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Advancing the development and implementation of regional, national tuberculosis control programs in livestock in africa, asia, and latin america

Edited by
Douwe Bakker, Joram Josephat Buza, Julio Alvarez and Vivek Kapur

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Advancing the development and implementation of regional, national tuberculosis control programs in livestock in africa, asia, and latin america

Topic editors

Douwe Bakker — Independent researcher, Netherlands

Joram Josephat Buza — Nelson Mandela African Institution of Science and Technology, Tanzania

Julio Alvarez — VISAVET Health Surveillance Centre (UCM), Spain

Vivek Kapur — The Pennsylvania State University (PSU), United States

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EDITED AND REVIEWED BY
Salome Dürr,
University of Bern, Switzerland

*CORRESPONDENCE
Douwe Bakker
douwe.bakker@kpnmail.nl

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Editorial: Advancing the development and implementation of regional, national tuberculosis control programs in livestock in Africa, Asia, and Latin America

Douwe Bakker[1,2]*, Joram Josephat Buza[3], Julio Álvarez[4,5] and Vivek Kapur[2,3]

[1]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain, [2]School of Life Sciences and Bioengineering, The Pennsylvania State University (PSU), University Park, State College, PA, United States, [3]Huck Institutes of the Life Sciences, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania, [4]VISAVET Health Surveillance Centre (UCM), Madrid, Spain, [5]Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States

KEYWORDS

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Editorial on the Research Topic
Advancing the development and implementation of regional, national tuberculosis control programs in livestock in Africa, Asia, and Latin America

Introduction

Tuberculosis in livestock caused by members of the *Mycobacterium tuberculosis* (MTb) complex is a notifiable zoonotic animal disease (1), which has been eradicated or held to very low prevalence levels in many high-income economies. Successful campaigns were all build on a very strict test-and-slaughter strategy using the tuberculin PPD skin tests as diagnostic tool. However, tuberculosis in livestock remains endemic in most Low- and Middle-Income Countries (LMICs). This not only represents a threat to public health in those countries but also places a significant burden on their economies due to a negative impact on livestock productivity and the resources invested in healthcare, prevention, surveillance, and, when present, control and/or eradication programs. Moreover, tuberculosis in livestock affects a wide variety of species as well as breeds, raised in a wide variety of farming systems, in a broad range of different climates, thus ruling out a "one size fits all" approach for disease control. Since "traditional" test and cull programs are costly, very demanding on the livestock holder and may be ruled out as option for religious reasons, such programs must be tailored to ensure they are fit for purpose considering the respective socio-economic context in which they have to be implemented in each country.

This Research Topic includes a variety of articles addressing some of the aspects of the design, description, evaluation and monitoring economic benefits/costs of surveillance and/or eradication programs targeting tuberculosis in livestock in LMICs.

Mammalian tuberculosis

In 2022 the World Organization for Animal Health (WOAH) replaced the chapter for Bovine Tuberculosis in their Terrestrial Animal Health Manual with a new chapter on Mammalian Tuberculosis, thereby recognizing the fact the original definition of bovine tuberculosis, which was restricted to tuberculosis in bovines caused by *Mycobacterium bovis* (Mb), was no longer fit for purpose. Tuberculosis in bovines was shown to be also caused by other recently characterized new members of the MTb complex whereas at the same time Mb was shown to cause tuberculosis in wide variety of other mammalian hosts.

Two articles in this Research Topic further illustrate this issue (Ambaw et al.; Barandiaran et al.). Barandiaran et al. investigates the epidemiology of tuberculosis in pigs in Argentina caused by a molecular diverse population of Mb strains, which share 60% of their genotypes with Mb strains isolated from local cattle and wild boar, suggesting transmission at the interface between pigs, cattle and wild boar. This is a further indication that the control of Tb in other susceptible mammalian hosts should not be ignored while attempting to control and eradicate Tb from bovines. In the second article by Ambaw et al. evidence is provided for a difference in disease susceptibility between three cattle breeds in Ethiopia. A significant difference in skin test reactivity and pathology severity between infected animals belonging to the respective breeds housed on the same farm was observed. Moreover, in addition to the 14 Mb isolates recovered from the lesions, two *M. tuberculosis* (M. tb) isolates were recovered from lesions as well. Thereby it is illustrating the fact that other members of the MTb-complex can cause tuberculosis in cattle with an identical pathology.

Diagnostics and epidemiology

The diagnosis of tuberculosis in livestock as well as the detection and the characterization of its causative agents are described in detail in the aforementioned chapter of the WOAH Terrestrial Animal Health Manual (1).

The prescribed *ante mortem* tests used are the tuberculin intradermal tests, respectively the comparative cervical test (CCT), the single cervical test (SCT) and the caudal fold test (CFT), and the *in vitro* interferon gamma release assay (IGRA). Two of the articles in this Research Topic [Kelly et al. (a); Kelly et al. (b)] evaluate the use of both tests and the application of one of them, the IGRA, in sub-Saharan Cameroon. In Kelly et al. (a) cattle were sampled in two different regions and the results of the IGRA and the CCT were compared: in one of the regions the agreement between both tests was fair, whereas in the other region the agreement was poor-moderate. The main source of test disagreement was animals testing positive by IGRA and negative by CCT. The authors concluded that an individual or combined use of these tests could lead to a large variation in TB estimates in different settings and/or trials. The same authors subsequently used the IGRA in Kelly et al. (b) to describe the epidemiology of Tb in dairy herds in Cameroon and the potential public health risk from those dairy herds.

In a longitudinal study by Tschopp, Gemechu et al. the productivity of intensive dairy herds in Central Ethiopia was assessed and found to be sub-optimal. Subsequently, Tschopp, Conlan et al. used the CCT as a proxy of disease status to analyse the effect of tuberculosis on the productivity and movement of dairy cattle in and between those herds. Test negative bulls were shown to be heavier than reactor bulls indicating a production loss due to Tb infection. Interestingly, it was also noted that test-positive animals were eliminated faster from the herd than test-negative animals. Not by slaughter, or by being separated from the test-negative animals but often by selling those test-positive animals directly to other farms, sometimes further away to other regions, posing a significant risk for further spread of Tb. This highlights the need for stricter guidelines on the handling of Tb test positive animals and the urgent need for an animal identification and tracing system.

Traditional control and alternative approaches

As mentioned before, the traditional way to control and eradicate tuberculosis from a livestock holding is the test- and-cull approach using the tuberculin skin test (1). To assess the feasibility of such an approach in Ethiopia, a pilot study was performed by Lakew et al. Over a period of 6 years all cattle in an infected farm were tested by CCT and all test-positive animals were culled. In the fifth and final round of testing the number of CCT positives had dropped to almost zero. However, analyses of the skin test data applying the single cervical test (SCT) cut-off's, indicated that there was still a considerable level of residual Tb infection present in this herd, indicating that a prolonged control by test-and-cull would be need. Moreover, the high costs of culling and replacing considerable numbers of test positive animals confirmed that the test-and-cull strategy may be an impractical approach for a nationwide implementation in Ethiopia as well in most other LMICs.

In two articles, potential alternatives for the test-and-cull approach are described. Mazorra-Carrillo et al. compared serum proteins from Tb infected and non-infected cows and identified three proteins involved in inflammatory/immunomodulatory responses to infections being expressed at a higher level in non-infected or potentially resistant cows. These proteins might be useful as novel biomarkers for the breeding of cattle resistant to TB. Finally, Sirak et al. looked at the difference in the expression of immunological markers and gross pathology between *M. bovis BCG* vaccinated and non-vaccinated cross-breed calves in Ethiopia. Findings indicated stronger responses of a set of immunological cells and markers at local granulomas as well as a reduction of the severity of the gross pathology at the primary site of infection in vaccinated animals, thus indicating that (repeated) BCG vaccination could play a role in the control of tuberculosis by a gradual reduction of Tb infection in vaccinated herds.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Effect of Bovine Tuberculosis on Selected Productivity Parameters and Trading in Dairy Cattle Kept Under Intensive Husbandry in Central Ethiopia

Rea Tschopp [1,2,3], Andrew J. K. Conlan [2,4], Gizachew Gemechu [3], Gizat Almaw [5], Jan Hattendorf [1,2], Jakob Zinsstag [1,2], the ETHICOBOTS consortium and James L. N. Wood [4]*

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [2] University of Basel, Basel, Switzerland, [3] One-Health Unit, Armauer Hansen Research Institute, Addis Ababa, Ethiopia, [4] Disease Dynamics Unit, Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom, [5] National Animal Health Diagnostic and Investigation Center, Sebeta, Ethiopia

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Edited by:
Joram Josephat Buza, Nelson Mandela African Institution of Science and Technology, Tanzania

Reviewed by:
Lucy Brunton, Royal Veterinary College (RVC), United Kingdom
Rebecca Lee Smith, University of Illinois at Urbana-Champaign, United States

***Correspondence:**
Rea Tschopp
rea.tschopp@swisstph.ch

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Bovine tuberculosis (BTB) has substantial impact on fertility, milk, and meat productivity in cattle. However, these assumptions are based on outdated data. Recent global studies on the impact of BTB on cattle productivity are scarce and show sometimes inconclusive and/or contradicting results. This pilot study is the first longitudinal study performed in urban upgraded dairy cattle in Ethiopia that are kept under intensive husbandry. We assessed whether BTB has an impact on various animal productivity parameters and animal movement. Animals ($N = 890$) included in the study were tested for BTB at least once using the comparative intradermal tuberculin test (CIDT). Fertility, mortality, and offtake were assessed in 21 dairy farms where herd follow-ups over 3 years were performed. In addition, an independent abattoir survey was conducted to assess carcass weight and visible TB-like lesions upon meat inspection. Animal movements (purchasing and offtakes) were documented for each farm. The impact of BTB status on the intervals been birth, service, and calving times and the intercalving intervals was analyzed using a Cox proportional hazards model. The hazard ratio associated with BTB-positive animals was smaller than 1 for all fertility parameters, suggesting that BTB status increases the time between events; however, the effect was small and only statistically significant (95% level) for the time between calving and service. Offtakes included a higher percentage of reactor animals (58%) as compared with non-reactor animals (42%) ($p = 0.0001$). Overall, reactors were eliminated from the farms within 238.6 days after receiving test results, which was 54.9 days faster than for negative animals. The majority of owners purchased animals within their town or its surrounding. Nearly a quarter of reactors were sold directly to other farms. Animals were also sold further away, including other regions, raising the question of disease spread and the need for an animal tracing mechanism. In the abattoir survey, a total of 349 carcasses were weighed, of which 8% showed visible TB-like lesions and 53.6% had fasciolosis. Negative adult bull carcasses were 7.5 kg heavier than reactor bulls.

Keywords: Ethiopia, dairy cattle, productivity, bovine tuberculosis, disease impact

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic bacterial zoonotic disease caused by *Mycobacterium bovis*, with cattle being the primary host. However, a wide-range of domesticated and wild mammal species as well as humans can become infected with BTB through direct contact with a positive host or consumption of raw animal products (1, 2).

Besides being an animal and public health concern, BTB has globally substantial direct and indirect economic implications related to direct impacts on animal health and productivity, surveillance-control and eradication programs, market losses, and animal movement restrictions.

Costs of BTB control and interventions have been quantified in some contexts (3–6). The economic cost of BTB on animal level and productivity, on the other hand, has rarely been captured. The literature mentions that BTB causes increased mortality, reduced milk and meat productivity, reduced fertility, and organ or carcass condemnations at abattoirs due to visible TB-like lesions. However, references of quantifications are sparse. The most extensive analysis on the BTB impact on animal productivity was carried out by Meisinger (7), who followed up 8,000 cows over 5 years and conducted an abattoir survey in former East Germany in the 1970s. He found an average milk loss of 10% and a meat production loss of between 6 and 12%. Bernues et al. (8) assumed a milk loss of 12% and a reduction in fertility of 5%. The Meisinger and Bernues figures were since then extensively used to describe productivity and economic losses in cattle due to BTB. However, these figures are now outdated and do not take into account other factors such as breed, husbandry, and environment.

More recent small-scale studies showed diverging or sometimes inconclusive results. Boland et al. (9) showed a significant decrease in milk production in BTB reactors in Irish dairy farms ranging from 120 to 573 kg milk loss per lactation. Rahman and Samad (10) showed a 17% decrease in milk yield in Bangladesh, but their study included only 17 lactating cows. On the other hand, more recently, a study done in Mexico (11) showed only marginal decrease of some reproductive performances and milk yields in BTB-positive animals. Organ and carcass condemnation due to TB-like visible lesions has a cost implication at abattoir level (12–15). However, studies focused generally on TB visible lesions during meat inspection and rarely followed up on laboratory diagnostics to confirm *M. bovis*.

Cattle productivity analyses have rarely been performed. Such analysis require large animal numbers, longitudinal studies, or very well kept detailed records on productivity parameters over years. Studies in the scale of the Meisinger study have never been replicated in recent times. In Africa, only very rare longitudinal studies were performed to estimate fertility and mortality of local zebu cattle under traditional husbandry management (16–19).

BTB is endemic in Ethiopia (20, 21). Overall, low prevalence was described in local zebu cattle (2, 22). In contrast, BTB has been shown to be an important disease in exotic breeds and their crosses in Ethiopia particularly when kept under intensive husbandry systems. In and around Addis Ababa, BTB prevalence in dairy cattle ranged between 11.4 and 53.6% (23–26). BTB prevalence in emerging intensive dairy areas in the country ranged between 1.4 and 12% (27). Few studies exist in Africa at large and in Ethiopia in particular on BTB impacts on animal productivity. A small retrospective record-based study investigated the impact on calving rates (28).

This present study is the first longitudinal study performed in urban dairy cattle in Ethiopia that are being kept under intensive husbandry. We aimed in this pilot study to assess whether BTB has an impact on animal movement and on various animal productivity parameters and, through establishing biological effect sizes, inform the necessary sampling scale for future larger studies.

MATERIALS AND METHODS

The methods used are similar to the ones described in Tschopp et al. (29) and are therefore summarized here for brevity.

Study Sites

This study was carried out in Central Ethiopia within the greater milk production belt, namely, Addis Ababa (Kaliti, Kolfe, Yeka, and Gulele districts), as well as Debre Zeit and Sendafa, 50 and 40 km away from the capital, respectively. Data were collected between November 2015 and September 2018. The area is conducive to dairy farming and has high production potential. It benefits from a temperate climate and an abundant rainfall (1,000–1,900 mm/year) with good animal fodder potential and holds the largest high-yielding dairy cow numbers in the country. The milk shed of the study areas has access to big markets including Addis Ababa (30).

Study Farms and Animals

Intensive urban dairy farms were selected based on the willingness of the owners to collaborate on a 3-year longitudinal study. Animals were high milk-yielding Holstein–Frisian cross-breed cattle. All farm sizes were represented and categorized into small farms (three to 19 animals), medium farms (20–49 animals), and large farms (50 and more animals). Except for one government farm, all farms were privately owned. Husbandry was similar within farm size categories.

Animals were categorized into sex and age class. Young stock were animals younger than 12 months, replacement stock were animals from 12 months to around 3 years, and breeders were from 3 years onwards.

Tools and Parameters Recorded

An initial registration of all animals was performed in each farm at the start of the study. A herd-book was prepared capturing parameters such as any new animal entry (purchase, birth, and gift), animal exit (selling, slaughtering, and death), detailed data related to selling and purchasing of animals (e.g., cost, location, and reason), morbidities including mastitis, mortalities, and fertility [artificial insemination (AI) dates and calving dates]. The farms were visited twice a month by the same investigators. Herd-book information was updated on hard copies during each visit,

and data were entered in a Microsoft Access table. All animals were dewormed once a year with albendazole.

Animals were tested early in the study (spring 2016) using the comparative intradermal tuberculin test (CIDT). They were tested again toward the end of the study (2018) by a trained veterinarian. Two sites of 12–15 cm apart in the middle of the neck were shaved and cleaned. Skinfold thickness was measured with a digital caliper at both sites. Animals were then injected intradermally with 0.1 ml (2,500 IU/ml) avian PPD and 0.1 ml (3,000 IU/ml) bovine PPD (Lelystad B.V., Overijssel, the Netherlands) using insulin syringes. The injections sites were examined, and the skin thicknesses measured 72 h post-injection. The difference in the increase of skin thickness at the bovine and avian sites before and after inoculation was measured and recorded by the same veterinarian. A reaction was considered positive if the increase in skin thickness at the bovine site of injection was 4 mm greater than at the reaction shown at the site of the avian injection. The reaction was inconclusive if the increase was between 1 and 4 mm (31) and negative if the increase was <1 mm.

Abattoir Study

An abattoir study was carried out independently from the above longitudinal study (different animal group as above). Carcasses from cattle slaughtered in two abattoirs in the study area (Addis Ababa and Sululta) were weighed by quarters. Liver was examined for *Fasciola hepatica* infection and graded as + low infection, ++ moderate infection, and + + + heavy infection, leading to organ confiscation. Carcasses were also assessed for visible TB lesions, and samples from these lesions were collected and cultured in duplicate on glycerol and 0.4% pyruvate LJ medium (Loewenstein-Jensen TB Medium Base, 500G; BD Cat No. 283813; Sigma-Aldrich Corp., St. Louis, MO, USA) and incubated at 37°C. Cultures were observed daily for growing colonies during the first week and then weekly from the second week onwards. Cultures with no growth at week 8 were considered negative.

Data Analysis

Raw log book data from farms within the study were entered in Microsoft Access tables. Each fertility parameter can be considered as a time interval, measuring the time to either calving or service from a given reference time (birth or previous calving date). As such, each of these measures is potentially right censored either through the removal of the animal from the herd before the end of the study or by the end of the study itself. Survival analyses specifically account for this censoring and are therefore the most appropriate way to assess the impact of BTB status, or other explanatory factors, on each of these parameters (32). To this end, extracts from the study database were imported into R (33) and reshaped to create tables of time intervals for each fertility parameter (34). For censored observations, the censoring date was taken to either the date of removal of the animal from the herd or the end of the study taken as the date of the final BTB test on December 14, 2018. All R code and data tables are provided as **Supplementary Material**.

We performed an initial exploratory analysis by plotting the Kaplan–Meier survival curves for each fertility measure in turn, stratified by farm size, farm identification, and TB test status using the survival package in R (35). These revealed larger variations in the hazard rate for each fertility parameter with respect to farm size and between farms than with BTB status, suggesting that these must be adjusted for in assessing the impact of BTB status. We therefore fit a Cox proportional hazards model for each parameter, with BTB status and farm size (categorical) as explanatory variables. Robust errors were calculated using an infinitesimal jackknife variance estimate to adjust for clustering of animals within herds (32, 35). The proportional hazards assumption was assessed by graphical inspection of the scaled Schoenfeld residuals.

As the Cox model only provides the hazard ratio with respect to BTB status, we also present summary statistics for each fertility parameter in terms of the median value and 95% quantiles (which are of intrinsic interest with respect to understanding the baseline productivity within commercial dairy herds in Ethiopia).

Ethical Clearance

This research study was approved by the Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (reference number IRB/ALIPB/2018) and the IRB of AHRI (AAERC) (reference number PO46/14) and supported by the Ethiopian Ministry of Livestock and Fisheries. Skin testing of cattle was based on the international standards (31). Verbal informed consent was given by farmer owners, following the disclosure of the project objectives, benefits, and possible limitations. Each owner was informed about the results of their animals on the day when skin reactions were measured.

RESULTS

Bovine Tuberculosis Prevalence

A total of 890 animals were tested at least once for BTB in 21 farms. Sixteen farms (76.2%) had at least one reactor animal. Overall, animal crude apparent prevalence for BTB was 40% ($N = 356$) and 31.7% after adjusting by herd size (95% CI: 19.5–47%). Overall, 53.2% ($N = 474$) were negative and 6.8% ($N = 60$) were inconclusive. Three-hundred twenty-seven animals were tested twice over a period of 3 years, of which 237 (72.5%) kept the same BTB status, whereas 42 animals (12.8%) converted from inconclusive or negative to being reactors. An additional eight animals (2.4%) converted from negative into inconclusive. The remaining 40 animals that were initially positive either became inconclusive or negative.

Bovine Tuberculosis Impact on Reproductive Parameters

The following reproductive parameters were analyzed in regard to BTB status: time between animal birth to the first service, time between animal birth and their first calving, calving interval, and time interval between calving and next first service. Farm

TABLE 1 | Hazard ratios for fertility parameters among BTB-positive cows.

Parameter	Nb of positive animals	Nb of negative animals	HR	95% CI	*p*-value
Calving to next service	397	354	0.753	0.58–0.98	0.037
Calving interval	398	354	0.973	0.75–1.26	0.834
Birth to first calving	148	58	0.896	0.28–2.98	0.852
Birth to first service	148	58	0.809	0.47–1.40	0.450

BTB, bovine tuberculosis.

TABLE 2 | Description of fertility parameters by farm size and BTB status.

Parameter	Farm size	BTB status	*N*	Median days	95% quantiles
Calving to next AI	Small	Neg	14	126.5	87.6–229.0
		Pos	8	188.0	72.0–295.2
	Medium	Neg	53	139.0	59.5–263.4
		Pos	23	133.0	55.1–269.2
	Large	Neg	124	136.5	27.3–303.4
		Pos	125	133.0	35.3–316.6
	Overall	Neg	191	137.0	34.0–294.5
		Pos	156	133.5	37.6–313.5
Calving interval	Small	Neg	10	435.5	303.6–620.8
		Pos	6	443.5	296.6–824.5
	Medium	Neg	46	431.5	327.7–776.1
		Pos	16	481.5	313.4–687.2
	Large	Neg	85	462.3	306.2–939.9
		Pos	108	473.5	319.4–907.5
	Overall	Neg	141	445.0	303.0–876.0
		Pos	130	474.0	301.1–889.8
Birth to first calving	Small	Neg	3	789.0	705.4–954.3
		Pos	18	–	–
	Medium	Neg	10	690.5	629.7–900.2
		Pos	5	766.0	635.5–1033.0
	Large	Neg	13	818.0	474.6–1066.6
		Pos	6	945.5	799.2–1156.0
	Overall	Neg	26	777.0	488.2–1034.7
		Pos	11	808.0	646.7–1151.0
Birth to first AI	Small	Neg	7	485.0	428.3–660.7
		Pos	26	–	–
	Medium	Neg	19	503.0	381.0–796.3
		Pos	5	511.0	416.8–590.0
	Large	Neg	30	618.0	435.7–847.7
		Pos	19	706.0	485.0–1083.7
	Overall	Neg	56	548.0	390.1–858.7
		Pos	24	666.0	420.6–1060.6

BTB, bovine tuberculosis; AI, artificial insemination.

size effects were all non-significant (and not reported here) but were forced into the model to adjust for this important source of variation identified by the exploratory analysis. The hazard ratio associated with BTB-positive animals was smaller than 1 for all fertility parameters, suggesting that BTB status increases the time between events. However, the estimated effect was small and only significant (at the 95% level) for the calving to service time (see **Table 1**). **Table 2** shows the median and 95% quantiles for the different fertility parameters stratified by BTB status and farm size.

Bovine Tuberculosis and Natural Mortality

Among the BTB tested animals, 34 animals died of a natural cause during the study period. Seventeen were reactors and

TABLE 3 | Time interval between CIDT result and offtake ($N = 236$) among reactor and non-reactor animals.

Farm size	TB status	Nb	Mean days test to offtake	Days difference	SE	95% CI	*p*-value
Small	Neg	0	–				
	Pos	5	154.4	–	72.5		
Medium	Neg	40	242.9		26.3	189.6–296.1	0.02
	Pos	45	170.2	72.7	17.9	134.2–206.2	
Large	Neg	56	329.7		24.6	280.3–379.1	0.14
	Pos	90	277.5	52.2	22.8	232.1–322.9	
Overall	Neg	96	293.5		18.5	256.7–330.3	0.03
	Pos	140	238.6	54.9	16.5	206.0–271.3	

CIDT, comparative intradermal tuberculin test.

TABLE 4 | TB status and carcass weight among 267 animals (based on visible TB-like lesions).

Animal category	TB status	Observations	Mean weight (kg)	Weight difference	SE for mean	CI for mean	*p*-value
Heifer	Pos	1	112.0		–	–	
	Neg	6	96.3	−15.7	17.4	51.6–141.0	
Young bull	Pos	1	123.0		–	–	
	Neg	26	135.5	+12.5	7.9	119.2–151–7	
Breeding cows	Pos	15	127.1		11.2	103.0–151.2	
	Neg	135	126.8	−0.3	2.6	121.6–131.9	0.96
Breeding bulls	Pos	9	143.0		17.6	102.4–183.6	
	Neg	74	155.5	+7.5	5.3	144.9–166.0	0.44

17 were BTB negative. The numbers were too small to justify further analyses.

Bovine Tuberculosis and Offtakes

Overall, at the end of the study, 245 animals among the 830 tested animals had been eliminated (selling or slaughtering); among them, 103 (42%) were BTB negative and 142 (58%) were BTB positive. Among all 356 BTB-positive animals, 142 were eliminated (39.9%) and 214 (60.1%) were still in the farms at the end of the study. Among the 474 BTB-negative animals, 103 (21.7%) were eliminated and 371 (78.3%) were kept.

Animals tested during the first round were followed up over time to assess whether owners would eliminate them and, if so, how long after the test results. Among the offtakes ($N = 236$), 96 (40.7%) were BTB negative and 140 (59.3%) BTB positive. Overall, for the 236 animals that were eliminated, median time between test result and elimination of the animal was 191.5 days (95% CI: 158–254) regardless of TB status. Overall, reactors were eliminated faster than BTB-negative animals (mean difference was 54.9 days). This was particularly true in medium farms ($p = 0.02$) (see **Table 3**).

Detailed information on where these positive animals went was available for 82 animals. The great majority was sent to abattoirs ($N = 60$; 73.2%) mainly in Addis Ababa, whereas a quarter were sold directly to other farms ($N = 21$, 25.6%), and one animal was sold to a broker.

Bovine Tuberculosis Impact on Carcass Weight

The abattoir study included 351 animals, of which 349 carcasses were weighed. One hundred forty (40.1%) and 209 (59.9%) carcasses were from Addis Ababa and Sululta abattoir, respectively. One hundred ten were male (41.2%) and 157 female (58.8). Replacements accounted for 48 animals (13.7%) and breeders for 299 animals (85.7%).

