the individual lobes were added up to determine the lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0, no gross lesion; 1, a small lesion at one focus (just starting); 2, small lesions at more than one focus; 3, extensive necrosis. Individual lymph node scores were added up to calculate the lymph score. Finally, both lymph node and lung pathology scores were added up to determine the total pathology score per animal.

Isolation of mycobacteria. Isolation of mycobacteria from tissue was performed in accordance with Office International des Epizooties protocols (23). Briefly, tissue specimens for culture were collected in sterile universal bottles in 5 ml of a 0.9% saline solution and then transported to the laboratory. At the laboratory, they were sectioned into pieces with sterile blades and then homogenized with a pestle and a mortar. The homogenate was decontaminated by adding an equal volume of 4% NaOH by centrifugation at 3,000 rpm for 15 min. The supernatant was discarded, while the sediment was neutralized with 1% (0.1 N) HCl with phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow (23). Thereafter, 0.1 ml of suspension from each sample was spread onto a slant of Lowenstein-Jensen medium. Duplicates of Lowenstein-Jensen medium were used; one was enriched with sodium pyruvate, while the other was enriched with glycerol. Cultures were incubated aerobically at 37°C for about 5 to 8 weeks with weekly observation for growth of colonies.

Data analysis. Student’s t test was used to compare the mean optical density values of IFN-γ, the mean pathology scores, and the mean skin indurations post-comparative intradermal tuberculosis test. The chi-square test was used to analyze the variation in mycobacterial growth in the tissues of Holstein.
and zebu cattle. Linear-correlation analysis, with a nonparametric test (Spearman’s $r$, two tailed with the GraphPad Stats function), was performed with Prism 4.0 (GraphPad Software, San Diego, CA) for determination of the degree of association between the IFN-$\gamma$ response level and the severity of pathology.

RESULTS

IFN-$\gamma$ responses to mycobacterial antigens. Samples were obtained from 91 skin test-positive reactor cattle, 37 Arsi zebu cattle, and 25 Holstein cattle kept under low-intensity farming conditions in a pasture (Selalle region), as well as 29 Holstein cattle kept under intensive farming conditions (Holeta region). Of the 91 reactors, 83 had visible lesions typical of bovine TB postmortem and/or were culture positive for $M. bovis$. No significant difference (chi-square test, $P = 0.18$) in mycobacterial growth between tissues of Holstein and zebu cattle was observed. When responses of tuberculous Arsi and Holstein cattle kept under identical husbandry conditions (low intensity, in a pasture) were compared, it was evident that IFN-$\gamma$ responses to avian PPD (0.49 ± 0.10 versus 0.39 ± 0.07), bovine PPD (0.63 ± 0.11 versus 0.43 ± 0.07), and the ESAT6-CFP10 protein cocktail (0.43 ± 0.01 versus 0.30 ± 0.05) were significantly higher (for all antigens, $P < 0.02$) in Holstein than in Arsi cattle, while responses to the positive control PHA or to saline (i.e., no antigen) control wells were not significantly different between the two breeds (Fig. 1). These results are in contrast to the skin test responses, where no significant differences in reaction sizes were observed between zebu and Holstein cattle (Fig. 2, $P > 0.099, 0.145,$ and 0.33 for responses to the bovine and avian PPDs and the difference between the two tuberculins, respectively).

When Holstein cattle under different husbandry practices were compared, Holstein cattle that were kept indoors (high-intensity farming) were shown to produce significantly higher IFN-$\gamma$ levels in response to avian PPD (0.63 ± 0.10 versus 0.49 ± 0.10), bovine PPD (0.85 ± 0.14 versus 0.63 ± 0.11), and the ESAT6-CFP10 protein cocktail (0.56 ± 0.11 versus 0.43 ± 0.10) than Holstein cattle that were kept in a pasture (Fig. 3; all

FIG. 2. Comparative intradermal tuberculin test results for Holstein and zebu cattle. Changes in skin thickness after application of avian PPD and bovine PPD were measured in millimeters. In addition, the differences between reaction sizes at the bovine PPD injection site and the avian PPD injection site (PPD B – PPD A) are also shown.

FIG. 3. IFN-$\gamma$ response to mycobacterial antigens in Holstein cattle kept indoors or in a pasture. Whole-blood cultures were stimulated with avian PPD, bovine PPD (both used at 10 $\mu$g/ml), or an ESAT6-CFP10 protein cocktail (each protein at 5 $\mu$g/ml). Positive control, PHA (5 $\mu$g/ml). IFN-$\gamma$ was determined by the Bovigam enzyme immunoassay, and results are expressed as the mean ± the standard error of the mean. *, $P < 0.01$. OD, optical density.
antigens, \( P < 0.01 \).