Overall *Fasciola* prevalence was 53.6% ($N = 187$). Half of the animals had moderate *Fasciola* infestation ($N = 95$; 50.8%), whereas 32.1% ($N = 60$) had severe and 17.1% ($N = 32$) had mild infestation. Twenty-eight (8%) animals had visible TB-like lesions upon meat inspection examination. Culture was done on 18 lesions, and 13/18 (72.2%) were culture positive for *Mycobacteria*.

BTB-negative breeding bull carcasses were overall 7.5 kg heavier than BTB-positive animals; however, there was no statistical difference at the 95% level (see **Table 4**).

Animal Trade

Overall, 102 animals were purchased. Among them, 53 were included in the original stock during registration, and 49 were further purchased during the study time for 48 of which detailed information was collected. The majority of animals were purchased directly from another farm (93.7%), while 6.2% were purchased from local markets. No brokers were involved. The majority of farmers bought their animals within their town or its surrounding. Addis Ababa farmers purchased 89.6% of the total purchased animals in the study.

Overall, 538 animals were sold during the study period. The majority were heifers and cows ($N = 275$; 51.1% of all animals sold) followed by male calves that were eliminated within the first 2 weeks of life ($N = 222$, 41.3% of all sold animals). The majority of sold animals originated in the Addis farms ($N = 299$; 55.6% of all sold animals) and the least in Sendafa ($N = 110$;

TABLE 5 | Purchase and selling locations of animals observed in the three study sites.

Locations of selling or purchase		Selling by study site				Purchasing by study site			
		Addis Ababa	Bishoftu	Sendafa	Total	Addis Ababa	Bishoftu	Sendafa	Total
Addis Ababa		121 (75.7)	33 (37.9)	57 (81.4)	211 (66.6)	35 (67.3)		1 (20)	36 (62)
Addis Ababa surroundings	Mukatori					8 (15.4)			8 (13.8)
	Lemma					6 (11.5)			6 (10.3)
Bishoftu			20 (23)	2 (2.8)	22 (6.9)		1 (100)		1 (1.7)
Bishoftu surroundings	Adama	32 (20)			7 (2.2)				
Sendafa			2 (2.3)	7 (10)	9 (2.8)			3 (60)	3 (5.2)
Sendafa surroundings	Girar							1 (20)	1 (1.7)
	Bake			3 (4.2)	3 (0.9)				
Debre Berhan (Amhara)				1 (1.4)	33 (10.4)				
Dessie (Amhara)			16 (18.4)		16 (5)				
Mekele (Tigray)			10 (11.5)		10 (3.1)				
Wellega (Oromia)			6 (6.9)		6 (1.9)				
Gondar (Amhara)						3 (5.8)			3 (5.2)
Total		160	87	70	317	52	1	5	58

20.4%). Reasons for selling were given for 467 animals, as follows: immediate need for cash (18.6%), underperforming animals (14.6%), depopulation (10.3%), diseased animals (6.8%), old animals (6%), and animals suffering a trauma such as broken legs (2.4%). The rest were young male calves eliminated within their first 2 weeks of life. TB was never given as a direct reason. Most animals were sold directly to abattoirs ($N = 264/375$, 70.4%). Nearly a quarter ($N = 90/375$, 24%) were sold directly to another farms, whereas 15 animals (4%) were sold *via* brokers. None were sold *via* a market system; 66.6% of all animals were sold to Addis Ababa, where large abattoirs are found. **Table 5** shows the locations where animals have been purchased from and/or sold to. The majority of the trade—particularly purchase of animals—remained local within the study site and its neighboring locations. Animals were sold in addition to other regions.

DISCUSSION

Overall, crude BTB prevalence was 40% at animal level, which is in line with previous studies performed in the area (23, 24). During the 3-year study time, 12.8% of initially negative animals converted into new reactors. An additional eight animals (2.45%) converted from negative to doubtful, possibly being in the process of becoming positive. In recent times, an increase in number of intensive dairy farms has been observed in several regions of the country (emerging dairy areas). Central Ethiopia remains often the source of animals for stocking up new farms or replacing animals, hence raising the question of possible risk for BTB spread into new areas, particularly in a national context where no BTB control measures exist (e.g., no compulsory testing and no movement restrictions). Our study showed that animal trading (purchasing and selling) was mostly occurring locally within the same town or within the same geographical area. The majority of sold animals were sold to local abattoirs (70.4%). However, nearly a quarter (24%) of animals were sold to other farms mainly in the surroundings but some—albeit few in numbers—also further away, even to other regions of the country (e.g., Tigray and Amhara), hence increasing the risk for further TB spread. Animal trade was observed between dairy farms belonging to universities (from Addis to Mekele and Gondar). Interestingly, none of the reactors included in our study were traded to the various universities. University dairy farms in the different regions would have the strong potential to become further animal stocking/re-stocking source for dairy farms in the respective regions and to act as a model farm that could provide awareness and good farm practice training to existing and emerging dairy farms.

Without institutional and legal support in BTB surveillance and control, further spread of the diseases could potentially be linked to farmers' attitude upon receiving test results. Overall, 39.9% of the reactors were eliminated as compared with 21.7% of the non-reactors. Our study showed that among all 356 BTB reactors, 60.1% were kept in the farms and 39.9% were eliminated during the study time. Regardless of BTB status, the median time interval between a test result and elimination date was 191.5 days (95% CI: 158–254). Overall, reactors were eliminated faster than non-reactors (mean 54.9 days quicker; $p = 0.03$). This result highlights potential substantial hidden economic impact of BTB on farms that cannot be easily measured. There is an indication that farmers are selling more reactors than non-reactors and that they are selling their reactors faster than they would in average. Therefore, they might lose on optimal market prices, affecting both farm dynamic and business through, for instance, economic and logistic challenge of animal sourcing for restocking; loss of markets; and a possible additional factor (among many others) of observed farm depopulation. In our study, nine out of the 24 study farm (37.5%) had their animal number decrease over the last 3 years including 80% of all large farms (29). This study showed also that nearly a quarter of the reactors were sold to other farms and not slaughtered, hence highlighting the risk of disease spread through uncontrolled animal movement.

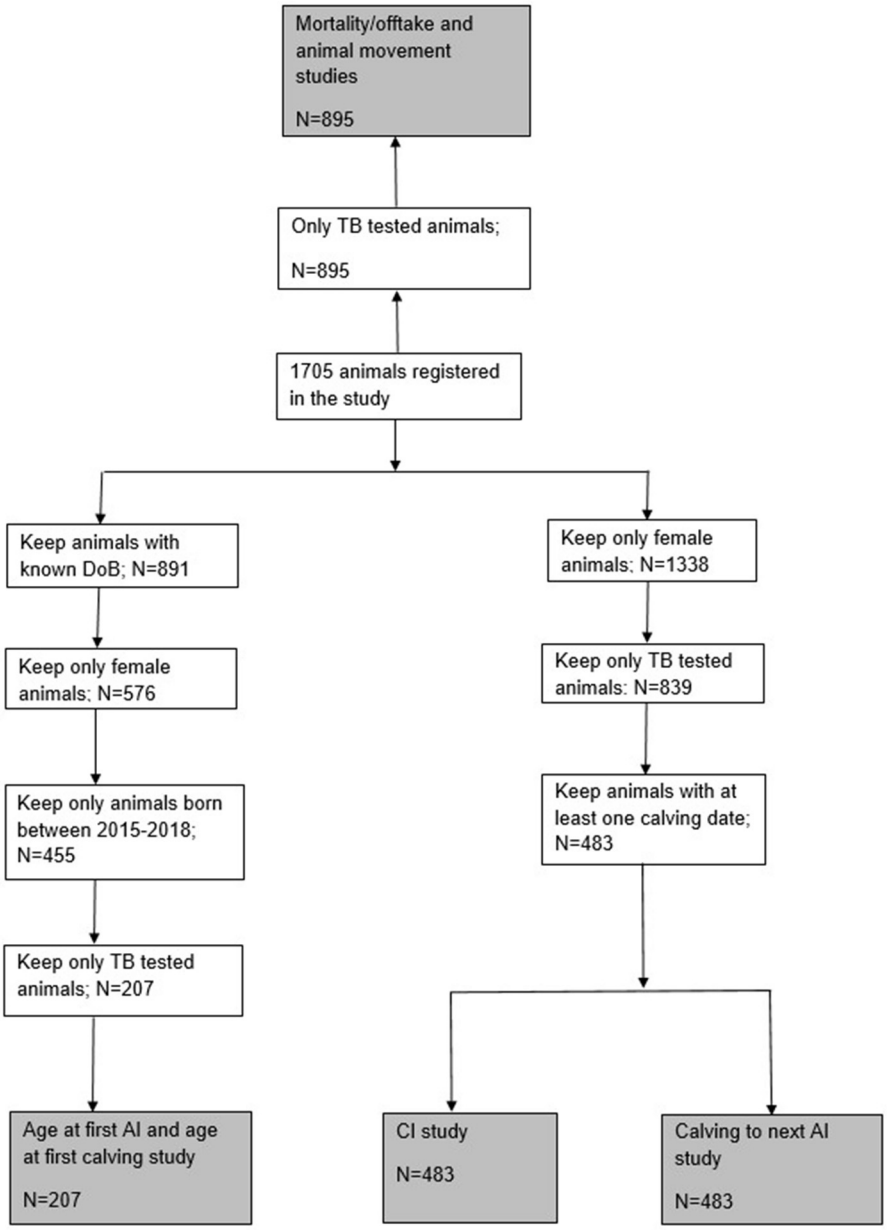

FIGURE 1 | Flowchart showing animals used for each study analysis.

Limited studies exist on BTB effect on various productivity parameters. BTB has negative impacts on animal fertility (7, 8); however, recent studies on quantifications are lacking. Interestingly, BTB impact on animal reproduction has been analyzed for wildlife bovine species such as the Wood bison in Canada (*Bison bison athabascae)* and the African buffalo (*Syncerus caffer*), where lower pregnancy probability was described (36, 37). But data from domestic cattle are lacking. A preliminary study in Ethiopia based on a retrospective record study showed that BTB status had no influence on age at puberty and age at first calving, but reactor cows in their third calving had significantly higher mean number of services (28). Our results showed that the hazard ratio associated with BTB-positive animals was smaller than 1 for all fertility parameters, suggesting that BTB status increases the time between events. The median interval was overall longer in BTB-positive animals as compared with negative animals (with the exception for the calving to next service parameter).

Our study did not take in account specific age of the animal and related lactation number when calculating the fertility parameters. A significant proportion of animals within our study population were lost due to the absence of a TB test result (**Figure 1**) due to either being too young at the date of the first test or having been removed from the herd by the second.

If there is an association between BTB infection status and removal of animals, then this may introduce biases that are difficult to quantify. This is an important, but unavoidable, limitation of carrying out our study under production conditions on commercial farms given the frequency of testing that was feasible (given the nature of the tuberculin test) and acceptable to the farmers.

Studies of the economic BTB impact on meat production have so far primarily focused on abattoir study with monetary translation of confiscation of carcasses and organs due to visible TB-like lesions (14, 15, 38–40). BTB (solely based on visible lesions) is one of the major reasons for meat and organ confiscation at abattoirs in Ethiopia besides parasitic diseases (12, 41, 42). However, the direct loss of meat (reduced life weight and reduced carcass weight) has to our knowledge never been assessed in Africa. Unfortunately, our sample size was small (N = 349), with 8% visible TB-like lesions upon meat inspection. Nevertheless, we could observe a trend that negative bulls produced in average 7.5 kg more meat than positive bulls.

Our study showed also that half of the animals (53.6%) were infested with *Fasciola* (*F. hepatica*), a common liver fluke found in cattle. A third (32.1%) showed even severe infestation of the liver. *F. hepatica* infections were shown to be linked with altered immune responsiveness to PPD, hence having implications with BTB diagnosis and disease progression (43–45). The high prevalence of *Fasciola* observed will need to be addressed before embarking in large-scale future BTB surveillance and control programs in Ethiopia. In a nation with no compulsory TB testing, a mechanism that can trace back carcasses from abattoirs with visible TB-lesions to the source farms would be an additional important step in TB surveillance.

CONCLUSION

This study is the first one in Ethiopia to assess the potential impact on BTB on dairy cattle productivity parameters in a holistic way (fertility, mortality, morbidity, and weight). Due to the lack of accurate record keeping of milk yield, we could not calculate the effect of BTB on milk productivity. Although monetary translation of productivity loss was difficult to perform at this stage, and although our sample size was too small for definitive conclusions, our pilot study indicated that BTB has likely an impact on productivity (lower fertility and weight loss) and has likely further invisible costs and indirect economic impacts such as forgoing access to better markets, time, and change in farm business. Besides a regular BTB testing follow-up of each animal over time, a much larger sample size including >5,000 animals is warranted in future follow-up studies to corroborate these findings and enable to control other factors influencing productivity such as husbandry factors or comorbidities. With the growing intensification of cattle production in Ethiopia, analysis of productivity and impact of diseases in productivity are important. The results of this study will provide important information to conduct further cost–benefit analysis of different control strategies for BTB.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because restrictions linked to project (EThicobots) regulations.

ETHICS STATEMENT

This research study was approved by the Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (reference number IRB/ALIPB/2018), the Institutional Review Board of AHRI (AAERC) (reference number PO46/14) and supported by the Ethiopian Ministry of Livestock and Fisheries. Written informed consent for participation was not obtained from the owners because Verbal informed consent was obtained by owners.

AUTHOR CONTRIBUTIONS

RT designed the research, performed the data analysis, and drafted the manuscript. GG and GA collected field data and contributed to drafting the manuscript. AC and JH contributed to the data analysis. JW and JZ provided critical comments to the manuscript. All authors have contributed to the manuscript and have approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.698768/full#supplementary-material

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Epidemiology of Pig Tuberculosis in Argentina

Soledad Barandiaran[1], María Jimena Marfil[2], Guillermo Capobianco[3], María Sol Pérez Aguirreburualde[4], Martín José Zumárraga[5], María Emilia Eirin[5], María Ximena Cuerda[5], Marina Winter[6], Marcela Martínez Vivot[2], Andres Maximiliano Perez[5] and Luciano Francisco La Sala[7]*

[1] Instituto de Investigaciones en Producción Animal (INPA), Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Cátedra de Enfermedades Infecciosas, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Departamento de Matemática, Instituto de Matemática de Bahía Blanca- Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional del Sur, Buenos Aires, Argentina, [4] Center for Animal Health and Food Safety, College of Veterinary Medicine, University of Minnesota, Minnesota City, MN, United States, [5] Instituto Nacional de Tecnología Agropecuaria (INTA), Instituto de Agrobiotecnología y Biología Molecular (IABIMO), Instituto Nacional de Tecnología Agropecuaria: Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [6] Centro de Investigaciones y Transferencia de Río Negro, Universidad Nacional de Río Negro, Bariloche, Argentina, [7] Instituto de Ciencias Biológicas y Biomédicas del Sur, Universidad Nacional del Sur – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

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Edited by:
Joram Josephat Buza, Nelson Mandela African Institution of Science and Technology, Tanzania

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Bernat Pérez de Val, Centre for Research on Animal Health, Spain

***Correspondence:**
Soledad Barandiaran
sbaran@fvet.uba.ar

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Bovine tuberculosis (bTB) is a disease caused mainly by the *Mycobacterium bovis* and that is endemic to livestock populations in most Latin American countries. Traditionally, bTB control programs are costly and targeted to cattle, largely disregarding other species such as swine and wildlife. According to official services, in Argentina disease prevalence in pigs is comparable to that observed in cattle, suggesting the need for efficient control programs to manage the disease in both species. Additionally, extensive farming systems, which are commonly practiced in Argentina, allow the interaction between livestock and wildlife such as wild boar (*Sus scrofa*), which is considered a natural host of the disease. Here, we evaluated the bTB pigs- cattle interface, studying the dynamics of *M. bovis* isolates in the pig population and identifying farm-level epidemiological variables associated with the disease confirmation at slaughterhouses. Additionally, to assess the potential multi-host systems in the transmission of bTB, the molecular characterization of wild boar mycobacterial strains was included in the study, as this interaction has not been previously evaluated in this region. Multivariable logistic regression models were used to assess the association between farm-level epidemiological variables (location, farm size, and co-existence with cattle and goats) and bTB confirmation in pig tuberculosis-like lesions samples. Results showed that when cattle were present, the odds of bTB in pigs decreased 0.3 or 0.6% for every additional sow when cattle were present or absent in the farm, respectively. Pigs shared 60% (18/30) of the genotypes with cattle and wild boar, suggesting transmission at the interface between pigs and cattle and highlighting the potential role of wild boar in bTB maintenance. These results provide novel information about the molecular diversity of *M. bovis* strains in pigs in Argentina and proposes the potential relevance of a multi-host system in the epidemiology of bTB in the region. The statistical models presented here may be used in the design of a low

cost, abattoir-based surveillance program for bTB in the pig industry in Argentina, with potential extension to other settings with similar epidemiological conditions.

Keywords: bovine tuberculosis, pigs, spoligotyping, surveillance, farm-level epidemiological variables, *Mycobacterium bovis*

INTRODUCTION

Bovine tuberculosis (bTB) is a widely spread disease that causes far-reaching economic losses through direct impact on animal health, restrictions to trade, confiscation and destruction of meat, and costs associated with the implementation of control programs (1). *Mycobacterium bovis* (*M. bovis*) is the most prevalent etiological agent causing bTB. In developing countries, bTB is often a neglected disease with reemergence periods in domestic animals, wildlife, and humans, thus representing a public health concern (2, 3). Because there are many potential hosts for *M. bovis* and disease incidence and distribution are wide, the implementation of effective control measures is complex in regions where susceptible livestock and wildlife coexist (4).

Domestic pigs (*Sus scrofa domestica*) are susceptible to different mycobacteria, mainly those species included in the *Mycobacterium tuberculosis* complex (MTC) and in the *Mycobacterium avium* complex (MAC). In countries in which infection is endemic, *M. bovis* is the most frequently reported *Mycobacterium* in pigs (5–7). Conversely, in countries where bTB is not endemic, MAC species become relevant (8, 9).

Due to geographic and climatic conditions, livestock production in Argentina is largely extensive based on a mixture of natural grazing and pastures. bTB is endemic in the country, and infection is believed to be associated with dairy cattle (10). Although cattle are the most affected domestic species and have the highest bTB prevalence, pigs can also be affected by the disease (7). The local pig production has experienced a significant growth in recent years, leading to an increment of both meat consumption and international trade (11). Few (11%) producers concentrate 54% of the sow's stock, contributing with nearly 80% of the national volume of produced pork. Therefore, a large proportion of small pig producers concentrate a minor number of sows under poor biosecurity and limited technification. This type of farms coexists with relatively large, intensive farms with high technology and stricter biosecurity levels. Often, this scenario makes the implementation of disease control extremely difficult (12) (SENASA, 2016).

Previous studies conducted in Argentina have suggested the role of pigs in the transmission of *M. bovis* to cattle, especially in farms where pigs and cattle coexist (7). Moreover, whereas bTB has been detected in wildlife populations in the county, including collared peccary (*Peccari tajacu*), axis deer (*Axis axis*), gray fox (*Lycalopex griseus*), brown rat (*Rattus norvegicus*), opossum (*Didelphis albiventris*), and wild boar (*Sus scrofa*), no studies have been conducted to assess the epidemiological role of these species as reservoirs hosts (13).

Wild boar may play a role in the epidemiology of the disease in Argentina, considering that their population is rapidly growing and expanding geographically, the infection is present in this species, and its distribution overlaps that of major livestock production areas (14). Also, it is worth noting that wild boar has been suggested as responsible for the maintenance and transmission of *M. bovis* to livestock in other countries (15, 16).

Argentina has a bTB control and eradication program in place since 1998, that is only mandatory in dairy cattle, goats, sheep and in animals bred for genetic purposes (SENASA, Res. 128/12). Contrarily, this program does not include swine, where bTB confirmation relies entirely on meat inspection at the abattoir, which arguably leads to an underestimation of *M. bovis* prevalence.

Worldwide, several studies have addressed the association between bTB infection and farm-level epidemiological variables in livestock (17–19), but only a handful of studies have explored transmission in pig farming systems (20, 21). With this background, disease surveillance and control strategies for bTB could benefit from the development and implementation of a predictive tool that estimates, with adequate confidence, the probability that a slaughtered pig with tuberculosis like lesions (TBL) is truly infected, given the presence of certain farm-level epidemiological variables.

With this background, an observational (cross-sectional) analytic study was conducted under the hypotheses that: (1) the interface between pigs and cattle plays an important role in the dynamics of bTB in Argentina, and (2) wild boar may act as key maintenance host for bTB in the region.

The aim of this study was to evaluate the dynamics of mycobacteria in the pig population and to identify farm-level epidemiological variables associated with *M. bovis* confirmation in TBL samples detected during slaughter. Additionally, the diversity and frequency of spoligotypes was investigated to elucidate potential bTB transmission among pigs, cattle, and wild boar at the interface between these species. Results presented here will contribute to the evaluation and design of surveillance programs for bTB in swine of Argentina.

MATERIALS AND METHODS

Study Population

The studied samples were collected from slaughtered pigs originated from 135 farms located in the main productive areas of Argentina, including the provinces of Buenos Aires, Córdoba, Santa Fe, and La Pampa. A few (3%) samples came from other

Abbreviations: bTB, bovine tuberculosis; TBL, tuberculosis-like lesions; MTC, Mycobacterium tuberculosis complex; MAC, Mycobacterium avium complex; PPD, protein purified derivative test; SENASA, National Service of Agri-Food Health and Quality, AFB, acid-fast bacilli; TB-TP, true positives; AIC, Akaike information criterion; wiAICc, Akaike weight; VIF, inflation factor; OR, odds ratios.

secondary production provinces such as San Luis, Entre Ríos, and Santiago del Estero. Farms of origin were representative of the two major production systems in Argentina; specifically, 77% of the studied farms were small pig producers and had <1,000 pigs, whereas the remaining 23% were intensive pig producers with 1,000–4,000 pigs.

Sample Collection

Special permits for accessing abattoirs were issued by the official authorities (SENASA, National Service of Agri-Food Health and Quality). Samples were collected at four annual visits to a large abattoir in the province of Buenos Aires during an 8-year period (2010–2017). Lymph node sections (∼4 × 4 cm) with TBL were collected from slaughtered pigs ($n = 191$). Between 20 and 29 samples were collected annually at each of the four visits. Tissue samples were stored at −20°C until bacteriological and molecular analyses were conducted. Samples were collected after meat inspection by SENASA and according to national regulations; therefore, no ethical consent approval was required.

Mycobacterial Detection and Molecular Confirmation

Bacteriological Culture

Samples were decontaminated using the Petrof's method and cultured in Löwenstein-Jensen and Stonebrink media at 37°C for 60 days (22). Ziehl-Neelsen staining of colonies was performed for the detection of acid-fast bacilli (AFB). Briefly, a loopful of AFB colonies was suspended in 300 μL of bidestilled water, then heated for 40 min. at 95°C and centrifuged at 12,000 rpm for 10 min. The DNA obtained was stored at −20°C until processed.

Molecular Confirmation

A total of 5 μL of the supernatant was used as template for PCR. IS*6110*-PCR (23) and IS*1245*-PCR (24) were performed to detect the MTC and MAC, respectively. Isolates positive to IS*6110*-PCR were subjected to spoligotyping (25).

Statistical Analysis

Epidemiological Information

Each slaughtered pig was traced-back to the farm of origin, via the National Sanitary Registry of Agricultural Producers (RENSPA), and epidemiological data related to each farm were obtained from SENASA's official database. Farm-level epidemiological data included location (geographic coordinates), size (total number of pigs and number of sows), and co-existence with cattle. When multiple samples were taken from the same farm in different dates, only the one collected closest to date of epidemiological data collection were considered for the statistical analysis.

Multivariable Analysis

TBL samples characterized and confirmed as *M. bovis* were considered as cases (true positives; TB-TP), whereas *M. bovis*-negative samples were considered as controls. Multivariable logistic regression was used to assess the association between farm-level epidemiological variables and the confirmation of TB-TP. First, the linearity assumption for continuous predictor variables was checked following Hosmer and Lemeshow (26). Following, a model was fitted including farm location (geographical coordinates), number of sows (continuous), co-existence with cattle (categorical: yes/no) and the interaction between the last two variables (number of sows × co-existence with cattle) as independent variables. The confirmed status of each sample (case, control as outlined above) was included as dependent variable. The presence of influential data was detected using the Cook's distance (27) for each observation in the model. Two farms were identified as strongly influential on the model and were removed before refitting the model ($n = 133$).

Model selection was streamlined by generating models including all possible combinations of variables using the R package "MuMIn" (28). Here, our main focus was to generate a set of best models for making predictions given new data, while ascribing the principle of parsimony (29). Therefore, the Akaike information criterion [AIC; (30)] corrected for small sample sizes (AICc) was used for model selection. The lowest AICc value was considered evidence of best model fit, and models with AIC_c values within 2 units of distance were also considered as competing models. Akaike weights ($w_i AIC_c$) were calculated and interpreted as the probability that a model was the best-fitting given the data and the set of candidate models. The strength of evidence in favor of one model over the other was obtained by dividing their $w_i AIC_c$. Within each model, variable importance was assessed by evaluating the increment in AIC value if the single term was dropped.

First-order spatial effects were investigated by including the location of each farm in the final model, as "latitude," "longitude," and their interaction term. Second-order spatial effects were assessed using scan statistic (31) in the R package "satscan" (32) and fitting a Bernoulli model, where "cases" were represented by farms with TB-TP and controls were those with TBL samples which could not be confirmed. Potential multicollinearity in the models was evaluated using the variance inflation factor (VIF) for each variable using the R package "car" (33), where VIF values ≥5 indicate potential multicollinearity problems.

The association between TB status and independent variables was assessed by estimation of the odds ratios (OR) and their 95% confidence intervals (95% CI). The OR for the interaction term was calculated and interpreted following Hosmer and Lemeshow (26). The presence of influential data points in the multivariable model was assessed using the Cook's Distance. All analyses were performed using the statistical program R (34) and the mentioned packages.

The multivariable model was evaluated using a leave-one-out cross-validation procedure, which allowed the estimation of model accuracy (the model's ability to correctly predict sample status), sensitivity (proportion of correctly classified TB-TP), and specificity (proportion of correctly classified *M. bovis*-negative samples) (35). In all models, a decision probability boundary was set at 0.5, to classify samples as positive or negative.

Genotyping and Distribution Among Species

Spoligotyping was performed following Kamerbeek et al. (25) using a commercial kit (Ocimum Biosolutions Company, Hyderabad, India). The scanned images of the films obtained were analyzed by BioNumerics (Version 3.5, Applied Maths, Sint-Martens-Latem, Belgium). The observed patterns were compared with those in national and international databases (INTA-CONICET and VISAVET Health Surveillance Center, available at: www.mbovis.org). The spoligotypes obtained in this study and those previously reported for pigs in Argentina (7) were compared to spoligotypes reported in cattle (36) and wild boar in the country. The logical relationships between spoligotypes reported in each species were analyzed using a Venn diagram in the R package "Venn" (37).

RESULTS

Mycobacteria Detection and Molecular Confirmation

Out of 191 TBL samples, 130 (68%) were TB-TP and 61 (32%) were TB-negative (included negative cultures and MAC positive cultures). In the TB-TP samples, 6.92% (9/130) exhibited co-infections with MAC species. Of the 61 negative TBL samples, 52 were culture-negative and nine were MAC culture-positive.

Statistical Analyses

Multivariable Models

Three competing logistic models were selected (**Table 1**). Their corresponding accuracy, sensitivity and specificity are shown in **Table 2**. Model 1 showed that the odds of bTB decreased 0.6% ($\beta = -0.006$) for every additional sow in the farm but were twice as high (OR: 2.02; 95% CI: 0.88–4.65) in farms with cattle, compared with those without cattle. Model 2 showed that, when only the number of sows was included as independent variable, the odds of bTB dropped 0.7% ($\beta = -0.007$) for every additional sow in the farm. Model 3 included an interaction term, indicating that the effect of the number of sows on the odds of bTB depended on the levels of the interacting variable (presence/absence of cattle). In this model, the odds of bTB decreased 0.3% for every unit increase in the number of sows when cattle were present in the farm. When cattle were absent, the trend in the odds of bTB also decreased as the number of sows increased, but the magnitude was 2-fold (0.6%) compared to that observed when cattle was present (**Figure 1**). No first or second-order spatial effects were detected in the data.

Spoligotype Diversity, Isolation Frequency, and Hosts

The 130 *M. bovis* isolates were classified in 15 different spoligotypes (**Figure 2**). Unique spoligotypes represented 33.3% (5/15) of the total, whereas 66.6% (10/15) were detected in more than one isolate. The SB0140 was the most frequent ($n = 71$; 54.6%) spoligotype. The distribution of spoligotypes among pigs, wild boar, and cattle is shown in **Figure 3**. Out of all 78 spoligotypes described here, seven (9%) were shared by the tree species. The frequency of shared spoligotypes between any two species was led by wild boar and pigs (37.5%; 9/24), followed by cattle and pigs (22.7%, 17/75), and cattle and wild boar (10.6%; 7/66).

DISCUSSION

Despite significant improvement in the last 20 years, bTB and zoonotic TB (zTB) remains a major health challenge for many developing countries. One of the main goals of bTB national control programs is to decrease the incidence of zTB, an objective that can only be achieved through improving food safety and controlling bTB in domestic and wild animal reservoirs (38, 39).

Our study suggests an association between bTB infection in pigs and cattle, through the analyses of the genotypes present in these species and farm-level epidemiological variables. The identification of farms at highest probability of being infected will allow the implementation of effective preventive and control measures in Argentina. Additionally, the diversity of spoligotypes

TABLE 1 | Parameters of the three candidate logistic models showing the association between risk factors and confirmation of sample TB positive status ($n = 133$).

Variables	Coefficient	SE	OR (95%CI)	ΔAIC_c
Model 1 = TB ~ No. of sows + Cattle				
Intercept	0.874	0.339	-	-
Cattle $_{present}$	0.701	0.426	2.02 (0.88–4.65)	0.69
No. of sows	−0.006	0.002	0.994 (0.990–0.998)	11.7
Model 2 = TB ~ No. of Sows				
Intercept	1.234	0.272	-	-
No. of sows	−0.007	0.002	0.993 (0.989–0.997)	18.6
Model 3 = TB ~ No. of Sows + Cattle + No. of Sows × Cattle				
Intercept	0.921	0.357	-	-
Cattle $_{present}$	0.500	0.590	1.65 (0.52–5.24)	1.2
No. of sows	−0.006	0.002	0.994 (0.990–0.998)	9.9
No. of Sows × Cattle $_{present}$	0.003	0.006	0.994 (0.56–1.78)	−1.9

ΔAICc represents AICc value change when the single variable is removed from the model.

TABLE 2 | Logistic models with their accuracy, sensitivity, and specificity reported as percentage and their 95% confidence interval in parenthesis.

Model	Accuracy %	Se %	Sp%	AICc	ΔAICc	w_iAICc
M1: TB ~ No. of sows + Cattle $_{(presence)}$	72.2	92.7	39.2	159.8	0.0	0.477
M2: TB ~ No. of sows	69.2	92.6	33.3	160.5	0.69	0.338
M3: TB ~ No. of sows + Cattle $_{(presence)}$ +No. of sows × Cattle $_{(presence)}$	71.4	91.5	39.2	161.7	1.89	0.186

AICc values, differences (ΔAICc), and AICc weights (wAICc) are presented.

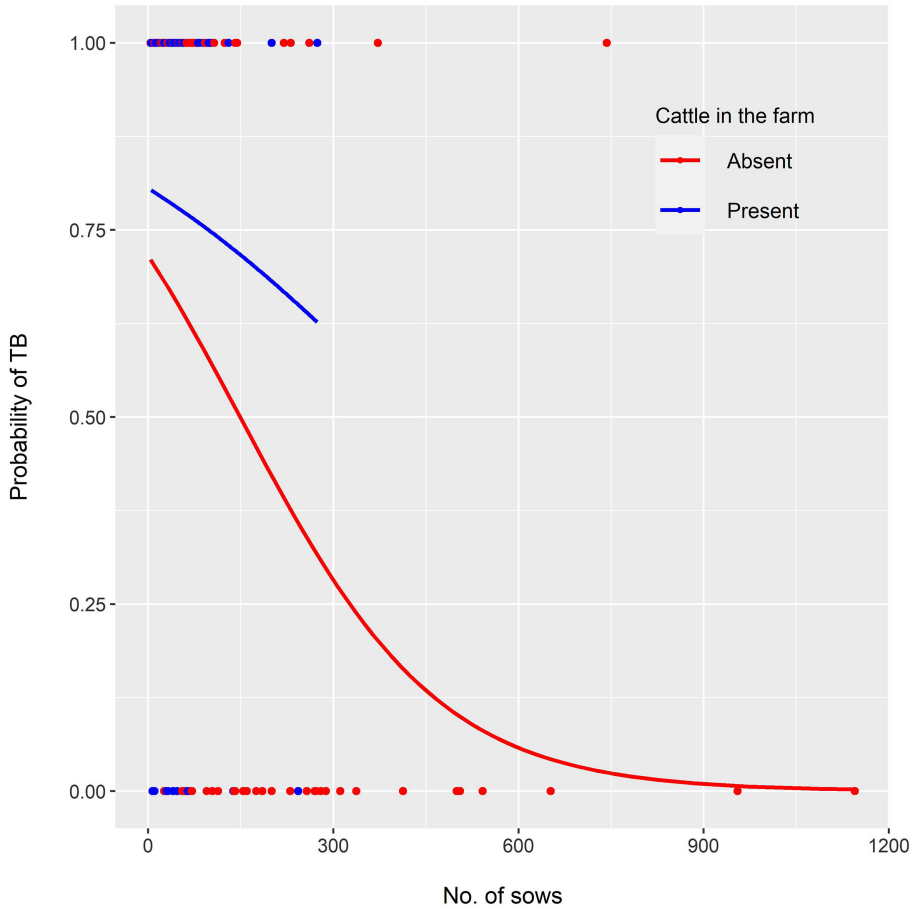

FIGURE 1 | Multivariable logistic model showing the probability of TB confirmation in TBL samples as a function of the number of sows in the farm of origin, when cattle were present or absent (red and blue lines, respectively). Dots along the *x* axis represent samples with ($y = 1$) and without ($y = 0$) TB confirmation, in farms where cattle were present (red) or absent (blue).

detected in these species strongly suggests the possibility of bTB transmission between livestock and wildlife in this region.

A large proportion (68%) of the collected TBL samples was confirmed as TB-TP. Studies conducted in Spain reported similar results, with over 63% of MTC isolates in TBL samples (40). In South America, epidemiological studies in pigs are scarce, with one study in Brazil reporting that all TBL samples from pigs were negative to MTC isolates (41). Studies conducted in other countries reported lower infection to MTC rates in pigs, such as Ethiopia (10%), Uganda (2%) and Norway (0.3%) (6, 20, 42). Interestingly, Di Marco et al. (43) reported 50% prevalence for MTC isolates in black pigs from Italy and suggested that Sicilian black pigs might act as reservoirs of bTB. The latter seems relevant considering that this breed is produced in extensive farming systems, similar to most pigs in Argentina.

MAC species were isolated in 9.1% (18/191) of TBL samples, similar to previous research in pigs in Argentina (7). In contrast, the percentage of MAC obtained from TBL samples was higher in developed countries compared to the results obtained in the present study (8, 44, 45). Such finding might be related to intensive farming and high biosecurity measures employed in developed countries, where MAC outbreaks are generally associated with contaminated feed or bedding, rather than interaction with domestic animals or wildlife (46). The number of negative samples for both MTC and MAC in this study could

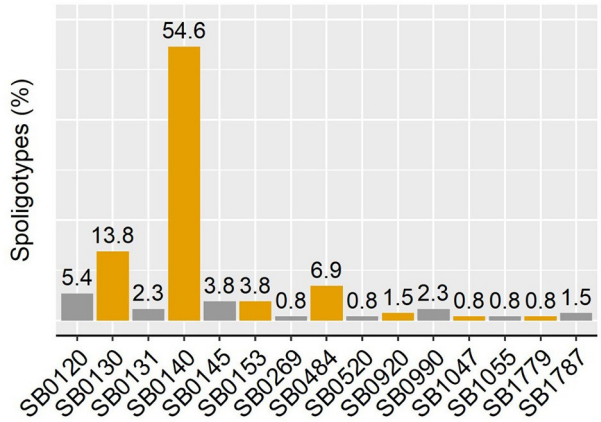

FIGURE 2 | Spoligotype diversity and frequency of occurrence in pigs from this study.

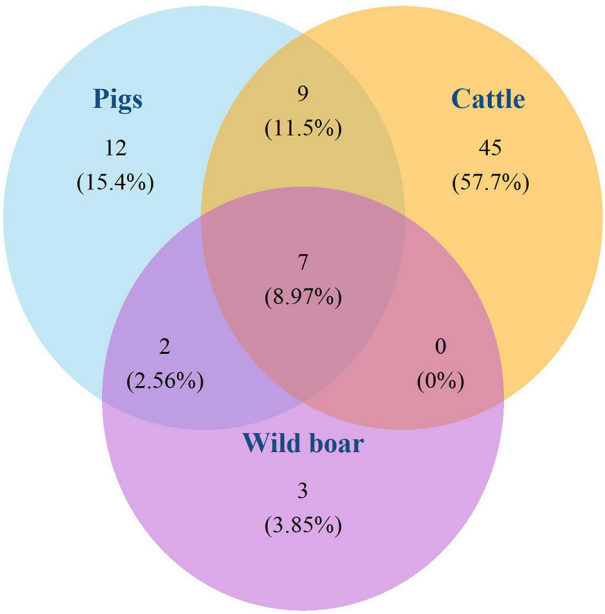

FIGURE 3 | Absolute and relative (%) number of spoligotypes isolated in pigs, wild boars, and cattle, and spoligotypes shared by these species. INTA-CONICET and VISAVET Health Surveillance Center, available at: www.mbovis.org.

be associated with non-infectious causes other non-pathogenic mycobacteria, or different infectious agents (44).

The association between farm-level epidemiological variables and bTB prevalence in pig systems has been assessed in other countries, such as Ethiopia and Norway (20, 42). In our study, the third best model (Model 3) suggests that the odds of bTB confirmation decrease as the number of sows' increases, and that this drop is strongly associated with the absence of cattle. This finding may be explained by stricter health control measures adopted by larger pig producers. Accordingly, in Argentina, small pig producers (50 sows or less) comprise 98% of the registered producers (SENASA 2020). Basic sanitary measures are often neglected by small producers' worldwide (20, 42), and Argentina is not an exception, where only 21% of this stratum is aware of national programs for the control of the most important pig diseases (47). Also, our results highlight the role of cattle in the epidemiology of bTB in pig production systems, and support previous findings suggesting an epidemiological link between coexisting pigs and cattle in the transmission of bTB in Argentina (7).

Genotyping analysis is a valuable tool for the study of bTB dynamics and the role of pigs and wildlife in the maintenance of infection (4, 48, 49). In this study, all MTC isolates were confirmed as *M. bovis*, and 15 different spoligotypes were detected. Here, the most frequent spoligotype was SB0140, which is also the most frequent in Argentina, being detected in zTB and several host species (36).

Our results showed that the frequency of shared spoligotypes was largest between pigs and cattle, followed by those shared between pigs and wild boar. This supports our hypothesis of bTB transmission in a multi-host context, as also previously suggested by others in Argentina and other countries (7, 50–52). Contrarily, the finding of spoligotypes that are not shared with other species (12 in pigs, 45 in cattle, and 3 in wild boar) suggests the relevance of intra-species transmission.

Wild boar shared a large proportion (75%) of its spoligotypes with pigs, suggesting a bTB spillover phenomenon between these species. Wild boar is widely distributed in Argentina, with large populations coexisting in vast areas with pigs and cattle (53). In Argentina, pigs are produced mainly under extensive farming conditions, where cattle presence is also prominent and interaction between wild boar, pigs and cattle can be intense (53, 54). Therefore, wild boar may act as a reservoir in the bTB epidemiology in regions with similar conditions as those in Argentina.

Health research and policy addressing wild boar diseases have received increased attention in recent years (55, 56); however, the role of this species as spreader of bTB and other pathogens is far from being fully acknowledged (54). The surveillance strategy currently in place for bTB in cattle creates a complex scenario where pigs and wild boar continue to play their role as bTB sources for cattle, thus limiting the success of control programs. More in-depth studies are necessary to stablish the role of reservoir hosts in the transmission and maintenance of bTB.

Spoligotyping has limitations regarding its discriminatory power among isolates, and more accurate techniques, such as MIRU-VNTR or Whole Genome Sequencing, would be key to establish infection directionality under the epidemiological context presented here. Also, spatially explicit modeling of different spoligotypes over time might shed additional light on the potential for cross-species transmission of bTB, and specifically on the role of wild boar as reservoir and spillover host. A spatially broader sampling scheme including abattoirs from other geographical regions might reveal spatial patterns in the associations between farm-level epidemiological variables and TB-TP.

In conclusion, the study here suggests an association between presence of cattle and increased odds for bTB in domestic pigs, and also, transmission between domestic pigs and wild boars in Argentina. Results contribute to understanding the epidemiology of bTB in Argentine swine. These results will ultimately contribute to the design and implementation of surveillance and control programs for the disease in Argentina and other settings in which the disease is endemic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SB: data curation, formal analysis, methodology, funding acquisition, writing—review, and editing. MM: data curation, methodology, writing-review, and editing. GC: formal analysis and methodology. MA: data curation and methodology. ME and MV: writing—review and editing. MZ, MC, and MW: methodology. AP: methodology, writing—review, and editing. LS: data curation, formal analysis, writing—review, and editing. All authors contributed to the article and approved the submitted version.

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A Longitudinal Study of Cattle Productivity in Intensive Dairy Farms in Central Ethiopia

Rea Tschopp[1,2,3]*, *Gizachew Gemechu*[3], *James L. N. Wood*[4] and *The ETHICOBOTS Consortium*

[1] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [2] University of Basel, Basel, Switzerland, [3] One-Health Unit, Armauer Hansen Research Institute, Addis Ababa, Ethiopia, [4] Disease Dynamics Unit, Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom

Ethiopia is witnessing an emergence of intensive urban dairy farming. The aims of this study were to capture cattle productivity parameters in selected intensive dairy farms in and around Addis Ababa (Central Ethiopia). The study is a pre-requisite and baseline for further economic analysis of diseases such as bovine tuberculosis (BTB) and to assess some of the current challenges faced by farm owners for optimal animal performances. Hence, a 3-year longitudinal observational study was conducted for the first time in Ethiopia, in 24 dairy farms with intensive husbandry, including a total of 1,705 dairy animals. Herd characteristics, animal movement, and productivity parameters (fertility, morbidity, mortality) were recorded in a herd-book. Whereas, half the farms saw their animals increase in number over the 3 years, 37.5% (mainly large farms) saw their herd size decrease. Offtakes accounted for 76.6% of all animal exits. One hundred and ninety (11.1%) animals died of natural causes. Highest mortality was observed in young stock (13.9%). Overall, diseases were the leading cause for death (57.5%). The majority of calves (69%) that died, did so within the first week of life. Mean calving interval (CI) was 483.2 days. Successful conception after artificial insemination (AI) was 66.1% with Addis Ababa and smaller farms faring worst. Mean time interval from calving to first service was 152 days. Date of birth to first service was 592.2 days and date of birth to first calving was 794.7 days. In conclusion, the study showed sub-optimal productivity performances in intensive dairy cattle and highlighted some of the current gaps and challenges in urban dairy productivity.

Keywords: intensive dairy, Ethiopia, productivity, fertility, mortality, morbidity

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***Correspondence:**
Rea Tschopp
rea.tschopp@swisstph.ch

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INTRODUCTION

Ethiopia has the largest livestock population in Africa, with an estimated 60 million cattle (1). The great majority of cattle are local zebus while upgraded cattle (exotic breeds and crossbreeds) account for 0.22 and 1.54%, respectively (1). Ethiopia, thanks to its temperate climate, holds large potential for dairy development. Numerous program and policy interventions were implemented in the last decades to develop the dairy sector. As a result the milk production increased steadily from about 927 million liters in 1996 to 3.3 billion liters in 2018 (1, 2).

The Ethiopian Government has promoted a national development strategy of agricultural-led industrialization encouraging the private sector to get more involved in dairy farming (3). This

resulted in the emergence of private intensive dairy farms of various sizes run as a business, which focused on peri-urban and urban areas in the central highlands, taking advantage of urbanized large markets and the rapidly increasing demand for animal products such as milk (4, 5). Traditionally mainly located in and around Addis Ababa, intensive dairy farms are currently emerging in various locations in the Ethiopian Highlands (6, 7). Intensive commercial dairy farming is based on upgraded cattle breed, mainly Holstein-Friesian × Zebu, improved services and purchased conserved feed (8, 9). Almost all of the fluid milk supplied to major urban and peri-urban centers in Ethiopia, comes from these urban and peri-urban smallholder and commercial dairy producers (10, 11).

Despite the overall increased milk production, average milk yield per dairy cow remains low (1). In addition, fertility issues have been highlighted as a major problem in both local and crossbreed cattle in Ethiopia (12–14). Several factors contribute to this low productivity; among them diseases, nutrition, poor management, lack of infrastructure, and veterinary service provision (15, 16).

Longitudinal productivity studies are valuable tools to assess herd productivity, carry out cost-benefit analysis, evaluate potential health, and/or economic impacts of diseases and assess efficacy of interventions. These kind of studies provide a more accurate picture than rapid appraisals or cross-sectional studies. A longitudinal study on productivity of cattle held under traditional husbandry systems was performed in Ethiopia (17), which described herd dynamics, productivity parameters, and main constraints to better productivity. No such studies have so far been performed in the context of the Ethiopian urban dairy industry. Numerous research on dairy cattle productivity were done in Ethiopia but these either included small holders in peri-urban and rural areas, or focused only on a specific productivity parameter or are very much outdated (13, 14, 18, 19).

Current information on productivity in dairy cattle kept under intensive dairy systems in urban areas is lacking. The aims of this longitudinal study were to capture productivity parameters over a period of 3 years in selected intensive dairy farms in and around Addis Ababa (Central Ethiopia) and to assess some of the current challenges faced by farm owners for optimal animal performances.

MATERIALS AND METHODS

Study Sites

This study was carried out in Central Ethiopia within the greater milk production belt, namely Addis Ababa (Kaliti, Kolfe, Yeka, Gulele districts), as well as Debre Zeit (also known as Bishoftu) and Sendafa, 50 and 40 km away from the capital, respectively. Data were collected between November 2015 and September 2018. The area is conducive to dairy farming and has high production potential. It benefits from a temperate climate, abundant rainfall (1,000–1,900 mm/year) with good animal fodder potential and holds the largest high yielding dairy cow numbers in the country (1). The milk shed of the study areas has access to big markets including Addis Ababa (20).

Study Farms and Animals

This study was part of a larger study assessing Bovine tuberculosis (BTB) in dairy cattle in Central Ethiopia. A list of farms was obtained by district veterinary officers. For our study though, intensive urban dairy farms were purposively selected based on the willingness of the owners to collaborate on a 3-year longitudinal study. These owners were involved in the overall BTB testing program. Budget and logistics allowed for the selection of maximum 30 farms. Purposive sampling can lead to some degree of selection bias. Analysis of herd structure of the overall project in the area and of these selected farms were however very similar, hence giving evidence of representativity. Animals were high milk yielding Holstein-Friesian crossbreed cattle. Farm sizes were represented, and categorized into small farms (3–19 animals), medium farms (20–49 animals), and large farms (50 and more animals). Except for one government farm, all farms were privately owned. Husbandry was similar within farm size categories. Data from farms leaving the study for any reason before year 3 were not taken into account in the final data analysis.

Animals were categorized into sex and age class. Young stock were animals younger than 12 months, replacement stock were animals from 12 months to around 3 years, and breeders from 3 years onwards.

Tools and Parameters Recorded

An initial registration of all animals was performed in each farm at the start of the study. A herd-book was prepared capturing parameters such as any new animal entry (purchase, birth, gift), animal exit (selling, slaughtering, death), detailed data related to selling and purchasing of animals (e.g., cost, location, reason), morbidities including mastitis, mortalities, and fertility (AI dates, calving dates). The farms were visited twice a month by the same investigators. Herd-book information were updated on hard copies during each visit, and data entered in a Microsoft Access table. General observations were made on-farm during each visit (e.g., husbandry, fodder, discussion with farmers about any problems encountered). All animals were dewormed once a year with Albendazole by the farmers.

During the bi-monthly visits, the investigators who were all trained veterinarians offered advise on diverse husbandry issues and disease management, and provided training on particular topics on those farms that requested it (e.g., better heat detection, husbandry and fodder improvement, calf mortality, diseases).

Data Analysis

Data were entered into Microsoft Access, from which smaller subsets of data were transferred into Microsoft Excel tables to analyse particular parameters as needed. All data were analyzed using Stata 15 (StataCorp, Texas, USA). Data were analyzed using descriptive statistics. One-way analysis of variance (ANOVA) was used to analyze fertility parameters and results showed as Standard Error (SE), Standard Deviation (SD), 95%CI, and *p*-value. Calving rates, mortality and morbidity rates, offtake rates, and sales rates were derived from the collected data. Offtake rates were the proportion of sold or slaughtered animals in one particular year. Net offtake rates were calculated as the number

TABLE 1 | Overall cumulative herd structure categorized by sex and age class.

Overall herd structure	Female	Male	Total
Young stock (<1 year)	481 (36)	333 (90.5)	814 (47.7)
Replacement (1–3 years)	313 (23.4)	23 (6.2)	336 (19.7)
Breeder (>3 years)	543 (40.6)	12 (3.3)	555 (32.6)
Total	1,337	368	1,705

of animals that were removed from the herd (slaughter/sold) minus the number of animals that were brought into the herd (purchase/gift) divided by the initial herd size (opening number) multiplied by 100.

Ethical Clearance

This research study was approved by the Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (reference number IRB/ALIPB/2018), the Institutional Review Board of AHRI (AAERC) (reference number PO46/14), and supported by the Ethiopian Ministry of Livestock and Fisheries.

RESULTS

Herd Structure and Dynamic

Out of the 30 farms originally included in the study, six farms left either due to unwillingness to continue the study ($N = 2$), difficulty maintaining a herd-book follow-up (high staff rotation; poor data availability/accuracy) ($N = 3$) or due to farm closure ($N = 1$). Data were collected from 24 dairy farms from November 2015 to September 2018 and included in the final data analysis. Eight farms were categorized as small with a mean herd size of 13.2 animals (95%CI: 10.3–16.1) at initial registration. Eleven farms were medium sized with a mean herd size of 31 animals (95%CI: 24.1–37.9). Five were large farms with a mean herd size of 93.8 animals (95%CI: 65.5–122). A cumulative total of 1,705 animals were included in the study. The overall cumulative herd structure over the entire study duration is shown in **Table 1**. Female breeders accounted for 40.6% of all females. Of these, 10.9% were older than 10 years. Herd structure was similar in all three farm categories.

Overall, 789 new animals entered the study during the 3 years. The majority were calves born on farms (724; 91.8%), whereas 49 (6.2%) animals were purchased and 16 (2%) were either shared between farms or given as gifts. Females represented 57.5% of all newborn calves. Heifers ($N = 27$) and cows ($N = 13$) represented the majority (81.6%) of all purchased animals.

Overall, 830 animals left the study herds. The majority of exit were due to offtakes ($N = 638$; 76.6%) followed by natural death ($N = 190$; 23%), whereas 3 (0.4%) animals were lost through divorce asset sharing and 1 (0.1%) animal was stolen. Half of all exited animals (offtake and death) were calves ($N = 417$; 50.2%), a third were adult breeders ($N = 281$; 33.9%), and the others replacements ($N = 132$; 15.9%).

Half of the farms (50%) saw their animal numbers increase over the 3 years (total herd increase by 42%), 3 (12.5%) remained stable whereas 9 farms (37.5%) saw their animal numbers decrease (total herd decrease by 30.6%), including 4 out of the 5 large farms (80%). Geographical location ($p = 0.33$) and farm size ($p = 0.163$) were not statistically associated with change in herd size.

Offtakes and Natural Death

During the study, 190 (11.1%) animals died of natural causes. **Table 2** shows the mortality and offtakes by age and sex during the entire study.

The highest mortality was observed in young stock (13.9%), where mortality in males (15%) was higher than in females (13.1%).

The cause of death was known for 134 animals (see **Figure 1**). Overall, infectious diseases were the leading cause of animal mortality.

Among the 39 calves for which detailed information on the cause of death existed, besides infectious diseases, diarrhea accounted for the highest mortality ($N = 17$; 43.6%). Unexplained progressive weakness, emaciation, and refusal to drink caused death in 10 calves (25.7%), 5 (12.8%) died at birth, 4 (10.2%) animals died of respiratory distress, 2 of bone deformation/lameness, 1 had swallowed a foreign body and choked, another 3 died suddenly.