**Pathology of bovine TB in relation to cattle breed and husbandry.** We next determined whether the severity of the pathology caused by bovine TB was affected by cattle husbandry. This was done by tabulating the disease severity with a semi-quantitative pathology scoring system (29). Our analysis demonstrated that disease severity was significantly greater in cattle kept indoors than in those kept at pasture (mean lesion scores, 9.77 ± 0.20 versus 6.0 ± 0.28; \( P = 0.001 \)), regardless of the cattle breed (data not shown). In addition, intrabreed variation in disease severity and lesion distribution was observed in that the severity of pathology was significantly greater in housed Holstein cattle than in Holstein cattle that were kept in a pasture (mean lesion scores, 9.13 ± 2.02 versus 7.44 ± 0.54; \( P = 0.002 \)). In contrast, the disease severities of tuberculous Holstein and Arsi cattle both kept in a pasture were not significantly different (mean lesion scores, 7.44 ± 0.54 versus 6.41 ± 0.55; \( P = 0.23 \)). Furthermore, while the lesion distributions between Holstein and Arsi cattle kept in a pasture were identical and more concentrated in the alimentary tract (i.e., mesenteric lymph nodes; see Fig. 4 for Holstein cattle in a pasture), we observed a significant difference in the lesion distribution in animals kept under high-intensity management (indoors) compared to those kept in a pasture. Figure 4 illustrates this point by comparing the pathology scores of Holstein cattle kept in a pasture or kept mainly indoors under high-intensity management. The Holstein cattle kept indoors had lesions predominantly in the lungs and in the lymph nodes draining the upper and lower respiratory tracts (\( P < 0.01 \) compared to pastured Holstein cattle), while, as stated above, digestive tract lesions were more prominently found in Holstein cattle kept in a pasture (\( P < 0.001 \) compared to housed Holstein cattle).

**Relationship between IFN-γ responses and pathology in cattle kept under different husbandry conditions.** Different relationships between in vitro IFN-γ responses and the pathology of bovine TB were observed between cattle kept mainly indoors and cattle grazing in a pasture. Whereas the pathology of bovine TB in grazing Holstein cattle (Fig. 5A) and pastured zebus (not shown) positively correlated with IFN-γ responses after stimulation with bovine PPD (Spearman’s \( r = 0.487 \), \( P = 0.018 \)), avian PPD, and the ESAT6 and CFP10 proteins (\( P < 0.05 \), data not shown), no positive correlation was found between pathology and IFN-γ production when Holstein cattle housed indoors were assessed, although this correlation was not statistically significant (Spearman’s \( r = -0.262 \), \( P = 0.1945 \)) (Fig. 5B). Interestingly, skin test results—assessing either PPD B-induced results or the differences between PPD B- and PPD A-induced responses—did not correlate in pastured or housed Holstein cattle (Spearman’s \( r \) values of –0.124 to 0.112, \( P \) values of 0.488 to 0.803).
DISCUSSION

The level of IFN-γ responses to the tested mycobacterial antigens was significantly lower in Arsi cattle, a zebu B. taurus indicus breed, than in Holstein cattle (B. taurus taurus) kept under the same husbandry conditions. The difference in IFN-γ responses in zebu and Holstein cattle maintained under identical conditions could be due to the different BoLA alleles in the two breeds affecting the recognition of mycobacterial antigens. Although this appears not to be the most likely explanation as tuberculin responses (i.e., responses to a complex mixture of mycobacterial antigens) were equally lower in the zebu cattle studied, further investigation of the repertoire of mycobacterial antigens recognized by these two breeds, as well as the qualitative nature of the immune responses with respect to, e.g., other cytokines, will nevertheless be interesting. It is also noteworthy that the IFN-γ responses observed in Holstein cows in Ethiopia were considerably lower than those reported for Holstein cattle in the United Kingdom, Ireland, or New Zealand (6, 29). A likely explanation could be that a higher proportion of Holstein cattle in Ethiopia suffer from far advanced disease. Since the test-and-slaughter-based control method is not applied in Ethiopia, the disease could progress longer with a greater proportion of animals reaching a more severe disease status. In addition, multiple parasitic infections, which prevail in the study population (personal observation), could also modulate the IFN-γ responses to mycobacterial antigens. For example, a previous study in Ethiopia showed that infection with either Fasciola sp. or Strongylus sp. significantly reduced skin inductions in response to bovine PPD in M. bovis-infected heifers compared to M. bovis-infected heifers that had been dewormed before skin testing (1).

Our data also suggest that by lowering the cutoffs for sensitivity of the IFN-γ test, one could achieve comparable sensitivity levels between zebu and Holstein cattle (see below), although this will inevitably have an impact on test specificity. However, assessing absolute test sensitivity and specificity was not an objective of the present study, nor are the animal groups studied useful to pursue such objectives. Our data, however, did allow us to estimate the sensitivity of the IFN-γ test in relation to that of the comparative tuberculin test (relative sensitivity). Around 52% of the skin test-positive Holstein cattle (with an optical density at 450 nm of 0.1 as the cutoff) and around 46% of the skin test-positive zebu cattle (with an optical density at 450 nm of 0.05 as the cutoff) tested positive when tuberculin was used as the test antigen in the IFN-γ test. These values are relatively low compared to those of studies in other countries, which were conducted mainly in high-income countries in Europe, Australia, and North America (reviewed in reference 14). The full impact of these data cannot be assessed, however, without further studies, which are needed to determine absolute sensitivity and specificity. This is particularly important as the skin test and IFN-γ test are known to target not completely overlapping populations of infected animals (17), and it is therefore likely that a higher percentage of infected animals would be detected by the IFN-γ test than the around 50% of skin test-positive cattle (encompassing both skin test-positive and skin test-negative populations); i.e., its true sensitivity is likely to be higher. The notion that not completely overlapping populations of animals were detected by tuberculin skin testing and the IFN-γ test is also highlighted in this study because no correlation between the two responses was found (data not shown). Nor did the severity of disease (pathology scores) correlate with the extent of skin test responses, while a statistically significant correlation was observed between pathology scores and the IFN-γ test in the pastured Holstein cows (see also below). Therefore, arguably the most beneficial use of these tests would be in parallel to maximize the detection of infected animals (17). Thus, to determine the true sensitivities and specificities of both tests under Ethiopian conditions, further large-scale studies including both truly TB-free animals and skin test-negative and/or IFN-γ test-negative M. bovis-infected cattle are needed to test performance.