The majority ($N = 78$; 69%) of all calves died during their first week of life, whereas 87.6% ($N = 99$) died before the age of 6 months.

Among breeders, a third (34%) of the deaths were caused by digestive problems including metabolic problems, 25% were caused by parturition related problems and another 25% by infectious diseases [rabies, Foot- and Mouth Disease (FMD), and Bovine Viral Diarrhea (BVD)]. Hypocalcaemia (milk fever) was the cause of 13.6% of all female breeder's death. Another 13.6% died after experiencing progressive emaciation and weakness.

Table 3 shows the crude offtake rates (COR) and the net offtake rates (NOR) per age group and sex for year 1 and year 2. Year 3 was an incomplete 12 months period and hence data were not used in this table. The biggest offtake rates were observed in male calves (NOR = 70.6%). The majority of male calf offtakes happened in the first month of life (81.6%). A 185 (77.4%) calves were removed from the herd between the age of one and 15 days. Nineteen (79%) female calves were removed when aged 5 months or older.

Morbidity

Overall, 297 animals were reported sick during the study. Infectious diseases (33.7%), leg problems (lameness, arthritis, wounds, edema, abscess) (19.9%), and diarrhea (13.1%) were the three most recorded problem categories, followed by fertility/genital tract related problems (7.4%), respiratory (2.7%), and others (23.2%).

Detailed information on morbidity was recorded in 225 replacement/breeders and 63 young stock (see **Figures 2A,B**).

In replacements and breeders, the leading causes of morbidity were known infectious diseases ($N =$

TABLE 2 | Mortality and offtake numbers by age and sex during the entire study period.

Age and sex category	Total cumulative numbers	Natural death		Offtakes	
		Total animal number	% of category	Total animal number	% of category
Young stock	814	113	13.9	263	32.3
Female	*481*	*63*	*13.1*	*24*	*4.9*
Male	*333*	*50*	*15*	*239*	*71.7*
Replacement	273	17	6.2	53	19.4
Female	*238*	*15*	*6.3*	*38*	*15.9*
Male	*35*	*2*	*5.7*	*15*	*42.8*
Breeder	657	60	9.1	323	49.1
Female	*645*	*60*	*9.3*	*320*	*49.6*
Male	*12*	*0*	*0*	*3*	*25*
Total	1705	190	11.1	639	37.4

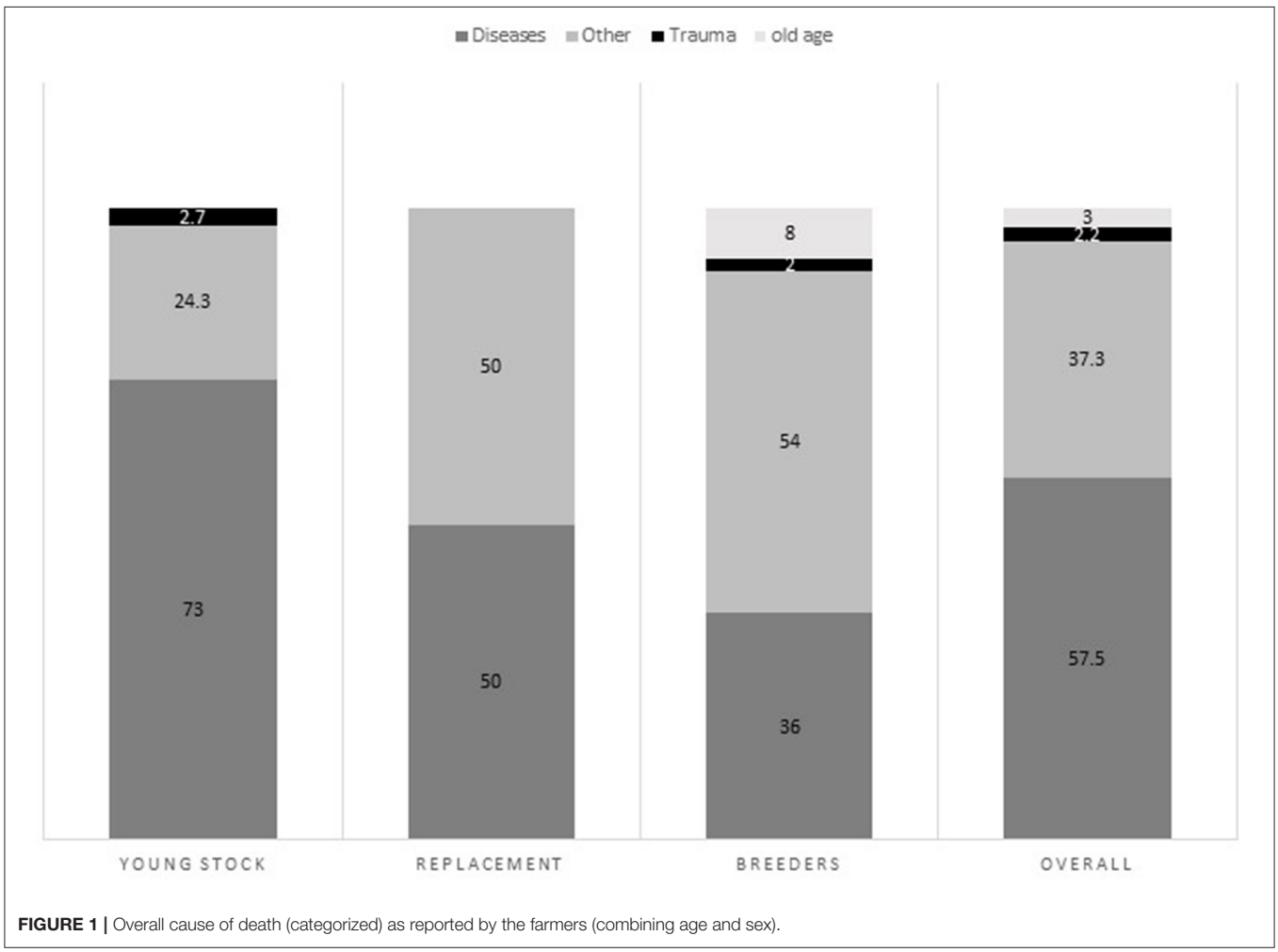

FIGURE 1 | Overall cause of death (categorized) as reported by the farmers (combining age and sex).

58; 25.8%) such as BVD, FMD, Lumpy skin disease (LSD), and rabies. Skin diseases ranked second with 40 cases (17.7%), followed by musculoskeletal problems (16.4%).

In addition, clinical mastitis was recorded in 76 animals (heifers and breeders), which accounts for approximately 9% morbidity. This number is likely severely underestimating the true mastitis incidence (personal observation).

FIGURE 2 | Detailed health problems as recorded in 288 animals (**A** in 225 replacement/breeder animals; **B** in 63 young stock).

TABLE 3 | Crude offtake rates (COR) and Net offtake rates (NOR) by age and gender for the first and second year of the study.

	Number animals		Number offtakes		Number purchases		COR (%)		NOR (%)			
Age	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Average COR (%)**	**Average NOR (%)**
Young stock	365	457	137	115	0	2	37.5	25.1	37.5	24.7	31.3	31.1
Female	230	316	38	18	0	1	16.5	5.7	16.5	5.4	11.1	10.9
Male	135	141	99	97	0	1	73.3	68.8	73.3	68	71	70.6
Replacement	308	273	112	71	11	21	36.4	26	32.8	18.3	31.2	25.5
Female	289	255	110	71	9	20	38	27.8	35	20	32.9	27.5
Male	19	18	2	0	2	1	10.5	0	0		5.2	0
Breeder	538	433	240	143	49	48	44.6	33	35.5	21.9	38.8	28.7
Female	528	422	219	140	47	45	41.5	33.2	32.6	22.5	37.3	27.5
Male	10	11	3	3	2	3	30	27.3	10	0	28.6	5
Total	1,211	1,163	471	329	60	71	38.9	28.3	33.9	22.2	33.6	28

Fertility

Table 4 shows the results for four fertility parameters for each farm size category. Generally, larger farms tended to show better performances with a clear statistical difference ($p = 0.007$) for calving to next service interval as compared with the other farms.

Forty-six animals out of 870 animals that had received at least one artificial insemination (AI) had aborted (5.3%).

Out of 610 animals that received AI, 341 had calved successfully (55.9%), whereas 175 failed to calve after 9 months (28.7%). Ninety-four were slaughtered or sold before the end of the 9 month pregnancy period (15.4%).

Overall, one-third of the AI did not lead to a successful calving. The conception rate decreased as the farm size decreased, and was lower in Addis Ababa as compared to the other geographical locations (see **Table 5**).

The numbers of AI provided per animal ranged from one to ten, with a median (50th percentile) of one (**Table 6**). Indeed, the majority of animals (65.4%) only had one AI; 83.9% received one or two; 14.2% received between three and five; and 1.9% received six or more AIs (**Table 6**).

DISCUSSION

Knowledge of herd structure, fertility, mortality, and offtake rates are key parameters for determining the population dynamics and herd productivity in the dairy sector at both farm and country level.

Long-term longitudinal studies have pros and cons. There is always the risk of participation bias, drop-outs during the study and delayed data analysis and results. They require a substantial time effort, which is also often linked to logistical and financial challenges and limitations. However, these type of studies produce more accurate and reliable data, reduce recall biases and are closer to the reality of event timelines, allowing to assess cause-effects more accurately. Representativeness of the larger population is always a limitation. In our study, we compared herd structure and farm size with the ones provided by the larger project for Central Ethiopia and they were very similar, thus providing some indication of representativeness. However, results translation to the larger national dairy cattle population should be done with care.

Major challenges faced in our study to record productivity were: lack of animal identification, lack of record keeping, high staff turn-over, lack of interest of owners, and depopulation or closing down of farms. Only a few farms (including all large farms) had their animals officially tagged despite the Ethiopian Government's efforts to implement a livestock identification system using plastic ear tags through the National Artificial Insemination Center (NAIC). The Ethiopian Ministry of Livestock and Fisheries launched a national livestock identification and traceability system (LITS) with the financial and technical support of the U.S. Agency for International Development (USAID) (21). Unfortunately, the system was not fully implemented during the time of our study. Owners would rely on their own identification system (individual name; own ear tag number) that were sometimes replaced entirely after some time with other ear tag numbers. Re-using ID tags of existing animals for a new animal was common. Accurate and updated record keeping of animals and productivity parameters were performed only in two large farms (8.3% of the farms). Two other large farms kept records on animal identification and some basic animal data but rarely updated the information on productivity. Hence the need for the study to introduce an independent animal registration and herd book that could be followed up for 3 years. In addition, dairy farm staff in medium and large farms were often untrained and showed high work turn-over. This likely contributed to poor animal management in many of the dairy farms. Nine farms (37.5%), including 4 out of the 5 large farms, decreased their number of animals. Reasons given included diseases, high running costs, poor availability of fodder, and poor animal productivity.

Dairy farms kept mainly female animals (78.4%). Among the males, the great majority were young calves (90.5%). Male calves represented also the biggest offtake (70.6%). They were largely removed from the farm during the first month of life, with 81.6% removed within the first 14 days of life. Currently

TABLE 4 | Fertility parameters during the study period.

Parameter	Farm size	Total number	Mean days	SE	CI for mean (days)	Min–Max (days)	*P*-value
DOB to first calving	Small	6	785	37.9	687.7–882.3	691–950	0.125
	Medium	14	740.1	32.1	670.7–809.5	617–1,044	
	Large	19	837.9	41.3	751–924.8	455–1,144	
	Overall	*39*	*794.7*	*24.5*	*744.9–844.4*	*455–1,144*	
DOB to first service	Small	10	592.2	14.7	563.1–621.4	422–675	*0.175*
	Medium	23	522.4	25.1	470.4–574.4	380–874	
	Large	53	642.9	17.4	607.9–678	428–967	
	Overall	*86*	*592.2*	*14.7*	*563.1–621.4*	*380–967*	
Calving to next service	Small	54	168.5	11.7	144.9–192	55–436	*0.007*
	Medium	84	158	11.8	134.6–181.5	21–669	
	Large	255	146.6	5.1	136.5–156.8	4–659	
	Overall	*393*	*152*	*4.5*	*143.2–160.9*	*4–669*	
Calving interval	Small	24	445.3	24.9	394.5–496.2	282–849	0.141
	Medium	67	462.5	15.3	431.9–493.2	292–967	
	Large	197	494.8	13.1	494.1–546	272–941	
	overall	*288*	*483.2*	*8.4*	*466.7–499.7*	*272–967*	

DOB, date of birth.

TABLE 5 | Number of successful AIs by farm size category and geographical locations.

Category		Total AI	Total failed conception after AI (%)	Total successful conception after AI (%)	Anova Bartlett's test *p*-value
Farm size	Small (3–19)	56	28 (50)	28 (50)	0.213
	Medium (20–49)	147	62 (42.2)	85 (57.8)	
	Large (50+)	313	85 (27.1)	228 (72.8)	
Location	Addis	257	112 (43.6)	145 (56.4)	0.06
	Debre Zeit	136	35 (25.7)	101 (74.3)	
	Sendafa	123	123 (22.8)	95 (77.2)	
Total		516	175 (33.9)	341 (66.1)	

TABLE 6 | Number of artificial insemination given per cow by farm-size category.

Number of AI/cow	Small farm	Medium farm	Large farm	Total
1	57 (58.8)	163 (72.8)	337 (63.5)	557 (65.4)
2	19 (19.6)	38 (17)	101 (19)	158 (18.5)
3–5	17 (17.5)	22 (9.8)	82 (15.4)	121 (14.2)
≥6	4 (4.1)	1 (0.4)	11 (2)	16 (1.9)
Total	97	224	531	852

not much veal meat is produced; it comes from very young calves (younger than 1 month old) with a market directed mainly to expatriate communities living in the capital city. Offtakes represented the majority of all animal exits, whereas natural death accounted for a quarter of the exits. Farms relied predominantly on own breeding rather than purchasing new animals ($N = 49$; 6.2%). Low numbers of purchased animals are ideal from a biosecurity point of view as the additional risk and burden of importing new diseases into the farm remains low at farm level.

Highest mortality was found among young stock (13.9%), which is higher than calf mortality of 9.3% found in small-holders in Hawassa (22) but in line with results found in calves from dairy farms by Romha et al. (23) in Debre Zeit (Bishoftu). The latter study observed that lack of colostrum provision was the major cause of mortality and diarrhea in calves. In our study, we did not follow up on the provision of colostrum. However, diarrhea accounted for 43.6% of calf mortality suggesting husbandry issues. Diarrhea was also observed by Megersa et al. (22) to be the leading cause of calf morbidity. A quarter of calves (25.7%) died following a period of progressive emaciation and reluctance to take milk, also suggestive of possible husbandry problems. Farmers acknowledged that calf mortality was one of the major issues on their farm in addition to fertility problems. Mortality in male calves (15%) was a slightly higher than in female calves (13.9%). This might reflect the fact that male calves were removed mostly within the first 2 weeks of life and were thus neglected. Death during birth or shortly after birth was

recorded in 12.8% of calves. High neonatal calf mortality was also observed in dairy farms by Fentie et al. (24). High calf mortality was one of the major complaints of the farm owners in our study. Among breeders, a quarter (25%) died due to parturition problems (e.g., birthing, endometritis, uterine prolapse). These results highlighted poor obstetric practices and husbandry as well as poor nutritional management (25). A third of all deaths in adults were caused by digestive/metabolic problems hinting again to poor husbandry and feeding practices. Milk fever is a metabolic disease caused by blood calcium deficiency around birth time, leading to poor labor activity (dystocia), birth of weak calves or stillborn, and can be fatal for cows. In our study, 13.6% of breeders who died had milk fever. Gammada (26) also observed 17.3% of milk fever cases in small-scale dairy farms in Jimma. Hypocalcaemia, which was probably under-reported, could also be a cause for the high level of parturition issues and high calf mortality at birth. The condition is more prevalent in pluriparous cows with higher milk production, and is strongly linked to feeding management during the weeks prior to calving. A previous study by GebreMichael et al. (9) showed that 70% of the study farms did not adapt the diet of their pregnant cows during the critical dry period.

Husbandry and fodder issues were reflected in several of the reported animal morbidities. Leg problems involving arthritis, wounds, and abscesses were reported in 19.9% of the animals. In addition, not recorded but observed by the researchers was the high prevalence of untrimmed hooves in all farms, leading to chronic hoof deformities and lameness. Chronic pains and inability to walk, jump, and display normal behavior can have an impact on fertility (poor heat detection) but also on milk productivity (27). The many metabolic issues reported such as bloating and milk fever are indications of inadequate fodder intake and feeding management. Anecdotally, some farmers were observed giving colostrum to drink to cows that had calved, leading to serious bloating. Infectious diseases were the top causes for animal morbidity (33.7%). Infectious diseases, such as Blackleg and LSD were also previously observed being leading causes of morbidities in small-scale dairy farms in Jimma (26). In our study, Besides bacterial septicemia, FMD, LSD, and BVD were recorded. Foot- and mouth disease is an endemic disease in Ethiopia with sero-prevalence in Central Ethiopia ranging between 14.5 and 30% (28–30). Lumpy skin disease prevalence in dairy cattle in Central Ethiopia ranges from 22.5 to 33.9% with mortality up to 7.4% (31, 32). Seropositivity of BVD was reported in dairy cattle as high as 32.6% (33). Infectious diseases can have a range of impacts on animal productivity. In our study, none of the farms had consistent appropriate biosecurity measures in place. The reasons were not specifically investigated as part of the study but included lack of awareness and cost. Infectious diseases were also the leading cause of death. In adults, rabies, FMD and BVD caused 25% of all deaths during the study period. LSD and FMD vaccines are produced in-country, though sometimes with limited capacity. Most farmers do not regularly vaccinate their cattle and rather wait for an outbreak to do so often worsening the outbreak situation and increasing complaints from farmers about the vaccine efficacy (NVI, personal communication).

Mean calving interval (CI) in our study was 483.2 days (16 months). This is a sub-optimal CI as compared to the ideal 365 days for dairy cattle (34). Studies on reproductive performances in intensive dairy cows in Ethiopia are sparse or outdated (35). Twenty years ago, studies done in dairy cattle in Central Ethiopia showed a CI ranging between 435.2 and 544.9 days (average 490 days) (18), which indicates the fertility performances have not much improved. Several factors can contribute to this long CI. Lobago et al. (19) showed that 67.4% of cows showed a delayed return to ovarian cyclicity after calving (longer than 55 days). Prolonged postpartum anestrus is most likely linked to inadequate nutrient intake. Other factors influencing the CI include the ideal timing of post breeding pregnancy testing (36) and proper service. In our study the majority of cows (65.4%) received only one AI. Besides financial constraints, this was mostly due to the lack of qualified insemination technicians or technicians not coming to the farm after the call and thus missing the estrus period. On the other hand, some animals in large farms received up to 10 AIs. Large farms sometimes have assigned AI technicians who are on call. The mean time between calving and the first AI given was 152 days (CI: 143.2–160.9). This was longer than the reported 115 days by Tadesse et al. (35) and much longer than the ideal calving-first insemination interval. Intervals between two AIs without a calf being produced ranged between 1 and 408 days. The median interval was 59.5 days (CI: 49.1–66 days). Animals were sometimes inseminated although not showing signs of estrus, e.g., during the first couple of weeks after calving (7.2% of AIs), during pregnancy, or the AI technician inseminated several animals the same day to reduce the numbers of visits even if outside the possible estrus period. 40.5% of AIs were repeated after 2.5 months. Poor estrus expression, detection by the owner, and poor AI techniques (timing, conception rate, pregnancy diagnosis, etc.), led to poor service per conception outputs. Infectious diseases are also known to affect fertility and cause diverse reproductive disorders. In intensive dairy cattle in central Ethiopia, high prevalence of *Neospora caninum* and Schmallenberg virus were both observed (37–39), whereas BVD was linked in Jimma (Western Ethiopia) with reproductive disorders in cattle (40).

Overall, 28.7% of all inseminations failed to conceive a calf after 9 months. The conception rate was better the bigger the farm. Small farms fared the worst as well as Addis Ababa (56.4%) as compared to the other sites. Abortion rate was in our study 5.3%. Previously reported abortion rates ranged between 1.7 and 20.2% (18, 41).

Our study showed overall sub-optimal productivity performances in intensive dairy farms, likely contributing to substantial financial losses at farm level. Keeping accurate herd book follow-up data would help identifying correctly and swiftly animals with reproductive problems, hence would help improve farm productivity. Record keeping helps managing the question of profitability to continue to inseminate animals with problems and this will increase farm profit. The problems highlighted in this study are complex and often interlinked. A holistic approach is needed in order to improve overall animal productivity. Poor husbandry was observed to be a major contributor to poor productivity, affecting among others calf

mortality, and animal fertility. These were also the two major topics that farmers would regularly raise during our entire study and questioning how to address them.

CONCLUSION

Longitudinal productivity studies and herd-book follow-ups are great tools to identify productivity challenges and establish baseline productivity data, on which further economic studies can be built upon. Despite the growing importance of the dairy industry in Ethiopia, accurate data on dairy cattle productivity under intensive farming is often lacking. Larger sample size would be warranted to corroborate our findings. Besides improving animal tracking (ear-tagging that is reliable and centralized), keeping accurate animal records and tackling prevalent infectious diseases, emphasis should be urgently given to improving overall animal husbandry and feeding, all of which would ultimately lead to better productivity and better animal welfare. Continuous training opportunities of farmers and their staff is a key step toward improving all the above mentioned aspects. Certifications of farms, and creation of model-farms can further help improve training quality and standard, and provide a positive deviance approach to better animal husbandry.

THE ETHICOBOTS CONSORTIUM

The members of the Ethiopia Control of Bovine Tuberculosis Strategies (ETHICOBOTS) consortium are: Abraham Aseffa, Adane Mihret, Bamlak Tessema, Bizuneh Belachew, Eshcolewyene Fekadu, Fantanesh Melese, Gizachew Gemechu, Hawult Taye, Rea Tschopp, Shewit Haile, Sosina Ayalew, Tsegaye Hailu, Armauer Hansen Research Institute, Ethiopia; Rea Tschopp from Swiss Tropical and Public Health Institute, Switzerland; Adam Bekele, Chilot Yirga, Mulualem Ambaw, Tadele Mamo, Tesfaye Solomon, Ethiopian Institute of Agricultural Research, Ethiopia; Tilaye Teklewold from Amhara Regional Agricultural Research Institute, Ethiopia; Solomon Gebre, Getachew Gari, Mesfin Sahle, Abde Aliy, Abebe Olani, Asegedech Sirak, Gizat Almaw, Getnet Mekonnen, Mekdes Tamiru, Sintayehu Guta, National Animal Health Diagnostic and Investigation Center, Ethiopia; James Wood, Andrew Conlan, Alan Clarke, Cambridge University, United Kingdom; Henrietta L. Moore and Catherine Hodge, both from University College London, United Kingdom; Constance Smith at University of Manchester, United Kingdom; R. Glyn Hewinson, Stefan Berg, Martin Vordermeier, Javier Nunez-Garcia, Animal and Plant Health Agency, United Kingdom; Gobena Ameni, Berecha Bayissa, Aboma Zewude, Adane Worku, Lemma Terfassa, Mahlet Chanyalew, Temesgen Mohammed, Miserach Zeleke, Addis ababa University, Ethiopia.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because Project regulations of the Ethicobots consortium project (PI: JW). Requests to access the datasets should be directed to JW (jlnw2@cam.ac.uk).

ETHICS STATEMENT

This research study was approved by the Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (reference number IRB/ALIPB/2018), the Institutional Review Board of AHRI (AAERC) (reference number PO46/14), and supported by the Ethiopian Ministry of Livestock and Fisheries. Written informed consent for participation was not obtained from the owners because a verbal informed consent was provided by the participants.

AUTHOR CONTRIBUTIONS

RT has designed the research, performed the data analysis, and drafted the manuscript. GG has collected field data and contributed to drafting the manuscript. JW provided critical comments to the Manuscript. All authors have contributed to the manuscript, and have approved the submitted version.

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Cellular and Cytokine Responses in Lymph Node Granulomas of Bacillus Calmette Guérin (BCG)-Vaccinated and Non-vaccinated Cross-Breed Calves Naturally Infected With *Mycobacterium bovis*

Asegedech Sirak[1,2], *Begna Tulu*[2,3], *Berecha Bayissa*[1,4], *Balako Gumi*[1], *Stefan Berg*[5], *Francisco J. Salguero*[6‡], *Gobena Ameni*[1,7*] *and The ETHICOBOTS Consortium*[†]

[1] *Animal Health and Zoonotic Research Unit, Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia,* [2] *National Animal Health Diagnostic and Investigation Centre, Sebeta, Ethiopia,* [3] *Medical Laboratory Science Department, Bahir Dar University, Bahir Dar, Ethiopia,* [4] *Vaccine Production and Drug Formulation Directorate, National Veterinary Institute, Bishoftu, Ethiopia,* [5] *Bacteriology Department, Animal and Plant Health Agency, Weybridge, United Kingdom,* [6] *Department of Pathology and Infectious Diseases, University of Surrey, Guildford, United Kingdom,* [7] *Department of Veterinary Medicine, College of Food and Agriculture, United Arab Emirates University, Al Ain, United Arab Emirates*

Local immunological responses at the site of infections, such as at the lymph nodes and lungs, do play a role in containing infection caused by *Mycobacterium bovis* (*M. bovis*). This bovine tuberculosis (bTB) study was conducted to evaluate cellular and cytokine responses in the lymph nodes and lungs of BCG-vaccinated and non-vaccinated calves that were naturally infected with *M. bovis*. Immunohistochemical assays were used for examination of the responses of macrophages, T cells, cytokines and chemical mediators of 40 (22 vaccinated and 18 non-vaccinated) Holstein-Friesian-zebu crossbred calves that were naturally exposed for 1 year to a known bTB positive cattle herd. The incidence rates of bTB visible lesion were 68.2% (15/22) and 89% (16/18) in vaccinated and non-vaccinated calves, respectively. The local responses of $CD4^+$ and $CD8^+$ T cells, and those of IFN-γ and TNF-α within the lesions, were stronger ($P < 0.05$) in BCG-vaccinated calves than in non-vaccinated calves. However, there was no statistically significant difference between the two groups ($P > 0.05$) in the response of $CD68^+$ cells. Thus, the findings of this study indicated stronger responses of a set of immunological cells and markers at the local granulomas of BCG-vaccinated calves than in non-vaccinated calves. Furthermore, BCG vaccination may also play a role in reducing the severity of the gross pathology at the primary site of infection.

Keywords: BCG vaccination, crossbred calves, immunological response, *Mycobacterium bovis*, natural infection

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Edited by:
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Agricultural Research Council of South Africa (ARC-SA), South Africa
Douwe Bakker,
Independent Researcher, Lelystad, Netherlands

****Correspondence:***
Gobena Ameni
gobena.ameni@aau.edu.et;
gobena.ameni@uaeu.ac.ae

[†]*Members of the ETHICOBOTS consortium are listed in the Acknowledgments*

‡Present address:
Francisco J. Salguero, National Infection Service, Public Health England, Salisbury, United Kingdom

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INTRODUCTION

Bovine tuberculosis (bTB) is a chronic progressive disease of cattle and other animals that is caused by *Mycobacterium bovis* (*M. bovis*) and is characterized by progressive development of granulomatous lesions, termed as tubercles, in different tissues. BTB poses a major economic problem worldwide costing US$3 billion annually through infecting over 50 million cattle (1). In addition, it causes zoonotic TB in humans particularly in developing countries in Africa, Asia and Latin America (2). The conventional control measures for bTB are based on a test-and-slaughter method, which is less likely to be implemented by the developing countries because of socioeconomic reasons (3). Furthermore, the test-and-slaughter control method have been shown not to be effective in some developed countries, like the United Kingdom and New Zealand, because of the interference of reservoirs of *M. bovis* in infected wildlife (4, 5). Therefore, alternative control methods such as the use of vaccination are required for improved control of bTB.

BCG is the only vaccine approved against human TB (6) and animal TB (7), and it has been used in field studies in cattle yielding variable protective efficacy levels (8–11). Most of these studies evaluated immunological responses induced by BCG vaccination in peripheral blood. In addition, data on the immunological responses induced by BCG-vaccination at the local site of infection (granulomatous lesion) are useful for understanding the roles of different cell types and immunological markers (12–14).

Cell-mediated immune response is crucial in the defense against intracellular bacterial pathogens including mycobacteria (15, 16). Immune cells like CD4, CD8 and γ/δ T cells are shown to be activated when exposed to mycobacterial antigens based on studies conducted in mice, humans, and cattle (17–19). These cells are recruited to the site of infection and are capable of producing various cytokines, including interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (20).

BTB granulomas are characterized by different developmental stages that are influenced by the interaction between the host immunological responses and virulence of *M. bovis* isolates (21, 22). The granuloma, the hallmark lesions of TB disease, is characterized by the accumulation of activated macrophages and a variety of other immune cells against the mycobacteria. The formation of the granuloma have long been regarded as the critical host-protective structures that wall off the bacteria, creating an immune microenvironment in which the infection can be controlled. Within the granuloma the mycobacteria are thought to undergo replicative arrest and in response to stress such as hypoxia (23). However, if immunological responses are weakened, the granulomas fail to contain the pathogen, and as a result, the bacilli disseminate to secondary sites of infection, producing multiple granulomas (24). Thus, the interaction between the immune response and the mycobacteria at the local lesion is determining the outcome of the disease. It can also be observed that granuloma protect the mycobacteria from the immune response and is probably responsible for the persistent infection (25, 26). Microscopically, bTB granulomas have been classified into four different stages; stage I being the least severe stage while stage IV being the most severe stage (27, 28). The present study was conducted along a BCG efficacy study (29), to evaluate the cellular and cytokine responses induced by BCG-vaccination in the granulomas of Holstein-Friesian (H-F) and zebu crossbred calves that were naturally exposed to *M. bovis* in Ethiopia.

MATERIALS AND METHODS

Experimental Setting

A BCG efficacy study against bovine TB conducted at the National Animal Health Diagnostic and Investigation Centre (NAHDIC) under natural challenge conditions was utilized for this study to generate immunohistochemical (IHC) information in the same animals. In short, male HF-zebu crossbreed calves were recruited within 2 weeks of age from known bTB free cattle farms as confirmed by the Single Intradermal Comparative Cervical Tuberculin (SICCT) test for which PPD-A and PPD-B were sourced from Thermo Fisher (Lelystad, the Netherlands). Calves were randomly assigned to either the unvaccinated control or the BCG vaccinated group. The latter group was vaccinated subcutaneously with 1×10^6 CFU BCG (InterVax, Canada), a vaccine based on the Russian type of *Mycobacterium bovis* BCG. Six weeks after vaccination, the two groups of calves were introduced into a herd of SICCT test positive cows at a ratio of 2:1 (Experimental calves: Reactor cows). After completion of 12 months exposure, 22 BCG vaccinated and 18 non-vaccinated calves had completed the experiment and they were slaughtered for post mortem examination and collection of tissue samples.

Sample Collection and Processing

Gross and microscopic pathological examinations as well as immunohistochemical examination were performed by a pathologist who was blinded to the vaccination status of the calves. The pathologist was trained on IHC at the Pathology Laboratory of Surrey University (UK) by Dr. Francisco J. Salguero before performing this experiment. All lymph nodes in the head and neck region (left and right lateral retropharyngeal, left and right medial retropharyngeal, left and right mandibular, and left and right parotid), thoracic (mediastinal, cranial and caudal, right and left bronchial and tracheobronchial) and abdominal (hepatic and mesenteric) cavities, together with the lungs were carefully examined for gross lesions of TB and for mycobacterial culturing by Bayissa et al. (29). For the present study, all TB-like gross lesions were collected into either 10% neutral buffered formalin or zinc salt fixative solution, depending on the type of markers, for further processing for IHC staining.

Immunohistochemical Staining

All tissue specimens were processed and embedded into paraffin wax in the histopathology laboratory at NAHDIC. Tissues for the investigation of CD68, IFN-γ, TNF-α, and iNOS were fixed in 10% neutral buffered formalin while tissues for the investigation of CD4 and CD8 cells were fixed in zinc salt by following the procedure used previously (30). All formalin fixed

TABLE 1 | Immune markers used for immunohistochemistry.

Primary antibody	Antibody type	Dilution	Supplier	Epitope demasking method	Link antibody (dilution)	Primary antibody incubation (Temp, Time)	Source	Buffer
IFN-γ	Mouse anti bovine IFN-γ (monoclonal)	1/200	BIO-RAD	NA	Horse anti-mouse (1/200)	RT, 1 h	Thermo Scientific	PBS
TNF-α	Mouse anti bovine TNF-α (monoclonal)	1/100	BIO-RAD	Trypsin	Horse anti-mouse (1/200)	RT, 1 h	Thermo Scientific	PBS
CD68	Mouse anti human CD68 (monoclonal)	1/50	BIO-RAD	Trypsin	Horse anti-mouse (1/200)	4°C (Overnight)*	Thermo Scientific	PBS
CD4	Mouse anti bovine CD4 (monoclonal)	1/30	BIO-RAD	NA	Goat vs. mouse (1/200)	RT, 1 h	BIO-RAD	PBS
CD8	Mouse anti bovine CD8 (monoclonal)	1/100	BIO-RAD	NA	Goat vs. mouse (1/200)	RT, 1 h	BIO-RAD	PBS
iNOS	Rabbit anti mouse iNOS (polyclonal)	1/500	BIO-RAD	NA	Goat anti-rabbit (1/1,000)	RT, 1 h	Thermo Scientific	PBS

**The optimization for anti-CD68 primary antibody was done overnight at 4°C.*
NA, Not Applicable; PBS, Phosphate buffered saline; RT, Room Temperature.

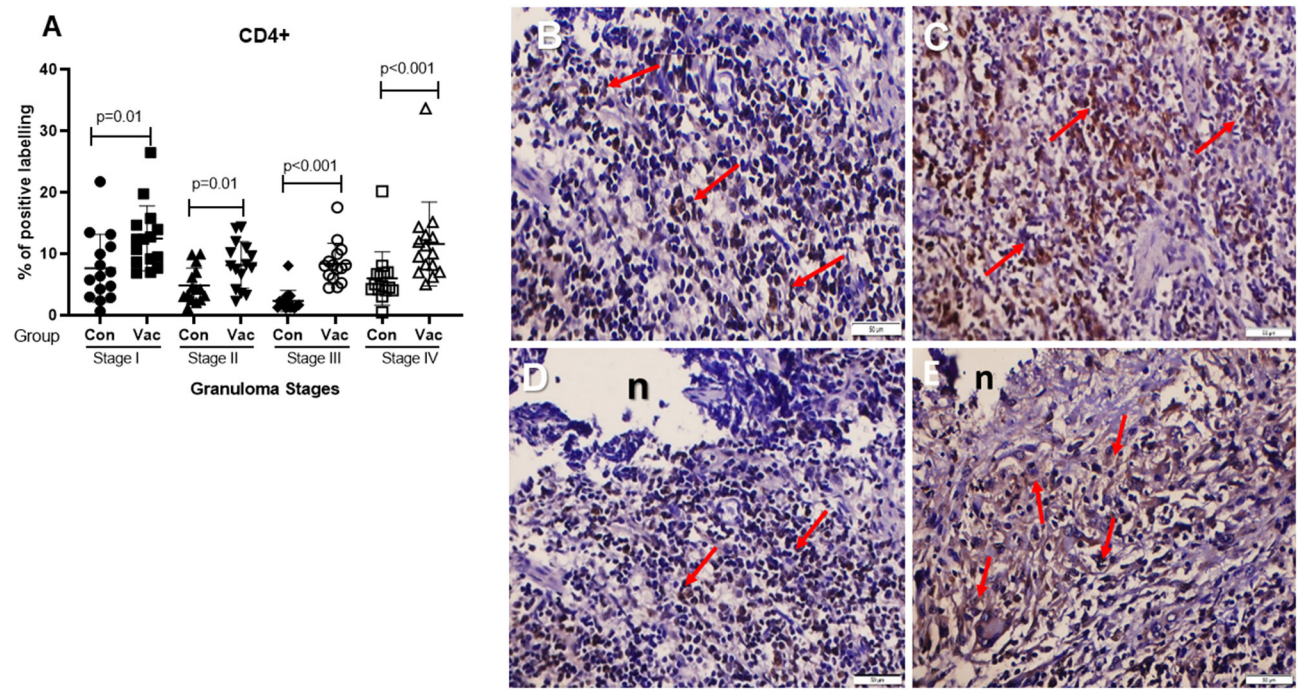

FIGURE 1 | Immunolabeling for $CD4^+$ cells (ABC IHC stain, DAB brown chromogen). **(A)** Comparison of $CD4^+$ cell distribution in different granuloma stages of BCG vaccinated (Vac, vaccinated) and non-vaccinated (Con, control) calves. **(B)** Stage I granuloma from non-vaccinated calves, showing stained $CD4^+$ cells (arrows). **(C)** Stage I granuloma from BCG vaccinated calf showing more $CD4^+$ cells (arrows). **(D)** Stage IV granuloma from non-vaccinated calf showing stained $CD4^+$ cells (arrows). **(E)** Stage IV granuloma from BCG vaccinated calf showing stained $CD4^+$ cells (arrows). Bar equals 50 μm in **(B–E)**. n, necrotic core.

tissues were processed within 7 days as previously described (31) whereas zinc solution fixation was carried out for 72 h. Specimens were trimmed and processed using a SHANDON Citadel 1000 tissue processor, dehydrated in six rounds using graded ethyl alcohol (at concentrations 70, 95, 95, and 3 × 100%) to remove excess water from the tissue, and cleared in three passes of xylene to clean alcohol from the tissue and to make the tissue clear/translucent. The tissue samples were then impregnated in melted paraffin (two passes) and embedded using the paraffin wax embedding method by the TEC2900 modular Tissue Embedding Center (Histo-line laboratories). Sections of 4 μm were prepared and placed on Vectabond-treated slides (Vector Laboratories, Peterborough, UK).

Lymph nodes and lung tissue sections from each animal were processed by IHC for cell types and chemical mediators using primary antibodies to label CD68, CD4, CD8, IFN-γ, TNF-α,

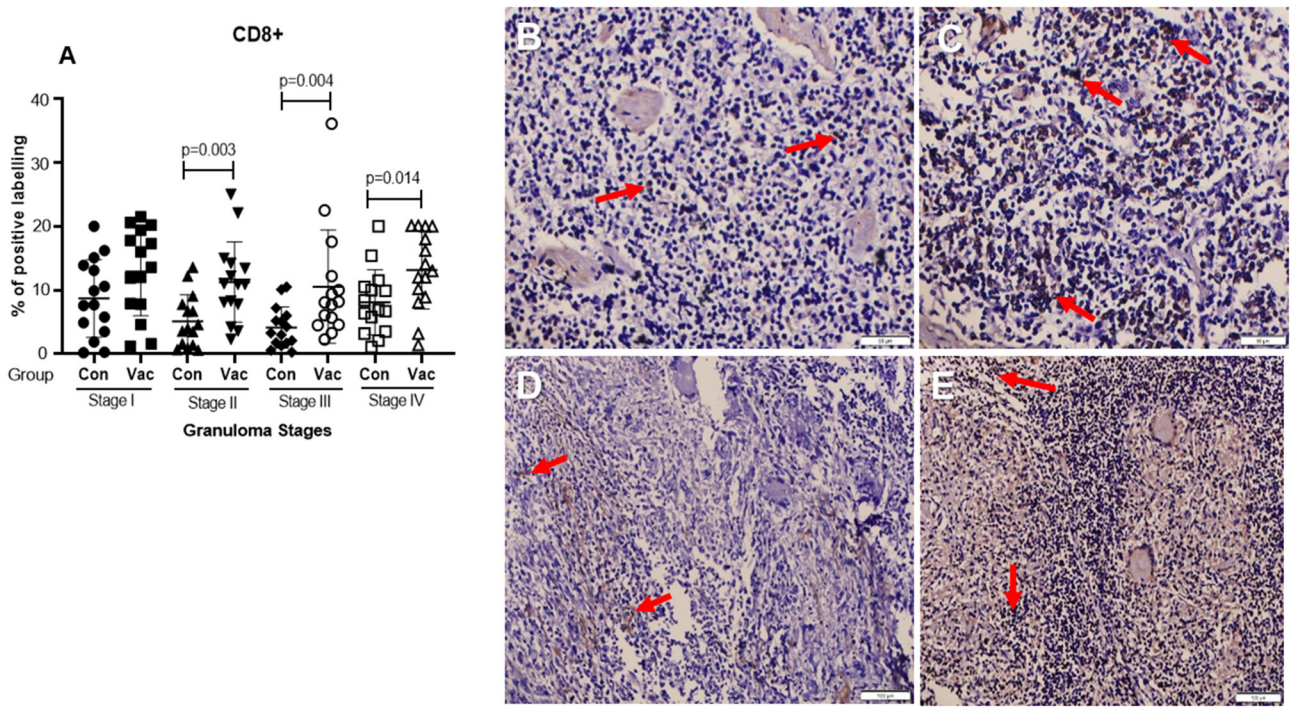

FIGURE 2 | Immunolabelling for $CD8^+$ cells (ABC IHC stain, DAB brown chromogen). **(A)** Comparison of $CD8^+$ cell distribution in different granuloma stages of BCG vaccinated (Vac) and non-vaccinated (Con) calves. **(B)** Stage I granuloma from non-vaccinated calf showing very few stained $CD8^+$ cells (arrows). **(C)** Stage I granuloma from BCG vaccinated calf showing stained $CD8^+$ cells (arrows). **(D)** Stage III granuloma from non-vaccinated calf showing few stained $CD8^+$ cells (arrows). **(E)** Stage III granuloma from vaccinated calf showing abundance of $CD8^+$ cells (arrows). Bar equals 50 μm in **(B,C)** and 100 μm in **(D,E)**.

or iNOS. The reagents and antibodies against each of these immunological markers are presented in **Table 1** and the staining was conducted as previously described (32). Enzymatic digestion was used as an epitope demasking technique for the formalin fixed material. Trypsin solution was prepared by measuring 0.5 g of trypsin, 0.5 g of chymotrypsin and 1 g of $CaCl_2$ and dissolving in 1 L of distilled water. A biotinylated secondary antibody (ABC Vector Elite; Vector Laboratories) was used at 1/200 (Ab + PBS) dilution for CD4 and CD8, and for IFN-γ and CD68 a biotinylated secondary antibody was used by adding 3 drops (135 μl) to 10 ml of normal horse serum (ABC Vector Elite; Vector Laboratories). For detection of TNF-α 1 drop (45 μl) of biotinylated secondary antibody from the ABC kit (ABC Vector Elite; Vector Laboratories) was added to 10 ml of PBS. For iNOS biotinylated Secondary antibody, the same dilution was prepared but instead of normal horse serum, normal goat serum was used from the ABC kit. A horseradish peroxidase-labeled avidin-biotin-complex was used with DAB as a chromogen (brown color) and Mayer's haematoxylin was used as counter-stain.

Image Analysis

Stained sections were subjected to digital image analysis to ascertain the percentage area of the tissue section positively labeled (brown color) for each marker. Images were captured using light microscopy (Nikon ECLIPSE E200 LED) and digital image analysis software (Nikon-NIS Br, Nikon, Japan). Consecutive sections were used and all granulomas observed within the tissue section were analyzed. The whole area of the granuloma was selected as Region of Interest (ROI), and the area with immunohistochemically-positive reaction within the ROI was calculated by the software after setting the thresholds. The results are expressed as the percentage of positively stain area within the total area of the granuloma. Necrotic or mineralized areas were not included in the ROI to be analyzed as previously described (32).

Data Analysis

Chi-square test for trend was used to compare the distribution of granulomas within the head and neck, thoracic, and abdominal lymph nodes as well as the lung sections. The presence of the different markers and cytokines were compared between the granulomas of vaccinated and non-vaccinated calves using the Mann-Whitney's *U*-test by Graph Pad Prism 8.0.2.263. This test was used to compare the medians and for all statistical analysis $P < 0.05$ was considered statistically significant.

RESULTS

Gross Pathology

BTB visible lesions were detected in 68.0% (15/22) of the BCG vaccinated and 89.0% (16/18) of the unvaccinated calves. Seven calves from the BCG vaccinated group and two calves from

the BCG non-vaccinated group were found to be free from bTB visible lesions. Most of the visible lesions were found in the thoracic lymph nodes of both BCG vaccinated and non-vaccinated calves. In addition, the 31 animals with visible lesions were all confirmed by culture to be *M. bovis* infected (29).

Histopathology

The histopathological examination was used to identify different stages of granulomas of both the 15 BCG-vaccinated and the 16 non-vaccinated calves that had TB lesions in lymph nodes and/or lung tissues. In total, 79 lymph nodes and lung tissues were taken forward and investigated by histopathology and IHC, of which 34 were from BCG-vaccinated and 45 were from non-vaccinated calves. The majority of granulomas were found within the thoracic lymph nodes, with more stage IV granulomas present in the control group when compared to the BCG vaccinated ($P < 0.05$). A similar trend was observed for the lung sections, with many granulomas observed (all stages) in the control group while only few stage II and III granulomas were found in animals from the BCG vaccinated group. In the head lymph nodes, only stage I granulomas were found in the BCG vaccinated group while granulomas of all developmental stages were found in the control group (**Supplementary Table 1**). In contrast, more granulomas were observed in the abdominal cavity lymph nodes from BCG vaccinated animals, including several stage IV granulomas, while control animals only showed a few stage I and II granulomas ($P < 0.05$).

Immunohistochemistry

IHC was used to detect CD68+ macrophages, CD4+ and CD8+ T cells, TNF-α, IFN-γ, and iNOS within tissue sections. The responses of these immunological markers were compared between controls and vaccinated calves and further stratified by different granuloma stages. The positive labeling was expressed as a fraction of the total area examined in each slide. The following results of cellular and cytokine responses were quantified:

CD4+ cells. Immunohistochemical staining of CD4 demonstrated an increase in the number of stained cells in the BCG vaccinated calves compared to non-vaccinated calves in different stages of granuloma. The fraction of immunolabeled $CD4^+$ cells among vaccinated calves was significantly higher ($P < 0.05$) in all four granuloma stages when compared with the non-vaccinated group (**Figure 1**).

CD8+ cells. Similarly, immunohistochemical staining of CD8 showed higher numbers of CD8+ cells in BCG vaccinated calves than in non-vaccinated calves and the difference was significant ($P < 0.05$) between the groups in stages II-IV (**Figure 2**).

Macrophages (CD68+). The immunohistochemical staining revealed no significant differences in CD68+ cell distribution in the different granuloma stages of the BCG vaccinated and non-vaccinated calves (**Figure 3**). The vaccinated group indicated a higher fraction of immune-labeled cells than the non-vaccinated group for stage I granulomas, but there was no statistically significant difference between the two groups ($P > 0.05$).

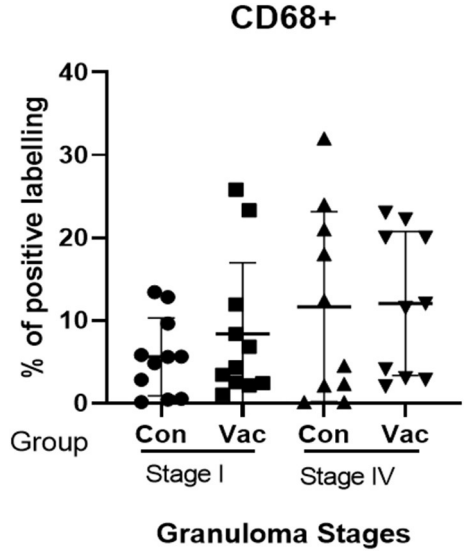

FIGURE 3 | Immunolabelling for $CD68^+$ cells. Comparison of $CD68^+$ cell distribution in granuloma stages I and IV of BCG vaccinated (Vac) and non-vaccinated (Con) calves.

TNF-α^+. The TNF-α^+ immunohistochemical staining showed higher TNF-α^+ response in BCG vaccinated as compared to the level of response in non-vaccinated calves (**Figure 4**), and that was the case in all four stages of granuloma ($P < 0.05$).

IFN-γ. Immunohistochemical staining for IFN-γ indicated an increased expression of IFN-γ in BCG vaccinated compared to non-vaccinated calves in all four granuloma stages, however, only in stage I-III were this increase statistically significant between the groups ($P < 0.05$) (**Figure 5**).

iNOS$^+$. Positive staining for $iNOS^+$ was also present at all stages of granulomas in both BCG vaccinated and non-vaccinated calves, and the analysis further demonstrated a statistically higher expression in BCG vaccinated calves at stage I, stage III, and stage IV (**Figure 6**).

DISCUSSION

In the present study, immunological responses were evaluated at local granuloma level in 15 BCG-vaccinated and 16 non-vaccinated calves that had been naturally infected with *M. bovis* and that showed gross visible lesions at the *post-mortem* examination. On the other hand, nine calves (seven from the vaccinated and two from the non-vaccinated group) had either not become infected or had no visible lesions. In the present study, more stage IV granuloma was observed in the mesenteric lymph node of vaccinated calves than in control calves. This observation can be considered as misleading, as such difference was more likely associated with the sampling procedure and preparation of tissue sections of the two groups of calves. As observed during post mortem examination, gross lesions were

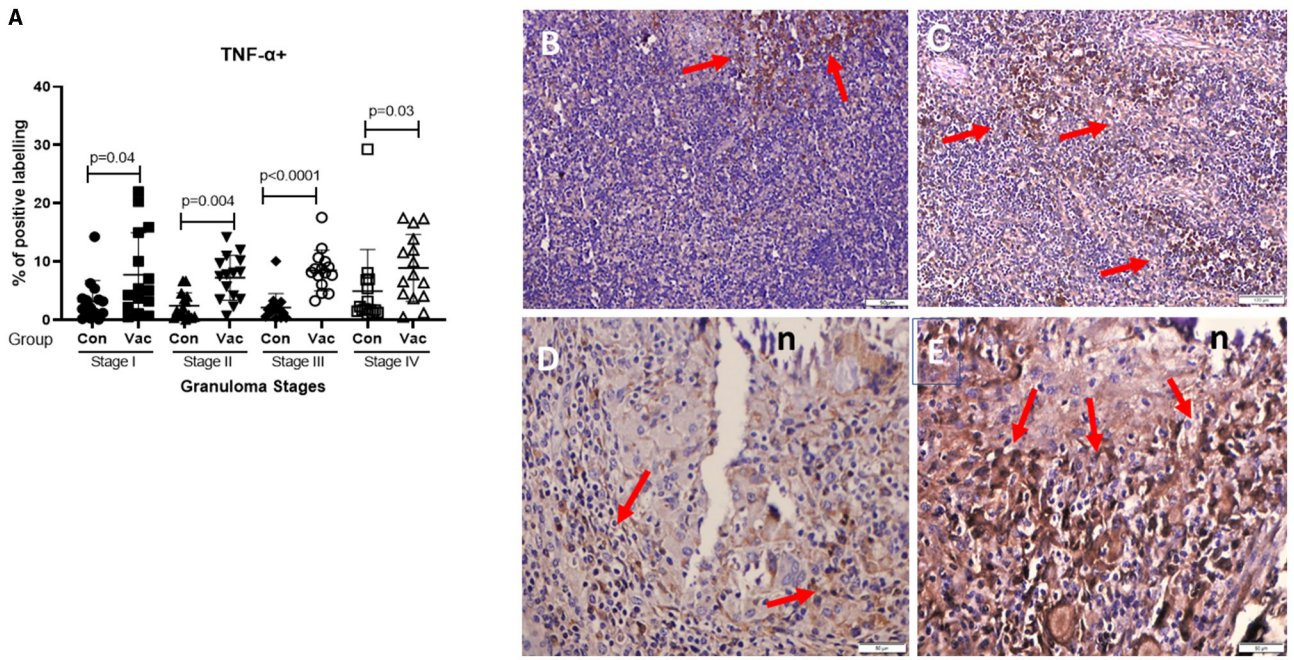

FIGURE 4 | Immunolabelling for TNF-α^+ (ABC IHC stain, DAB brown chromogen). **(A)** Comparison of TNF-α^+ distribution in different granuloma stages of BCG vaccinated (Vac) and non-vaccinated (Con) calves. **(B)** Stage I granuloma from non-vaccinated calf with few number of TNF-α stained macrophages (arrows), **(C)** Stage I from BCG vaccinated calf with more positively stained macrophages (arrows). **(D)** Stage IV granuloma from non-vaccinated calf with moderate number of positive cells (arrows). **(E)** Stage IV granuloma from BCG vaccinated calf with abundance of positively stained cells (arrows). Bar equals 100 μm in **(B,C)**, and 50 μm in **(D,E)**. n, necrotic core.