In addition to breed, cattle husbandry was found to be an important factor affecting the intensity and distribution of the pathology of bovine TB, as well as the strength of antigen-specific IFN-γ responses. The severity of bovine TB was significantly greater in cattle kept indoors at a higher population density than in those kept in a pasture. This is because, as previously stated (26), housing predisposes cattle to TB; the closer animals are packed together, the greater the chance that TB will be transmitted. Apart from physical factors like close contact facilitating the transmission of infective aerosols between animals, it is also possible that stress caused by overcrowding or nutritional differences between housed and pastured animals contributed to the severe disease found in housed Holstein cattle. Furthermore, a major difference in lesion distribution was observed between animals farmed intensively and those kept in a pasture under low-intensity management. Animals in intensive farms had lesions predominantly in the respiratory tract, whereas digestive tract lesions predominated in animals kept in a pasture. One could hypothesize different transmission routes of bovine TB depending on the methods of cattle husbandry used; inhalation could be the most likely route of infection in cattle kept indoors, and pathogen ingestion could be the most likely route of infection in cattle grazing outside in a pasture. Furthermore, one could hypothesize that fecal excretion of bacilli leads to pasture contamination, in contrast to respiratory shedding of aerosolized bacilli by cattle kept under high-intensity farming conditions at a higher population density. However, the present study cannot provide evidence for either hypothesis but rather highlights the need for further studies, for example, of environmental sampling for tubercle bacilli to test the validity of these hypotheses.

The relationship between the pathology of bovine TB and the antigen-specific IFN-γ response level varied between animals kept indoors and those kept in a pasture. A statistically significant positive correlation was observed between the IFN-γ response to mycobacterial antigens and the pathology of bovine TB in cattle kept in a pasture. In contrast, for Holstein cattle kept indoors, such a positive correlation was not observed. Instead, we found a negative trend between IFN-γ responses to mycobacterial antigens and the pathology of bovine TB, although this negative relationship was not statistically significant. Interestingly, in line with our results observed in cattle kept in a pasture, Vordermeier et al. (29) also reported a significant positive correlation between IFN-γ re-
sponses and the pathology of bovine TB in experimentally infected calves. The observed contrast between our findings in relation to animals kept indoors and the data reported by Vordermeier et al. (29) may be a reflection of differences in disease status and severity. The latter animals had considerably lower mean pathology scores than a proportion of the housed Holstein cattle in our study. However, in accordance with Vordermeier et al. (29), the proportion of housed Holstein cattle assessed in the present study with relatively low pathology scores (below 5) displayed vigorous IFN-γ responses, and consequently, the responses overall across the housed group were higher than those for the group of Holstein cattle kept in a pasture. In addition, the proportion of the animals kept indoors that had more severe disease and higher pathology scores displayed lower IFN-γ responses than the animals kept in a pasture and the lesions of these pastured Holstein cattle were relatively mild or contained. These latter findings are therefore more in line with those of other studies that described changes in the nature of the immune responses with disease progression, from a dominant Th1-type response at early stages of disease toward B-cell antibody production and lower T-cell responses at more-progressive disease stages (5, 9, 11, 15, 18, 19, 20), or a positive correlation between antibody responses and pathology (22, 27, 31). A consequence of lower cell-mediated immune responses in animals with severe disease could be their escape from detection by cell-mediated immune response-based tests like the IFN-γ test. However, our study was not aimed at assessing such “anergic” animals as all of the animals studied were skin test positive. Consequently, no negative relationship between skin test responses and pathology could be observed. Defining the epidemiological significance of the observed lower IFN-γ responses in cattle with higher pathology scores with respect to this allowing animals to escape diagnosis needs to be the subject of future large-scale studies.

In conclusion, the results of this study indicated that while the breed of cattle is a major factor influencing in vitro IFN-γ responses to mycobacterial antigens, cattle husbandry and cattle density are the dominant factors influencing disease severity and the distribution of the lesions in the Holstein cattle studied. Therefore, in parallel with efforts to develop effective novel diagnostic tools and vaccines, improvement of cattle husbandry systems by minimizing crowding and improving ventilation could also contribute significantly to the control of bovine TB.

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