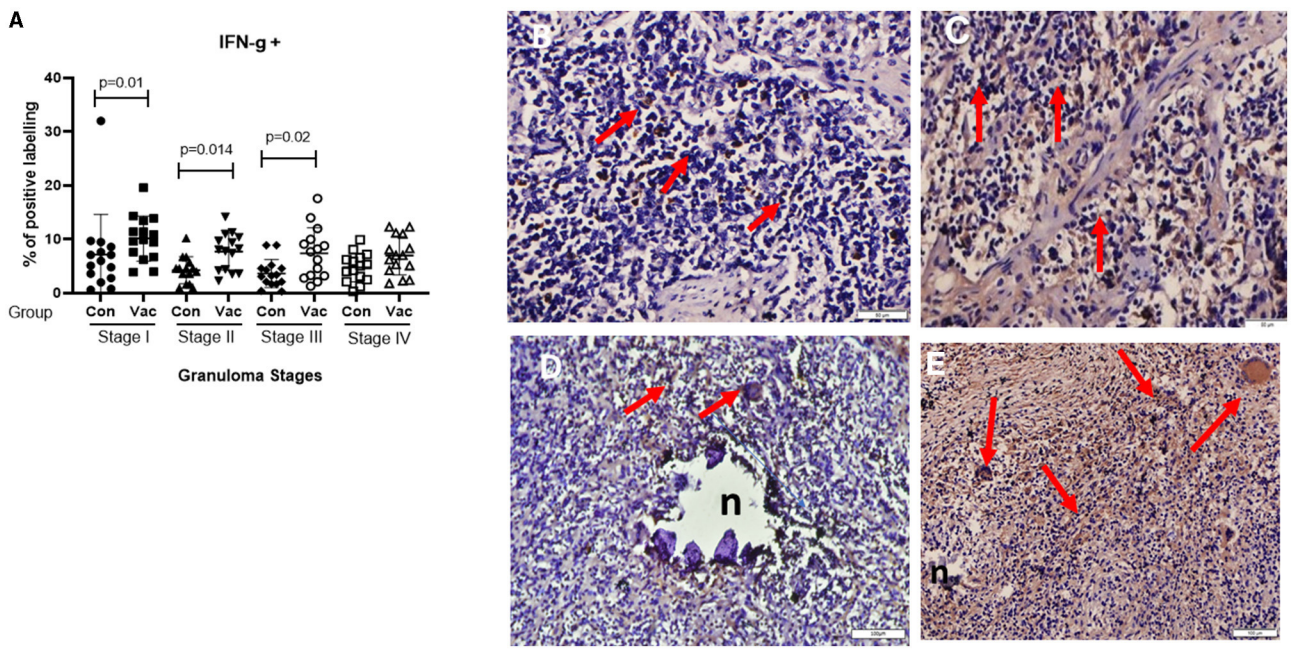

FIGURE 5 | Immunolabelling for IFN-γ^+. **(A)** Comparison of IFN-γ^+ cell distribution in different granuloma stages of BCG vaccinated (Vac) and non-vaccinated (Con) calves. **(B)** Stage I granuloma from non-vaccinated calf with some IFN-γ^+ positively stained cells (arrows). **(C)** Stage I granuloma from BCG vaccinated calf has more lymphocytes stained for IFN-γ^+ (arrows). **(D)** Stage IV granuloma showing IFN-γ^+ stained cells from non-vaccinated calf (arrows). **(E)** Stage IV granuloma showing IFN-γ^+ stained cells from BCG vaccinated calf (arrows). 50 μm for **(B,C)**, Bar equals 100 μm in **(D,E)**.

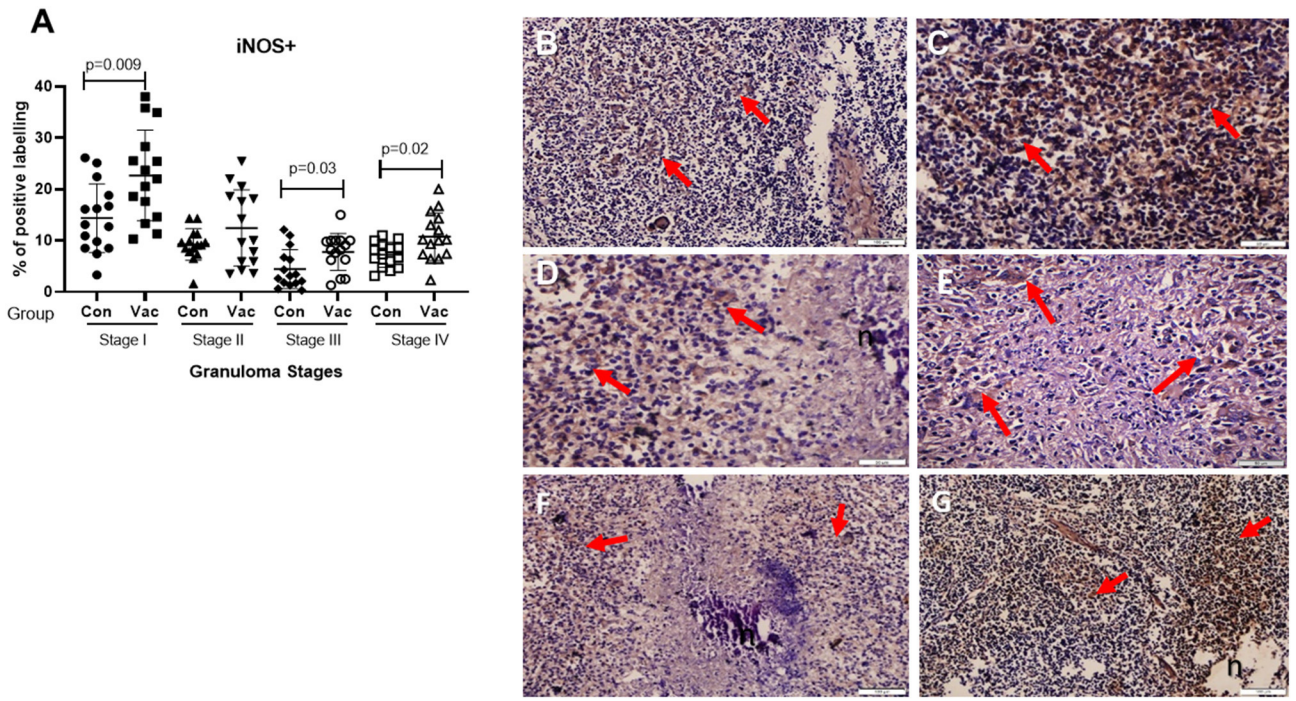

FIGURE 6 | Immunolabelling for iNOS$^+$. **(A)** Comparison of iNOS$^+$ cell distribution in different granuloma stages of BCG vaccinated (Vac) and non-vaccinated (Con) calves. **(B)** Stage I granuloma from non-vaccinated calf with some iNOS$^+$ positively stained cells (arrows). **(C)** Stage I granuloma from BCG vaccinated calf, many cells stained for iNOS$^+$ (arrows). **(D)** Stage III granuloma from non-vaccinated calf showing stained cells with iNOS$^+$ (arrows). **(E)** Stage III granuloma showing cells stained positively with iNOS$^+$ from BCG vaccinated calf (arrows). **(F)** Stage IV granuloma from non-vaccinated calf showing cells stained with iNOS$^+$ (arrows). **(G)** Stage IV granuloma from BCG vaccinated calf showing many cells stained with iNOS$^+$ (arrows). Bar equals in **(B,C)** 50 μm, Bar equals 100 μm **(D–G)**. n, necrotic core.

detected in the mesenteric lymph nodes of one vaccinated and one control calves. In both calves, the pathology score was 3, which is considered as the most severe for the lymph node score. This indicates that the severity of the gross pathology was similar in both the vaccinated and non-vaccinated calves. However, in microscopic pathological comparison, stage IV (the most severe microscopic pathology) was observed in the mesenteric lymph node of the vaccinated calf than in the mesenteric lymph node of the control calf. As the two calves had similar severity of gross pathology, the difference in microscopic pathology between the two calves is mainly attributed to the difference in the sampling of the two lymph nodes and or the difference in the sections of the two lymph nodes used for the evaluation of the microscopic lesions. In the case of the vaccinated calf, the part of the mesenteric lymph node that was sampled and examined was severely affected while in the case of the non-vaccinated calf, the part of the mesenteric lymph node that was sampled was less severely affected and could the peripheral part of the lymph node. But in general, it was observed that both gross and microscopic pathology were relatively less severe in vaccinated calves than in non-vaccinated calves. Substantiating this observation, a recently conducted meta-analysis suggested that BCG vaccination may help in accelerate control of bTB in endemic settings by inducing immunity against *M. bovis* and also limiting the progress TB lesion at the site of infection (33).

In the current study, the immunohistochemical staining of CD68, CD4, CD8, IFN-γ, TNF-α, and iNOS were evaluated and compared in BCG-vaccinated and non-vaccinated calves. The presence of these immunological markers was also compared between the different stages of granulomas using IHC staining on histological sections of granulomas from lymph nodes and lungs. The results showed stronger responses of CD4+ and CD8+ T cells in granulomas of the BCG vaccinated calves when compared to those from the unvaccinated group. This observation is consistent with the results in a study conducted by Hope et al. (34) who also reported higher induction of CD4+ and CD8+ T cell responses in BCG-vaccinated Holstein cattle compared to non-vaccinated (34). Studies have shown that the majority of CD8+ T cells in the local immunity outside the peripheral circulation are γδ T cells (35). In this regard, it is important to look into the expressions of γδ T cells at local lesion sites during natural *M. bovis* infection.

Phagocytic cells such as macrophages and dendritic cells, and T cells including CD4+, CD8+, and γδ T cells play important roles in controlling *M. bovis* infection in cattle (36). The observation of responses of these cells in bTB granulomas in the lymph nodes of BCG-vaccinated calves could suggest the significance of BCG in protecting against bTB (37). In the present study, the expression levels of CD68+ macrophages in the granulomas of vaccinated and non-vaccinated calves were not significantly different. On the contrary, Salguero et al. (38)

reported a significant reduction in the expression of CD68+ macrophages in granulomas of BCG-vaccinated Holstein calves as compared to its expression in non-vaccinated animals, after being experimentally inoculated intratracheally (38). On the other hand, a study conducted by Tulu et al. (39) on *M. bovis* naturally infected cross breed cows showed a significant increase in the immune-labeling of CD68+ macrophages as the level of granuloma increases from stage I to IV in culture positive animals as compared to culture negative animals. However, at the early stages of granuloma formation, the immune-labeling of CD68+ macrophages was significantly higher for *M. bovis* culture negative cows as compared to culture positive animals, which was explained in terms of the role of CD68+ macrophages geared toward the protection and elimination mycobacteria (39).

Furthermore, the result of the present study showed that the expressions of IFN-γ, TNF-α, and iNOS were stronger in granulomas of vaccinated calves than in non-vaccinated calves. The protective role of IFN-γ in the response to *M. bovis* infection in cattle is well-established (40). Previous experimental infection reported that IFN-γ, TNF-α, and iNOS showed increased expressions among the lymph node granulomas of BCG vaccinated cattle compared to non-vaccinated (38). Additionally, TNF-α is known to act in conjunction with IFN-γ to induce the release of reactive oxygen and nitrogen in infected macrophages, which are required for the killing of intracellular bacilli (41). On the other hand, such release can become toxic at high concentrations through an increase in necrosis and tissue damage (42). Previous studies have indicated that the macrophage and iNOS activity are more intense and sustained along the granuloma development (43, 44).

Limitation of the Study

The comparison of the microscopic pathology and immunological markers between the vaccinated and non-vaccinated calves were based on the examinations of one or few sections of tissues. The results of examinations of a single section or few sections does not give realistic comparison. Several sections should have been prepared and examined the comparisons of microscopic pathology and immunological markers between the vaccinated and control calves. The other limitation of this study is the consideration of only a few markers for comparison between the two groups of calves. Additional cell and cytokine, or chemokine markers could have been included in the study. In addition to the above two limitations, the use zinc solution fixed tissues for the histopathological examination and classification of granuloma. Tissue fixation with zinc solution is recommended for the investigation of CD4+ and CD8+ cells markers, but histopathological examination of tissues fixed with zinc solution is laborious and difficult to delineate the different stages of granuloma.

CONCLUSION

BTB continues to be a major animal and public health problem worldwide and new tools are required for the control of this disease, primarily in cattle. In the present study, vaccination of calves with BCG induced strong responses at the granulomas of CD4+ and CD8+ T cells, and the two cytokines TNF-α and IFN-γ as well as for the chemical mediator iNOS. These observations could suggest the potential of BCG vaccination in containing the spread of *M. bovis* and in reducing the severity of gross pathology at the primary sites of infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Review Board (IRB) of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Reference number IRB/ALIPB/013/1017/18).

AUTHOR CONTRIBUTIONS

AS and GA conceived the study. AS and BB conducted the field and laboratory aspects of the study. FS and GA supervised and lead the study. AS, BT, and FS analyzed the result of the study and interpreted the result. AS, BT, and BG drafted the manuscript while FS, SB, and GA edited the manuscript. All authors reviewed the final draft and agreed with its content and conclusions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.698800/full#supplementary-material

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Pathology of Bovine Tuberculosis in Three Breeds of Dairy Cattle and Spoligotyping of the Causative Mycobacteria in Ethiopia

Mulualem Ambaw[1,2], *Benti Deresa Gelalcha*[2], *Berecha Bayissa*[3], *Adane Worku*[4], *Aster Yohannis*[5], *Aboma Zewude*[6] *and Gobena Ameni*[3,7]*

[1] *Ethiopian Institute of Agricultural Research, Kulumsa Agricultural Research Center, Assela, Ethiopia,* [2] *School of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia,* [3] *Vaccine Production and Drug Formulation Directorate, National Veterinary Institute, Bishoftu, Ethiopia,* [4] *Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia,* [5] *Ethiopian Institutes of Agricultural Research, Holeta Agricultural Research Center, Holeta, Ethiopia,* [6] *Malaria and Neglected Tropical Diseases, Ethiopian Public Health Institute, Addis Ababa, Ethiopia,* [7] *Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, United Arab Emirates University, Al Ain, United Arab Emirates*

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***Correspondence:**
Gobena Ameni
gobena.ameni@aau.edu.et; gobena.ameni@uaeu.ac.ae

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Different breeds of cattle were observed to have a variable degree of susceptibility to bovine tuberculosis (bTB). The screening of bTB was conducted on 720 dairy cattle consisting of three breeds using the single intradermal cervical comparative tuberculin (SICCT) test. Besides this, 43 SICCT test-positive cattle were used to compare the severity of the pathology of bTB among the three breeds and to identify the causative mycobacteria using spoligotyping. The overall SICCT test positivity was 17.92% (129/720) by pooling all animals in the three farms. There was a significant difference in SICCT test positivity among the three breeds ($\chi^2 = 71.06$; $p < 0.001$); the highest (25.34%) was recorded in the crossbreed followed by the Boran breed (10.08%), while the least (3.14%) was recorded in the Jersey breed. On other hand, the highest median pathology score (10.0, interquartile range, IQR = 6.0–17.0) was recorded in Boran followed by cross (5.0, IQR = 3.5–7.5), while the least (3.0, IQR = 2.25–3.0) was recorded in Jersey. Thus, the difference in the median pathology scores was significant [Kruskal Wallis $\chi^2_{(2)} = 18.78$, $p < 0.001$] among the three breeds. Furthermore, multivariate analysis using ordinal logistic regression by considering age, sex, breed, reproductive status, and location of the farms also showed a significant [$\chi^2_{(2)} = 11.97$, $p < 0.01$] difference in pathology scores among the three breeds of cattle. Even at a single-herd level at Holeta, the difference in severity of pathology between the Boran and crossbreeds was significant ($U = 33.5$; $p < 0.01$). Culture positivity was 39% in 108 suspicious tissues. Fourteen *Mycobacterium bovis* (*M. bovis*) and two *Mycobacterium tuberculosis* (*M. tuberculosis*) were isolated from the lesions. All the 14 *M. bovis* isolates belonged to SB0912, while the two *M. tuberculosis* belonged to SIT54. In conclusion, although the frequency of the SICCT test positivity was high in the crossbreed, a more severe pathology was observed on the Boran (zebu) breed. In addition *M. tuberculosis* was isolated from TB lesions of dairy cattle, demonstrating the role of *M. tuberculosis* in causing TB in cattle.

Keywords: bovine tuberculosis, cattle breed, molecular typing, pathology, Ethiopia

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle and is characterized by the formation of tubercles in the lungs, lymph nodes, intestine, kidney, and other tissues (1). bTB is primarily caused by *Mycobacterium bovis* (*M. bovis*), although the other members of *Mycobacterium tuberculosis* complex (MTBC) have also been reported to cause bTB (2). *M. bovis* is most frequently isolated from cattle and less frequently from several other animals, including humans (2, 3). On the other hand *M. tuberculosis* is primarily adapted to human beings, though it is occasionally isolated from other mammals (2). Both *M. bovis* and *M. tuberculosis* belong to MTBC, which consist of mycobacterial species exhibiting a 99.9% sequence similarity with conserved 16SrRNA, with the exception of *M. canetti* (4), and are capable of causing a serious disease with a similar pathology (5). The members of MTBC known so far include *M. tuberculosis*, *Mycobacterium canettii*, *Mycobacterium africanum* subtypes I and II, *Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, the attenuated *M. bovis* Bacillus Calmette–Guerin (BCG*)* vaccine strain (6), *Mycobacterium orygis* (7), *Mycobacterium mungi* (8), *Dassie bacillus* (9), and *Chimpanzee bacillus* (10).

bTB is transmitted between animals primarily by inhalation in housed animals and through ingestion in animals grazing on a pasture contaminated with M. bovis. bTB causes a significant economic loss because of reduction in productivity, movement restrictions, screening costs, culling of affected animals, and trade restrictions (1). In addition, the disease is transmitted to humans, causing zoonotic TB in about 15% of the human population in developing countries (11).

Ethiopia is known to be the leading livestock-producing country in Africa and standing 10th in the world with its 59.5 million heads of cattle (12). Regarding breed composition, 98.20% of the total cattle in the country are zebu, while the remaining 1.8% was crossbreed and Holstein Friesian breeds (12). However, the huge livestock potential and the benefit that the country is getting from the livestock sector are not comparable because of the poor genetic potential of the zebu breed and the poor management and high prevalence of cattle diseases (13)—for example, the annual milk yield in the Boran zebu breed was observed to be 673 kg in Boran (zebu) breed as compared to 1,752 kg in the crossbreed of Holstein Friesian–Boran and to 2,678 kg in Holstein Friesian under Ethiopian conditions (14). Regarding the prevalence of diseases, 10 most important priority diseases have been identified based on their incidence and then ranked according to their impacts on (1) the livelihood of households, (2) markets and value chains, and (3) intensification pathways in the production system [(15); **Table 1**]. As indicated in **Table 1**, TB, brucellosis, echinococcosis, surra, and salmonellosis in species other than poultry do not have a control method, while all of the viral diseases and mycoplasma diseases are controlled by vaccination.

Regarding the estimation of the national prevalence of bTB in Ethiopia, a systematic review conducted on 56 articles demonstrated a pooled prevalence estimate of 5.8% (16), and according to this review, the prevalence of bTB was significantly ($p < 0.001$) higher in Holstein Friesian (21.6%) than in zebu (4.1%). In addition, the same review reported a higher prevalence (16.6%) of bTB in cattle kept under intensive and semi-intensive production systems than those kept in an extensive livestock production system (4.6%).

The government of Ethiopia has given attention to improve cattle production and productivity, including improving milk production, by raising the genetic potential of the zebu breed through crossbreeding with exotic dairy breeds using artificial insemination and synchronization in the dairy sheds and peri-urban areas of the country (15). The Boran breed (zebu) is the preferred zebu breed and is being used for crossbreeding with exotic breeds (mainly Holstein Friesian) by artificial insemination because of its better fitness in terms of size and productivity. The crossbreeding plan for the improvement of the productivity of dairy cattle should be integrated with the disease control strategy. bTB is endemic to Ethiopia and is one of the priority diseases of dairy cattle in the country that requires an appropriate control strategy. To this effect, generation of data on the comparative susceptibility of the different breeds of cattle to bTB is useful for identifying the relatively resistant and moderately productive breed. A few comparative studies conducted on the prevalence of bTB in Holstein Friesian, crossbreed of Holstein Friesian and zebu breeds, and zebu breed indicated that Holstein Friesian is the most susceptible breed as compared to either crossbreed or zebu breed, while the degree of susceptibility of a crossbreed was observed to be lower than of Holstein Friesian but higher than of the zebu breed (17–19). Although the reason behind the relative resistance of zebu to bTB has not been well-established, the innate immune responses, such as that of interleukin-6, could contribute a significant role in containing and clearing an infection with *M. bovis* (19). These studies were conducted in traditional farms in the three breeds that were kept on a grazing pasture. Similar studies are needed in dairy cattle that are kept in intensive and semi-intensive farms under modern dairy farming as the prevalence of bTB is influenced by cattle husbandry (18). Therefore, this study was conducted on three herds for screening 720 dairy cattle for bTB using the single intradermal cervical comparative tuberculin (SICCT) test, after which 43 SICCT test-positive dairy cattle were further recruited for slaughter and used for comparing the severity of pathology of bTB in the three breeds and identification of its causative mycobacteria using spoligotyping.

MATERIALS AND METHODS

Study Setting and Sampling of Study Animals

The study was conducted at three state dairy farms (Adaberga, Bishoftu, and Holeta) that are located in central Ethiopia at about 40 km in the west (Adaberga and Holeta) and east (Bishoftu) of Addis Ababa, the capital of Ethiopia. These farms are owned by the Ethiopian Institute of Agricultural Research and used for the genetic improvement of Ethiopian cattle breed. The dairy cattle management system is semi-intensive, where the animals are kept in the barns, feed on concentrate, and are watered and

TABLE 1 | The 10 top ranked livestock diseases in Ethiopia in descending order based on the degree of impact on the livelihood of households, market/value chains, and intensification of animal production (15) and their control methods.

Rank	Disease ranking based on the impact on the livelihood of households	Disease ranking based on the impact on market and value chain	Disease ranking based on the impact on the intensification of livestock production	Availability and type of control method
1st	Foot and mouth disease, FMD	FMD	Brucellosis	No control method
2nd	Contagious bovine pleuropneumonia, CBPP	LSD	PPR and FMD	Vaccination for both
3rd	Lumpy skin disease, LSD	Brucellosis	TB and Newcastle	TB: no control method Newcastle: vaccination
4th	Tuberculosis, TB	CCPP and Newcastle	CBPP	Vaccination
5th	Brucellosis	TB	LSD	Vaccination
6th	Contagious caprine pleuropneumonia, CCPP	Chicken pox	Gumboro	Vaccination
7th	Peste des Petits Ruminants, PPR	SGP	Salmonella	Vaccination only poultry
8th	Sheep and goat pox, SGP	Gumboro	SGP	Vaccination
9th	Newcastle disease	PPR	Chicken pox	Vaccination
10th	Surra	Echinococcosis	Surra	No control method

also allowed for open grazing in the field. This husbandry system was similar across the three farms, and management is done by the same management body centrally. Prior to the study on the comparative pathology in the three breeds, the three farms were screened for bTB using SICCT test. The SICCT test was conducted using bovine and avian purified protein derivatives (PPD-B and PPD-A) obtained from Thermo-Fisher (Prionics, Lelystad, the Netherlands). Briefly, 0.1 ml PPD-A (2,500 IU/ml) was administered intradermally on the left side of the middle of the neck on the cranial side, and the same volume of PPD-B (3,000 IU/ml) was injected at a site 12 cm distant from the PPD-A site in the shoulder direction. The skin thicknesses were measured just before injection and at 72 h post-injection by the same operator using the same digital caliper, and the results were presented as a change in skin thickness (mm) between the two readings. The interpretation of the result was according to the recommendation of the World Organization for Animal Health (20). The differences in the increase of skin thickness at the bovine and avian PPD injection sites were determined. An animal was considered to be positive when the increase in skin thickness at the bovine PPD site was >4 mm more than the increase in skin thickness at the site of the avian injection. If the differential increase between the two sites was equal to 1 mm or between 1 and 4 mm, the animal was considered negative or inconclusive, respectively.

The total number of animals in the three herds was 720. The farm at Adaberga consisted of 244 animals, which were comprised of crosses and Jersey breeds. The herd size of the farm at Bishoftu was 114, all of which were crossbreed, while there were 362 animals in the farm at Holeta and they were comprised of Boran zebu and crossbreeds. After screening the 720 animals with the SICCT test, 43 positive cattle were selected for slaughter from among the 129 SICCT test-positive cattle identified from the three farms. Thus, 12 Boran (zebu), 26 Holstein Friesian × Boran cross, and five Jersey breeds were used for studying the comparative pathology.

The 43 strong positive animals were transported by trucks under strict safety to the Addis Ababa City Abattoir and humanly killed using knife stunning. The butchers and the meat inspectors used a personal protective equipment while slaughtering and during the postmortem examination. Tissues with lesions were collected into universal bottles in 0.9% saline and transported to the TB Laboratory at the Aklilu Lemma Institute of Pathobiology, Addis Ababa University for culturing. The remaining tissues and carcasses with TB were incinerated and disposed. The tissues were processed for isolation of mycobacteria in Biosafety level III laboratory. The leftover infected samples were disposed after autoclaving at 121°C for 15 min.

Postmortem Examination and Pathology Scoring

The reactors were euthanized humanely and examined for gross lesion of bTB in detail by removing the lungs and lymph nodes (LNs). All the seven lobes of the lungs were inspected at the surface and palpated for the presence of TB lesions. Each lobe was then sectioned into slices to facilitate the detection of TB visible lesions (VLs). Similarly, LNs, including the left and right parotid, left and right mandibular, left and right lateral retropharyngeal, left and right medial retropharyngeal, cranial and caudal mediastinal, left and right bronchial, hepatic, and mesenteric LNs, as well as left and right tonsils, were sliced into thin sections and inspected for the presence of VLs. The severity of gross lesions was scored by a semi-quantitative scoring procedure as previously described by Vordermeier et al. (21). Briefly, the 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; and 4, gross coalescing lesions. The scores of the individual lobes were added up to calculate the lung score. Similarly, the severity of gross lesions in individual LN 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; and 3, extensive necrosis. Individual LN scores were added up to calculate the total LN score for each LN/tissue category. Finally, the LN and lung pathology scores were added together to give the total pathology score per animal.

Specimen Collection and Isolation of Mycobacteria

The standard operating procedure described by the World Organization for Animal Health (20) was used for the culturing of tissue lesions. Suspicious TB lesions were collected from 41 dairy cattle for mycobacterial isolation. A total of 108 suspected tuberculous lesions were aseptically collected into sterile universal bottles in 0.9% saline solution and transported to the laboratory in a cold chain. The specimens were sectioned into pieces with sterile blades, minced with scissors, and homogenized with sterile pestle and mortar. The homogenates were decontaminated by adding an equal volume of 4% NaOH and kept for 15 min and then neutralized with 1% (0.1 N) HCl (phenol red was used as an indicator). Then, the specimen was centrifuged at 3,000 rpg for 15 min, and the supernatant was discarded; finally, two drops of suspension from each sample were spread onto a slant of Löwenstein–Jensen (LJ) medium. Duplicates of LJ were used; one was enriched with sodium pyruvate, while the other was enriched with glycerol. The cultures were incubated aerobically at 37°C for about 8 weeks with weekly observation for the growth of colonies.

Spoligotyping

Spoligotyping was conducted following the protocol developed by Kamerbeek et al. (22), which involves amplification, hybridization, and detection steps. The colonies were heated at 85°C in a water bath for 1 h, and the released DNA was used as a template to amplify the direct repeat (DR) region of the *M. tuberculosis* complex by polymerase chain reaction (PCR) using oligonucleotide biotin-labeled primers derived from the DR sequence, RDa (5′GGTTTTGGGTTTGAACGAC3′), and RDb (5′CCGAGAGGGGACG GAAAC3′) (22).

A total volume of 25-μl reaction mixtures that consisted of 12.5 μl of HotStarTaq Master Mix (Qiagen), 2 μl of each primer (20 pmol each), 5-μl suspension of heat-killed cells, and 3.5 μl distilled water was used for running the PCR. The mixture was heated for 15 min at 96°C and then subjected to 30 cycles of 1-min denaturation at 96°C, annealing at 55°C for 1 min, and extension at 72°C for 30 s. Then, final stabilization was done at 72°C for 10 min. Thereafter, the PCR product was denatured using a thermocyler at 96°C for 10 min and then kept on ice so as to prevent the renaturing of the PCR products. The denatured PCR product was loaded onto a membrane covalently bonded with a set of 43 spacer oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus of the *M. tuberculosis* complex, and then hybridized at 60°C for 1 h. After hybridization, the membrane was washed twice for 10 min in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7)]−0.5% sodium dodecyl sulfate (SDS) at 60°C and then incubated in 1:4,000 diluted streptavidin peroxidase (Boehringer) for 1 h at 42°C. The membrane was washed twice for 10 min in 2× SSPE−0.5% SDS at 42°C and rinsed with 2× SSPE for 5 min at room temperature. The hybridizing DNA was detected by the enhanced chemiluminescence (ECL) method (Amersham, Biosciences, Amersham, UK) and exposure to an X-ray film (Hyperfilm ECL, Amersham). A mixture of 10 ml of ECL reagent 1 and 10 ml of ECL reagent 2 was prepared and then added onto the membrane, and the membrane was rinsed in the solution for 5 min at room temperature. Then, the membrane was attached onto a film in the dark room, placed in the cassette, and then incubated for 15 min at room temperature. The film was removed and placed in a developer solution for 2 min and then removed from the developer, rinsed with tap water for 15 s, and then placed in a fixer solution for 1 min. Finally, the film was dried and used for the interpretation of the result. The presence of the spacer was identified as a black square, while the absence of the spacer was identified as a white square on the film. The black squares were converted to 1, while the white squares were converted to 0 and then transferred to the Mycobacterium bovis Spoligotype Database for the identification *M. bovis* strains. Furthermore, the identification of strains of *M. tuberculosis* was done 1 and 0 into the spoligotype international type (SIT)–VNTR international type database.

Data Management and Analysis

Statistical Package for Social Sciences (SPSS) version 21 software was used for the analysis of the SICCT test data and part of the pathology data. Descriptive statistics was used for the analysis of the SICCT test data of the three herds. Univariate analysis was used to assess the associations of the risk factors with the SICCT test result, and chi-square (χ^2) test was used for the estimation of the significance of the association. In addition, ordinal regression analysis was used for the determination of the association of explanatory factors (breed, age, sex, reproductive status, and farm location) with the severity of pathology. The fitness of this model was diagnosed using−2 log likelihood model, and the result of the diagnosis demonstrated the fitness of the final model with independent variables [$\chi^2_{(11)} = 46.56$, $p < 0.001$]. Spearman correlation was to estimate the correlation between skin reaction to PPD (B-A) and the pathology score of the 43 reactors. Graph Pad Prism version 8 was used for the comparison of skin reactivity to the injection of purified protein derivatives, PPD (B-A), in the three breeds of cattle. Similarly, GraphPad Prism version 8 was used for the comparison of the severity of pathology in the three breeds. The comparison of the pathology among the three breeds was done by non-parametrical Kruskal–Wallis test for comparing the median pathology scores of the three breeds by assuming a non-pairing experimental design, while, on the other hand, non-parametric Mann–Whitney *U*-test was used for the comparison of the severity of pathology between the two breeds using the median scores.

RESULTS

Reactivity of the Three Herds to the SICCT Test

The SICCT test results of the three herds used for the recruitment of SICCT test-positive animals used for the pathology study are presented in **Table 2**. The overall SICCT test positivity was 17.92% (129/720) when all the animals were pooled together. When individual farms are considered, the SICCT test positivity were 27.2% (31/114), 25.41% (92/362), and 2.26% (6/244) in the farms located at Bishoftu, Holeta, and Adaberga, respectively. The difference in SICCT test positivity among the three farms was significant ($\chi^2 = 85.58$; $p < 0.001$; **Table 2**). Similarly, there was a significant difference in SICCT test positivity among the three breeds ($\chi^2 = 71.06$; $p < 0.001$); the highest SICCT test positivity (25.34%) was recorded in the crossbreed, followed by the Boran breed (10.08%), while the least (3.14%) was recorded in the Jersey breed. Forty-three reactors, including 12 Boran (zebu), 26 Holstein Friesian × Boran crosses, and five Jersey breeds, were slaughtered and used for studying the comparative pathology among the three breeds.

Skin Reactivity to the SICCT Test and Pathology Score in the Three Breeds

Typical TB lesions were observed in different lymph nodes (**Figure 1**) in 95% (41/43) of SICCT-positive cattle, but the correlation between the skin reaction to the SICCT test and the severity of pathology was not significant ($r = 0.23$; 95% CI, −0.13, 0.47, **Figure 2**). Change in skin thickness was compared among Boran, cross, and Jersey breeds using the Kruskal–Wallis test. The median skin thicknesses were 7.5 (interquartile range, IQR = 6.0–9.75), 10.0 (IQR = 8.0–14.75), and 8.0 (IQR = 5.0–12.5) in Boran, cross, and Jersey breeds, respectively. There was no significant [$\chi^2_{(2)} = 4.8$, $p > 0.05$] difference in skin reactivity observed among the three breeds (**Figure 3**).

Comparison of the Pathology of bTB in the Three Breeds of Cattle

The evaluation of the severity of pathology indicated that the median pathology scores were 10.0 (IQR = 6.0–17.0), 5.0 (IQR = 3.5–7.5), and 3.0 (IQR = 2.25–3.0) in Boran, cross, and Jersey breeds, respectively. There was a significant [$\chi^2_{(2)} = 18.78$, $p < 0.001$] difference in the pathology score among the three breeds using Kruskal–Wallis statistical test with Dunn's *post-hoc* multiple-comparison test (**Figure 4**). The difference was observed between the Boran and the crossbreeds ($U = 13.91$; $p < 0.01$) as well as between the Boran and the Jersey breeds ($U = 26.94$; $p < 0.001$). In addition, in order to control the cofounding factors, a multivariate analysis was performed using ordinal logistic regression by considering age, sex, breed, reproductive status, and the location of the farms in the analysis.

TABLE 2 | Association of different risk factors with the occurrence of bovine tuberculosis in three herds used for the selection of reactors for slaughtering for the investigation of pathology.

Factors		No. of tested animals	Single intradermal cervical comparative tuberculin test result			Prevalence	χ^2 test	*P*-value
			Negative	Doubtful	Positive			
Sex	Female	688	526	41	121	17.59	1.46	$p > 0.05$
	Male	32	22	2	8	25.00		
Age	<4 years	430	338	25	67	15.58	25.80	$p < 0.001$
	4–8 years	205	160	16	29	14.15		
	>8 years	85	50	2	33	38.82		
Breed	Boran	119	107	0	12	10.08	71.06	$p < 0.001$
	Cross	442	295	35	112	25.34		
	Jersey	159	146	8	5	3.14		
Body condition score	Poor	62	51	1	0	-	5.36	$p > 0.05$
	Medium	346	264	26	56	16.18		
	Good	312	233	16	63	20.19		
Reproductive status	Calf	22	22	0	0	-	[a]	
	Heifer	242	202	7	33	13.64		
	Pregnant	120	88	9	23	19.17		
	Lactating	250	185	20	45	18.00		
	Dry	65	39	5	21	32.31		
	Bull	21	12	2	7	33.33		
Location of the farm	Adaberga	244	230	8	6	2.46	85.58	$p < 0.001$
	Bishoftu	114	74	9	31	27.19		
	Holeta	362	244	26	92	25.41		
Total	720	548	43	129	17.92			

[a] χ^2 *could not be computed because the data did not support the analysis.*

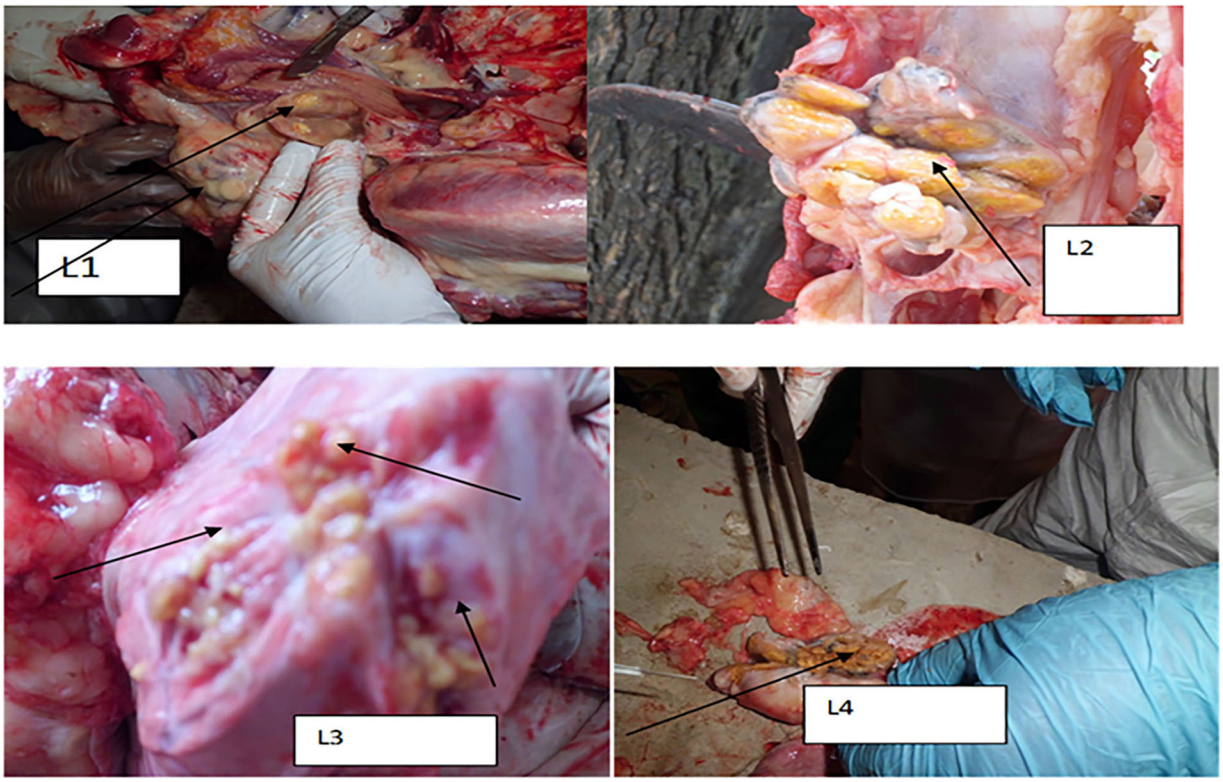

FIGURE 1 | Typical TB lesions obtained in the lymph nodes and lung lobs of selected dairy cattle in central Ethiopia. L1: TB lesion on retropharyngeal lymph node; L2: disseminated TB lesions on bronchial lymph node indicated by the arrow; L3: TB lesions on the diaphragmatic lobe of the lung; and L4: calcified lesion from the mesenteric lymph node.

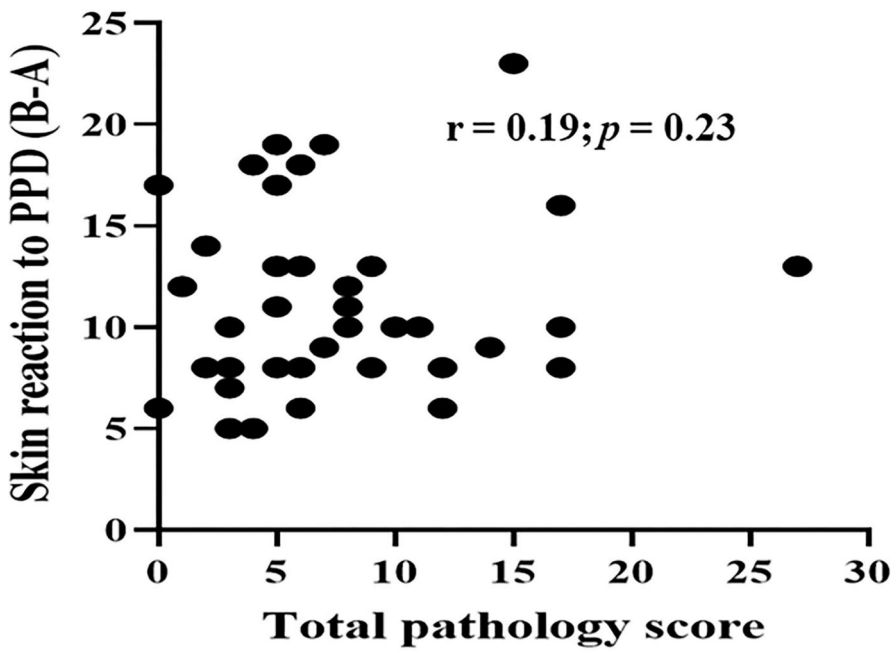

FIGURE 2 | Correlation between skin reactivity and pathology score of the 43 study animals. Spearman correlation was to estimate the correlation between skin reaction to PPD (B-A) and pathology score of the 43 reactors. The correlation between two was not significant ($r = 0.19$; $p = 0.23$).

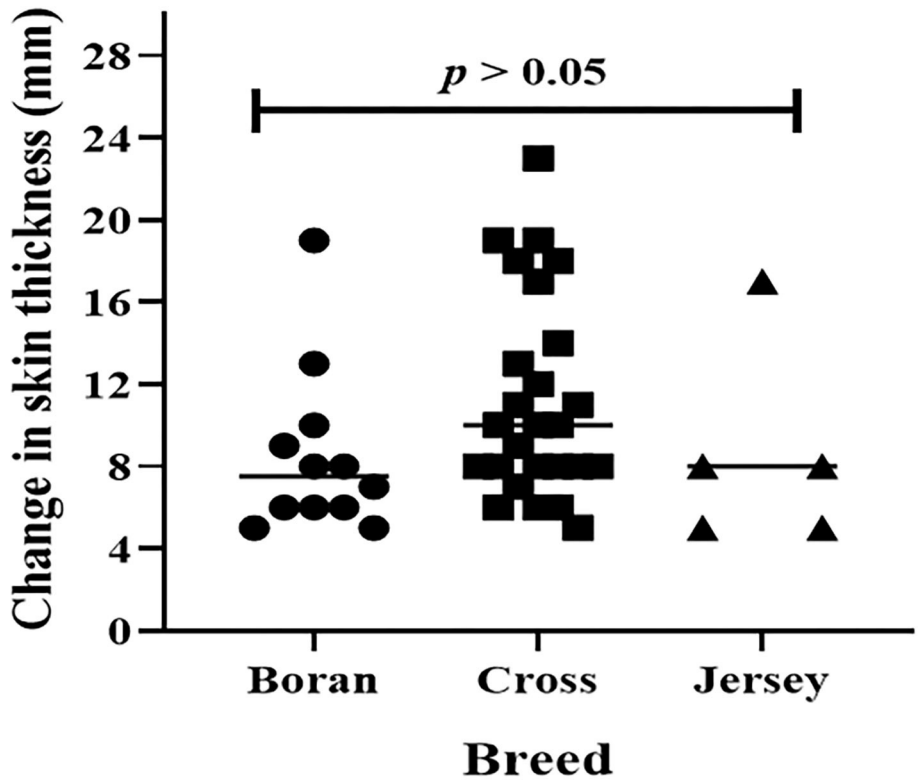

FIGURE 3 | Skin reaction to purified protein derivatives, PPD (B-A) in 43 dairy animals. The horizontal lines represent a median of skin reactions in each breed. Change in skin thickness was compared among Boran, cross, and Jersey breeds using Kruskal-Wallis statistical test. There was no significant [$\chi^2_{(2)} = 4.8$, $p > 0.05$] difference among the breeds.

The result of this analysis still showed a significant [$\chi^2_{(2)} = 11.97$, $p < 0.01$] difference in the pathology scores among the three breeds of cattle (**Table 3**). Thus, the difference in severity of pathology between the Boran breed and either cross ($p < 0.05$) or jersey ($p < 0.01$) breed was significant. However, no significant difference was observed in the severity of pathology between the cross and Jersey breeds ($p > 0.05$) (**Figure 4**). The comparison of severity of pathology was also performed in the herd located at Holeta, where two positive breeds were found, and the difference in the pathology score between the Boran and crossbreeds was significant ($U = 33.5$; $p < 0.01$) (**Figure 5**). However, a comparison of pathology between the breeds in the farms located at Adaberga and Bishoftu could not be performed as two or more positive breeds were not found in these farms.

Bacteriological Isolation and Identification of the Isolates by Spoligotyping

Culture positivity in the study animals was 39% (16/41) on the LJ medium. The highest (56.3%) and the lowest (2.4%) isolation rates were observed from lung lobes and mesenteric lymph nodes, respectively. From the 16 total isolates, 14 were confirmed to be *M. bovis*, and the remaining two were *M. tuberculosis* (**Table 4**). The 16 *M. bovis* isolates had a spoligotype pattern of B0912, while the two *M. tuberculosis* isolates had a spoligotype pattern of SIT54 (**Figure 6**).

DISCUSSION

Screening of bTB was conducted in three farms that are located at Adaberga, Bishoftu, and Holeta in central Ethiopia. These farms belong to the Ethiopian Institute of Agricultural Research, and testing for bTB was conducted using the SICCT test for the control of the disease in these farms. A further study was conducted on 43 SICCT test-positive dairy cattle for comparing the severity of pathology of bTB in Boran zebu, crossbreed of Holstein Friesian and zebu, and Jersey breeds. In addition, mycobacteria were isolated, and strain levels were identified using mycobacterial culturing and spoligotyping.

The SICCT test positivity was compared among the three breeds, and the result indicated the highest percentage in the crossbreed, followed by the Boran breed, while the least percentage was recorded in the Jersey breed. This observation is similar with the reports of earlier studies in central Ethiopia, which reported a higher prevalence of bTB in the Holstein Friesian and the crossbreeds than in zebu breed (18, 23–26). However, those studies were based on the SICCT test and did not involve the comparison of the severity of pathology in these

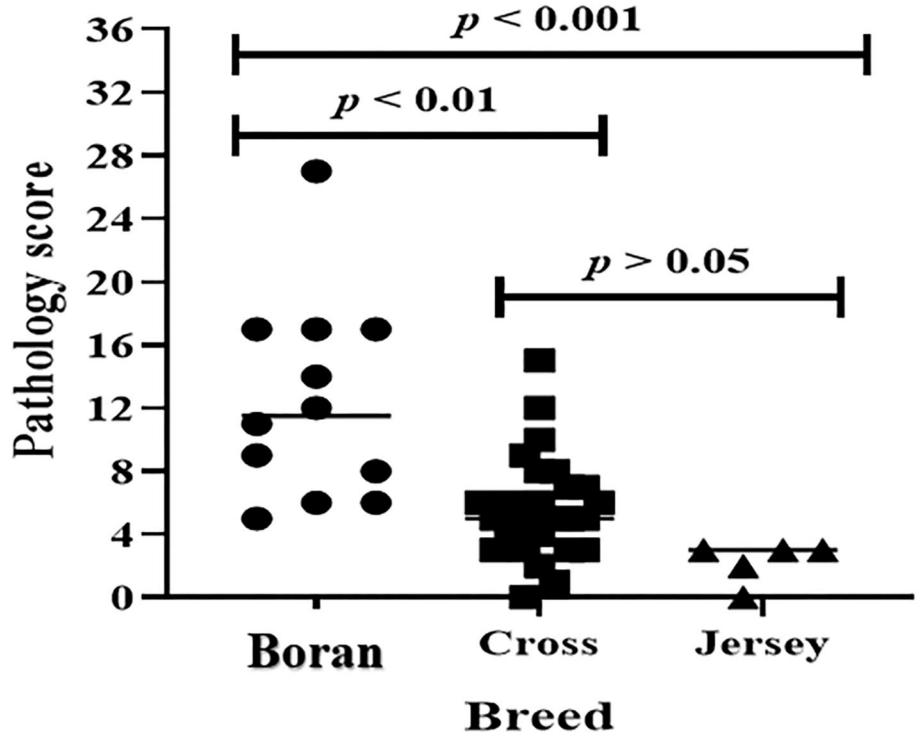

FIGURE 4 | Pathology score in 43 study animals. The horizontal lines show median pathology scores of each breed. The pathology score was compared in three breeds using Kruskal-Wallis statistical test with Dunn's *post-hoc* multiple comparison test. There was significant [$\chi^{2}_{(2)} = 18.78$, $p < 0.001$] difference in pathology score among the three breeds. The difference was observed between the Boran and the cross breeds ($U = 13.91$; $p < 0.01$) as well as between the Boran and the Jersey breeds ($U = 26.94$; $p < 0.001$).

TABLE 3 | Association of different risk factors with the severity of pathology in 41 (two animals with 0 pathology score were not considered in the analysis) cattle using multivariable ordinal logistic regression.

Risk factors	Category	No. of animals	Estimate (95% confidence interval)	Standard error	Wald χ^2 test	df	*P*-value
Breed	Borana	12	4.450 (1.621, 7.279)	1.444	9.503	1	0.002
	Cross	25	2.212 (0.077, 4.346)	1.089	4.124	1	0.042
	Jersey	4	-	-		-	-
Age	≤4 years	22	−0.090 (−1.641, 1.456)	0.790	0.140	1	0.907
	4–8 years	7	0.935 (−1.033, 2.903)	1.004	0.867	1	0.352
	≥8 years	12	-	-		-	-
Sex	Male	2	−1.333 (−4.833, 2.167)	1.786	0.557	1	0.455
	Female	39	-	-	-	-	-
Body score	Poor	6	−0.272 (−2.216, 1.672)	0.992	0.075	1	0.784
	Moderate	17	−0.658 (−2.075, 0.759)	0.723	0.827	1	0.363
	Good	18	-	-	-	-	-
Reproductive status	Heifer	10	−0.810 (−3.250, 1.631)	1.245	0.423	1	0.516
	Pregnant	8	−2.672 (−4.759, −0.584)	1.065	6.293	1	0.012
	Lactating	11	−0.677 (−3.063, 1.709)	1.217	0.309	1	0.578
	Dry	10	-	-	-	-	-
	Bull	2	-	-	-	-	-

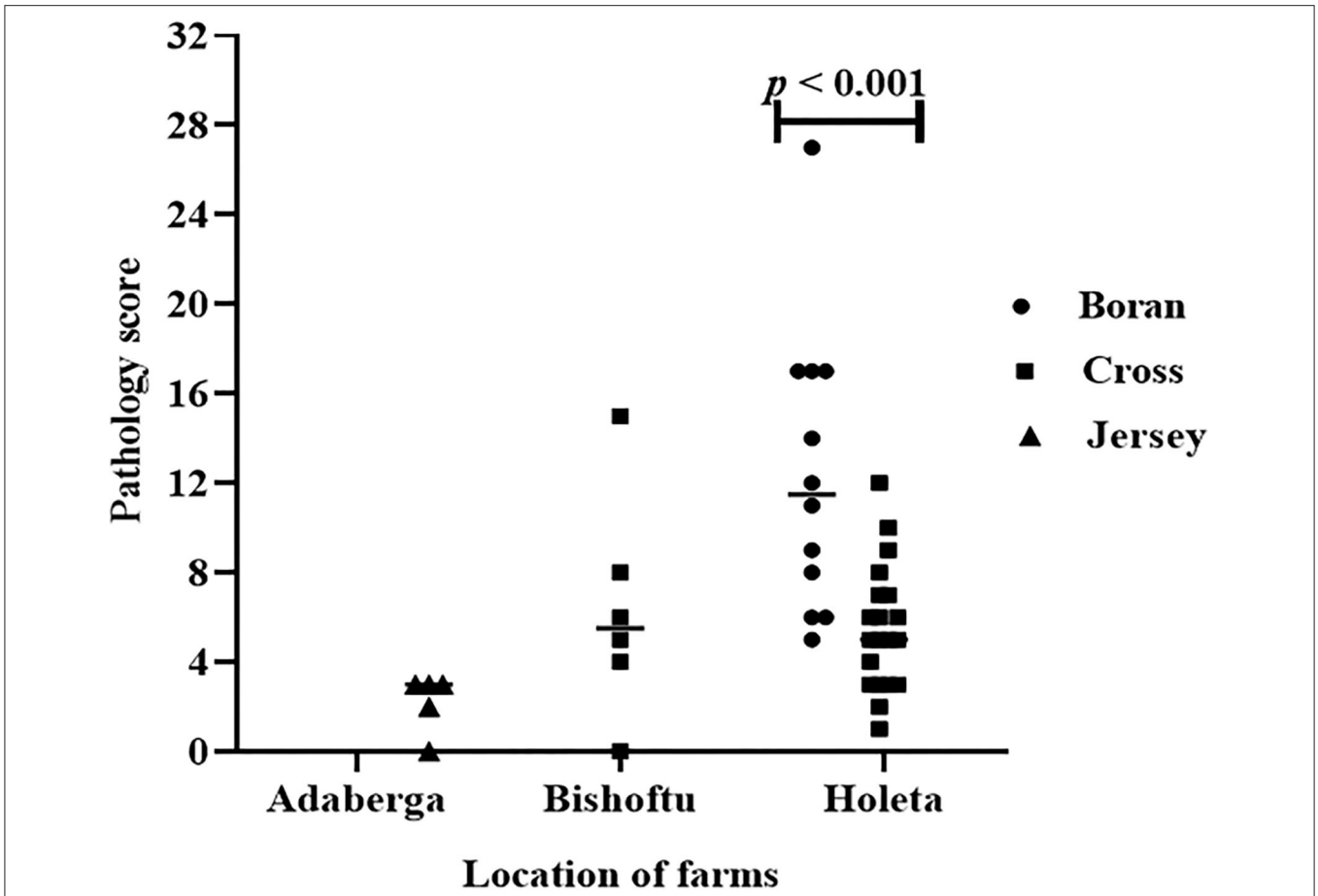

FIGURE 5 | Pathology score in the three breeds in three study farms. The difference in pathology score between the Boran and cross breeds was significant ($U = 33.5$; $p < 0.01$) at farm located at Holeta. However, comparison of pathology between the breeds was not possible at the farms located at the Adaberga and Bishoftu as the positive animals in these farms belong to a single breed; Jersey at Adaberga and cross at Bishoftu.

TABLE 4 | Distribution of tuberculosis lesion in the lymph nodes of single intradermal cervical comparative tuberculin test-positive animals and spoligotypes of *M. bovis* and *M. tuberculosis* isolated from the lesion.

ID	Breed	Age	Sex	Farm location	Lesion type	Infection site	Species	Strain
H211820	Cross	1 year	F	Holeta	Calcified	Right bronchial	*M. tuberculosis*	SIT54
H 20126	Cross	12 years	F	Holeta	Calcified	Left bronchial	*M. bovis*	SB0912
H 20146	Cross	12 years	F	Holeta	Spot like	Retropharyngeal	*M. bovis*	SB0912
H 25142	Cross	7 years	F	Holeta	Spot like	Mesenteric	*M. tuberculosis*	SIT54
H 21208	Boran	11 years	F	Holeta	Milliary	Caudal mediastinal	*M. bovis*	SB0912
H 21209	Boran	12 years	F	Holeta	Miliary	Retropharyngeal	*M. bovis*	SB0912
H 21206	Boran	9 years	F	Holeta	Milliary	Left bronchial	*M. bovis*	SB0912
H 29221	Boran	3 years	F	Holeta	Calcified	Right diaphragmatic lymph node	*M. bovis*	SB0912
H 21228	Boran	11 years	F	Holeta	Calcified	Right prescapular	*M. bovis*	SB0912
H 21244	Boran	11 years	F	Holeta	Calcified	Retropharyngeal	*M. bovis*	SB0912
H 26006	Boran	6 years	F	Holeta	Calcified	Retropharyngeal	*M. bovis*	SB0912
H 26006	Boran	6 years	F	Holeta	Calcified	Left bronchial	*M. bovis*	SB0912
H 25171	Cross	7 years	M	Holeta	Calcified	Mediastinal caudal	*M. bovis*	SB0912
H211146	Cross	1 year	F	Holeta	Calcified	Right bronchial	*M. bovis*	SB0912
D 102.2	Cross	4 years	F	Debrezeit	Calcified	Mammary lymph node	*M. bovis*	SB0912
D 92.5	Cross	5 years	F	Debrezeit	Calcified	Right accessory lung	*M. bovis*	SB0912

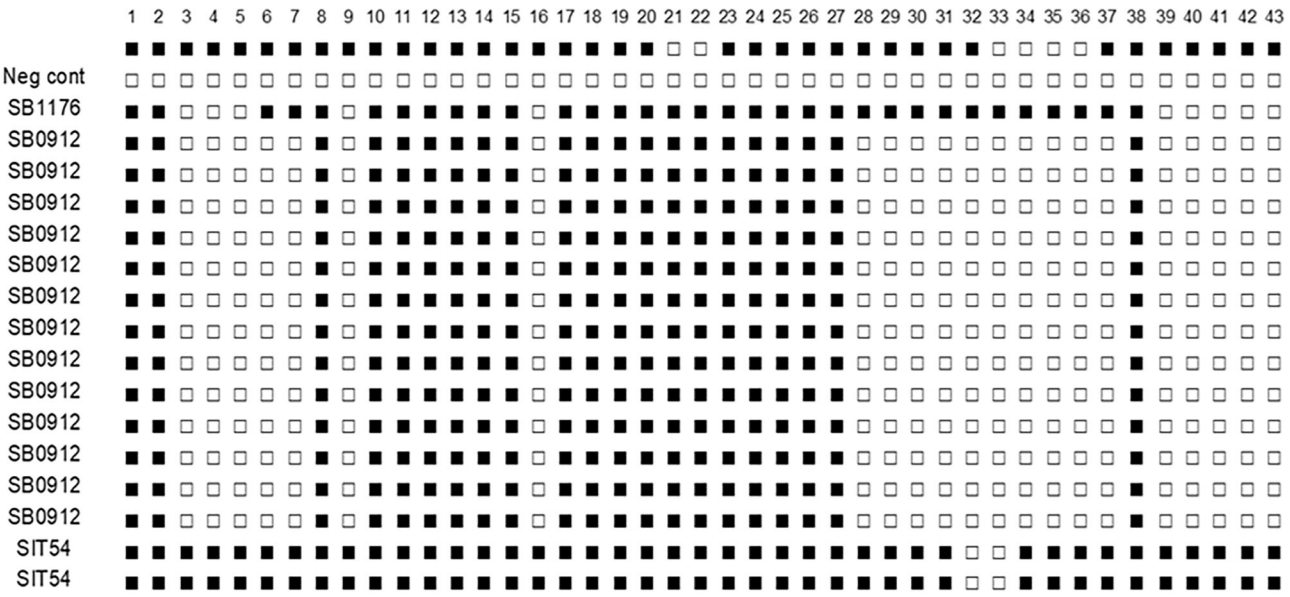

FIGURE 6 | Spoligotype patterns of isolates of *M. tuberculosis* and *M. bovis* from cattle TB lesions in central Ethiopia. All of *M. bovis* isolates had the spoligotype pattern of SB0912 while the two *M. tuberculosis* had the spoligotype pattern of SIT54. SB1176 is positive control and H_2O a negative control. The white rectangles indicate negative signals and the black rectangles represent a positive signal.

breeds since they were conducted in live animals. In the present study, the comparison was not only made in live animals on the basis of SICCT test but also conducted in slaughtered animals using detailed postmortem examination. The result of the SICCT test of this study agrees with the results of previous studies as indicated above.

However, the result of the detailed postmortem examination indicated a rather more severe pathology in the Boran breed than in either crossbreed or Jersey breed under a similar intensive management of cattle husbandry. This observation was disagreeing with a few studies which reported a more severe pathology in *Bos taurus* breeds such as Holstein Friesian or their crosses with zebu than in *Bos indicus* (17–19, 27). Additionally, historical comparative studies on the susceptibility of *B. taurus* and *B. indicus* in Asia and Africa indicated that *B. indicus* was resistant to bTB and also developed good protection against bTB by BCG vaccination (28–30). Carmichael (29) reported that the incidence and severity of pathological lesions of bTB were lower in zebu cattle than in Taurine breeds. However, in some of the previous studies, it was not well-elucidated if these comparative studies were conducted under similar environmental and husbandry conditions since husbandry plays a key role in the epidemiology of bTB. An intensive cattle husbandry is considered as the most important risk factor for facilitating the transmission of *M. bovis* infection [reviewed by Sihbat et al. (16) because of the close physical contact between animals, which can easily facilitate the transmission of the infection. Therefore, the comparison of the susceptibility of the two breeds to bTB should ideally be made under similar husbandry conditions so that the confounding factors can be avoided. In the present study, the three breeds were kept under a similar husbandry system, which is semi-intensive farming. In addition, the possible confounding factors such as sex, age, body condition, reproductive status, and location of the farms were considered in the analysis for controlling the effects of confounding factors. Although it is difficult to explain the reason why the pathology is more severe in zebu, keeping this breed indoors could cause stress to this breed and thereby increases the severity of TB lesions (31–33) since the zebu breed is usually kept on a pasture.

The other cause of the difference in severity of the disease could be the difference in the dates of infection, and in this case, the zebu breed had been infected earlier than the date of infection of the other two breeds. Obviously, the lesion is more severe in the late stage of infection than in the early stage of infection, and the dates of infection could be an important factor in affecting the severity of the pathology. On the other hand, no association was observed between the age of the animals and the severity of pathology as demonstrated using multiple logistic regression analysis.

The other possible reason for such difference in the severity of the lesion in these breeds could be attributed to the small number of study animals used for comparison. Additionally, the number of study animals in the three breeds was not similar which could have affected the result of the analysis. Therefore, a further study should be conducted on the comparative susceptibility of Boran breed (zebu) and Holstein–zebu crossbreed under a similar intensive husbandry system through the exposure of naïve TB-free cattle of the three different breeds of infected animals at the same time and then investigating the comparative susceptibility of the three breeds to bTB.

The proportion of gross lesion detected in SICCT test reactors was 95.4%, and it was higher than the proportions of gross lesions reported earlier by other studies (18, 23, 34, 35). The higher portion of lesion positivity could be due to the selection of strong reactors for slaughtering and postmortem examination, although there is a correlation between reactivity to PPD and the severity of pathology. The isolation rate of the bacteria from the 41 lesion-positive animals was 39% (16/41), which was higher than those reported by other workers in cattle and camel (36–38). In this study, better culture positivity was recorded in the thoracic lymph nodes and the lung lobes, followed by the retropharyngeal lymph nodes, which could suggest that both oral and respiratory routes could be important routes of infection in these dairy farms. Similarly, other authors had also reported a higher culture positivity in the lung and the thoracic lymph nodes than in the head lymph nodes (23, 39, 40). The low isolation rate in the mesenteric lymph nodes could be because most of the lesions that were seen in the mesenteric lymph node during postmortem inspection were calcified, which reduced the number of viable bacteria, leading to a low isolation rate.

The isolation of *M. bovis* from the mammary lymph node of lactating dairy cow implies public health risks as *M. bovis* can be excreted through milk and infect raw milk consumers. The isolation of *M. tuberculosis* from cattle TB lesions can suggest reverse zoonosis, suggesting the transmission of *M. tuberculosis* from animal attendants to cattle (41, 42). In addition to these two important species of mycobacteria, earlier studies reported the isolation of non-tuberculous mycobacteria from a TB suspicious lesion in cattle (26, 43). All *M. bovis* isolated by the present study had the spoligotype pattern of SB0912, suggesting the presence of an actively ongoing infection by SB0912. SB0912 was isolated from different regions of Ethiopia by other authors (38). In addition to SB0912, two *M. tuberculosis* with the spoligotype pattern of SIT54 were isolated, which agrees with the observation of other authors who isolated *M. tuberculosis* from a TB suspicious cattle lesion (38, 43–45). SIT54 is one of the frequent spoligotypes of *M. tuberculosis* being isolated from humans in central Ethiopia (46).

CONCLUSIONS

In conclusion, although the frequency of SICCT test positivity was high in the crossbreed, a more severe pathology was observed on the Boran (zebu) breed. In addition *M. tuberculosis* was isolated from TB lesions of dairy cattle, demonstrating the role of *M. tuberculosis* in causing TB in cattle.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the study was conducted on dairy farms of the Ethiopian Institute of Agriculture as part of the bTB control program and lead by the staff members of the same Institute.

AUTHOR CONTRIBUTIONS

MA, BG, and GA conceived and designed the experiments. MA, AW, AY, and AZ performed the field and laboratory work. MA, BG, and GA participated in the data analysis. MA drafted the manuscript, while BG and GA edited the manuscript. BB data analysis and edition of the revised copy of the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.715598/full#supplementary-material

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Host Serum Proteins as Potential Biomarkers of Bovine Tuberculosis Resistance Phenotype

Jorge Luis Mazorra-Carrillo[1†], *Omar Antonio Alcaraz-López*[1,2†], *Gonzalo López-Rincón*[1], *Bernardo Villarreal-Ramos*[3,4], *José A. Gutiérrez-Pabello*[2] *and Hugo Esquivel-Solís*[1*]

[1] *Biotecnología Médica y Farmacéutica, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, Guadalajara, Mexico,* [2] *Laboratorio de Investigación en Tuberculosis Bovina, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Mexico City, Mexico,* [3] *Department of Bacteriology, Animal and Plant Health Agency, Addlestone, United Kingdom,* [4] *Centre of Excellence for Bovine Tuberculosis, Institute for Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, United Kingdom*

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***Correspondence:**
Hugo Esquivel-Solís
hesquivel@ciatej.mx

[†]*Deceased*

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Eradication of bovine tuberculosis (bTB) continues to be a worldwide challenge. The lack of reliable vaccines dampens the control and eradication programs of *Mycobacterium bovis* infection and spread. Selection and breeding of cattle resistant to *M. bovis* infection would greatly enhance the effectiveness of bTB eradication programs. Here, we have evaluated the potential of serum proteins as biomarkers of cattle resistance to bTB in Holstein-Friesian cows, 6–8-year-old, born and raised in similar conditions in herds with bTB prevalence >30%. Serum proteins obtained from uninfected cows (bTB-resistant; R) were compared to those from infected cows (bTB-susceptible; S), defined by a negative or positive bTB diagnosis, respectively. bTB diagnosis included: (i) single intradermal (caudal fold) tuberculin test, (ii) whole blood IFN-gamma test, (iii) gross visible lesions in lymph nodes and lungs by inspection at the abattoir, and (iv) a bacteriological culture for *M. bovis.* Using 2D-GE and LC-ESI-MS/MS, we found higher expression levels of primary amine oxidase (AO), complement component 5 (C5), and serotransferrin (TF) in R cattle than S cattle. In-house developed and standardized ELISAs for these novel biomarkers showed the best sensitivities of 72, 77, 77%, and specificities of 94, 94, 83%, for AO, C5, and TF, respectively. AUC-ROC (95% CI) values of 0.8935 (0.7906–0.9964), 0.9290 (0.8484–1.010), and 0.8580 (0.7291–0.9869) were obtained at cut-off points of 192.0, 176.5 ng/ml, and 2.1 mg/ml for AO, C5, and TF, respectively. These proteins are involved in inflammatory/immunomodulatory responses to infections and may provide a novel avenue of research to determine the mechanisms of protection against bTB. Overall, our results indicate that these proteins could be novel biomarkers to help identify cattle resistant to bTB, which in turn could be used to strengthen the effectiveness of existing eradication programs against bTB.

Keywords: host resistance to infection, *Mycobacterium bovis*, bovine tuberculosis, biomarkers, proteome, ELISA, cattle

INTRODUCTION

Members of the *Mycobacterium tuberculosis* complex are a group of pathogenic mycobacteria responsible for tuberculosis (TB) in mammals. The principal causative agent of bovine TB (bTB) is *Mycobacterium bovis*, which affects the livestock industry worldwide (1). bTB is an ancient animal disease that represents a zoonotic public health concern (2). While test and slaughter programs using the tuberculin skin test have led to the eradication of bTB in several countries, bTB remains endemic in some geographic areas (3). In addition to the negative impact of bTB on livestock productivity, the surveillance program is costly and has placed an extra economic burden on farmers and governments (4). Moreover, the test-and-slaughter program is not compulsory in all countries, and in countries where this program is voluntary, the extra cost and the time it takes to eradicate the disease from the herd causes farmers to lose interest and withdraw from the program. Alongside is the pressure of other domesticated and wild animals infected with *M. bovis* that pose the ability to infect bTB-free cattle herds (5).

Currently, there is no licensed vaccine against bTB; the leading candidate is the live attenuated *M. bovis* bacilli Calmette-Guerin (BCG); however, BCG is not currently used as the protection it confers is variable and interferes with the current diagnostic tests (6). Therefore, new interventions are needed which may enhance the effectiveness of bTB eradication programs, which could eventually lead to the elimination of bTB. In this context, increasing the genetic resistance of cattle to bTB could help control bTB by reducing the susceptibility to infection of animals and by potentially allowing infected animals to contain the infection and therefore reducing environmental contamination (7). Genetic or natural disease resistance is the inherent capacity of an animal, involving both immune and non-immune mechanisms, to resist disease when exposed to pathogens (8). Natural resistance to bTB in cattle has been phenotypically observed (nonreaction to bTB tests, absence of lesions and negative *M. bovis* culture) and extensively evaluated in families and breeds exposed to *M. bovis* under field conditions (9–13). Based on bTB phenotypes, robust heritability estimates of genetic resistance to *M. bovis* in cattle has been statistically estimated over time and in different climatic zones, indicating that breeding for increased bTB resistance in cattle is a feasible strategy (9–15).

Implementing immunogenomics with genome editing for the generation of transgenic-resistant cattle to bTB is currently being tested as a promising strategy (16–19). Genetically modified cows with enhanced anti-mycobacterial capacity have been generated by insertion of the mouse intracellular pathogen resistance 1 gene (*Ipr1*), named as the Sp110 nuclear body protein (*SP110*), through transcription activator-like effector nuclease (TALEN) (16). In comparison, the insertion of the human defensin β-3 gene (*DEFB103A*) through plasmid transfection and somatic cell nuclear transfer has also achieved bTB-resistant cattle (17). The knock-in genome editing with the bovine gene natural resistance-associated macrophage protein-1 (NRAMP1), renamed as the functional solute carrier family 11A member 1 gene (*SLC11A1*), has produced cattle with increased resistance to bovine tuberculosis (18, 19). *SLC11A1* gene-edited bTB-resistant cattle have been successfully produced through the single clustered regulatory interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) nickase (Cas9n) and somatic cell nuclear transfer (18); system that has been improved implementing the homology-mediated end-joining (HMEJ)-based method (19). NRAMP1 is a divalent metal ion transporter whose expression occurring only in macrophages and other phagocytes is upregulated by cytokines and induces iron sequestration and the production of nitric oxide (NO), decreasing the survival of *M. bovis* and other intracellular pathogens (20, 21). The overexpression of bovine NRAMP1 provides cattle with improved resistance to bTB (18, 21). Polymorphisms of the *SLC11A1* gene influencing the NRAMP1 expression have been related to bTB-resistance in African Zebu cattle (22) and Chinese Holstein cattle (23).

M. bovis infects, resides, and replicates in monocytes-derived cells of infected cattle. Therefore, immunogenetic studies on those cells have been of particular interest for searching bTB-resistance candidate genes (24). Genomic microarray analysis in cDNA isolated from naïve bovine macrophages has identified the interleukin-1 receptor antagonist (IL1RN), a good candidate biomarker of bTB resistance of Mexican dairy Holstein-Friesian cattle (25). Genome-wide association studies (GWAS) in case-control matches have previously identified new SNPs and QTL regions associated with bTB resistance/susceptibility in dairy cattle from Mexico (10), Ireland (26, 27), UK (11, 14), and Cameroon (12). GWAS also revealed that bTB resistance is polygenic (12) and has no heterozygote advantage (28). Like genome analysis, serum proteomics is another reliable approach to predict biomarkers of bTB-resistance. Preliminary findings on the levels of serum proteins in cattle and their correlation with degrees of mycobacterial infection have led to the discrimination of clinical and subclinical bTB and different stages of bovine paratuberculosis (Johne's disease) from not infected cattle and exposed cattle (29–32). Furthermore, analyses of concentration in serum of proteins that impact the immune system and macrophage function, i.e., adiponectin, ceruloplasmin, and conglutinin (33–35), have revealed that those are under genetic control and heritability (36–38), and a negative association with the predisposition of respiratory infectious diseases in cattle (36, 37, 39) with implications in bTB (35). These findings suggest that identifying serum proteins for resistance against bTB is possible in cattle and since non-specific serum proteins have a role in protective immunity, measuring the level of serum proteins may be a helpful trait in such a breeding strategy. However, up until now, no serum proteins have been associated with cattle resistance to *M. bovis* infection. The present study aimed to investigate the differential expression in serum protein profiles between bTB-resistant and bTB-susceptible Mexican dairy Holstein-Friesian cattle following long-time *M. bovis* exposure (>6 years). Primary amine oxidase (AO), Complement component 5 (C5), and serotransferrin (TF), were found overexpressed in serum of bTB-resistant cattle compared to bTB-susceptible cattle. We then developed enzyme-linked immunosorbent assays (ELISAs) for each of these novel biomarkers and obtained an overall sensitivity higher than 77%

and a specificity of at least 83% in field diagnosis. Our results provide potential novel targets for breeding purposes to improve the resistance of cattle to bTB and contribute to the success of eradication campaigns.

MATERIALS AND METHODS

Cattle Population

The present study included Holstein-Friesan dairy cows, 6–8-year-old, born and raised in eight -different dairy farms located in Jalisco, Mexico, a geographic area with known prevalence and active transmission of *M. bovis* (40). Individuals were selected from herds with naturally *M. bovis*-infected animals confirmed by *M. bovis* positive culture, detected during on-going national bTB surveillance and eradication campaign of the Mexican National Service of Agro-alimentary Health, Safety, and Quality (SENASICA). Those herds were at active episodes of infection and were quarantined at the control campaign. All animals were tuberculin tested [single caudal fold (intradermal) test—CFT or intradermal comparative cervical tuberculin test] at intervals of 60–90 days, with restriction from trading and permanent identification and subsequent culling of reactors. The prevalence of bTB within those dairy farms was greater than 30% (some herds with a prevalence of up to 60%). All herds had similar husbandry conditions: a semi-intensive system, housing in barns and grasslands after milking, twice a day, fed with forage, grains, and supplemented during the lactating period. The size of herds was 70–180 milking cows (median of 90) with average milk production of 53,795 kg.

Phenotype and Exposure Definition

Infected cows (susceptible) were animals that had a positive CFT result (bTB reactors), and all these animals were also positive for the whole blood IFN-γ (interferon-gamma) release assay (IGRA, BOVIGAM Prionics, Thermo Fisher Scientific). When the IGRA is used in parallel with the CFT, the sensitivity for detecting *M. bovis* infected animals increases (41). Furthermore, all these animals were confirmed for bTB by detailed postmortem examination of visible lesions and *M. bovis* culture. They all had gross visible lesions in lymph nodes (head, thorax, and abdomen) or lungs and *M. bovis* positive culture. Lesions were processed for *M. bovis* culture on Lowenstein-Jensen and Stonebrink media at 37°C for 12 weeks. This conservative classification is consistent with the current formal definition of a potential bTB case of SENASICA from Mexico and the Animal and Plant Health Agency (APHA) from the UK. Cows with positive results for CFT or IGRA with no visible lesions and negative *M. bovis* culture were excluded from the study. Not infected animals (resistant) were cows that resulted negative for CFT and negative for IGRA (nonreactors). In addition, all these cattle were confirmed bTB-negative by detailed postmortem examination (i.e., absence of lesions and negative *M. bovis* culture) to avoid inclusion of *M. bovis*-infected but unresponsive (anergic) animals (41, 42).

According to Ring et al. (27), all the cattle included in the present study had a potential bTB exposure: cows within a herd were deemed exposed to bTB if (1) any herd-mate was identified with a lesion at slaughter after being removed as a bTB reactor, (2) any herd-mate was identified as bTB-infected by detailed postmortem examination, or (3) two or more herd-mates were removed from the herd as bTB reactors (27). Transmission of *M. bovis* infection from a reactor to an in-contact animal within a group can occur in a 12-mo period (43). However, tuberculin reactions are early detectable within 42 days following infection, and IFN-γ levels are correlates of infection as early as 14 days after challenge, regardless of the infective dose (41, 44, 45). In the present study, the random selection of cattle took into account infected and not infected in-contacts born and lived in the same herd (close-contacts) and were matched by year of birth: for each not infected cow, one or two infected cows of the same age were included. Cattle moved into herds were excluded from the study. Only animals older than 6 years but younger than 8 years were included in the study. Due to farmers' policies, culling of reactors was carried out only if the cattle were aged 6 years or older. Animals in which the gross pathology and *M. bovis* culture could not be performed were not included in the study. Therefore, we were not able to include younger animals.

Our design sought a long-term period of exposure to the pathogen, intending to reduce the probability of including animals that have yet to be exposed to an infective dose, which can be as minimum as one CFU of *M. bovis* (44), while avoided immunodeficiencies of the elderly. We included contemporary herd-mates potentially exposed to the pathogen with the same probability (same age, husbandry and environment, and lifelong exposure) but with a different outcome (infected or not infected) (46). These binary trait definitions have been used in cross-sectional studies seeking features of bTB resistance in cattle (9–12).

Samples

Two hundred thirty-four cows were blood sampled for the biomarkers' discovery study: 96 resistant and 138 susceptible to bTB. Animals were tuberculin tested at farms and blood sampled for sera and IGRA at slaughterhouse pens. Blood samples were taken into Vacutainer tubes (Becton and Dickinson, GDL, MX) with and without anticoagulant (lithium heparin). Samples were immediately protected from heat and sunlight after collection and processed within 2 h after sampling in the laboratory. After clot formation, serum was obtained by 15 mins centrifugation at 12,000 × *g*, 4°C, and stored at −20°C until use.

Although the sample size was not calculated, we had enough experimental power at ensuring accurate phenotype definitions by including not infected animals that have had a high probability of exposure to *M. bovis* and derived from epidemiologically comparable herds (11).

For biomarkers validation, a pilot study was performed individually with in-house ELISAs in another set of 72 serum samples collected from cattle without bTB ($n = 36$) and cattle with bTB ($n = 36$), both diagnosed with or without any other infectious disease (positive to specific RT-PCR tests in clinical samples) or metabolic disease from the same herds described above. Two cases each of the most frequent diseases in the geographical region of the sampled cattle were included per group: (1) *Mycobacterium avium* sp. *paratuberculosis* (MAP)/Johne's disease, (2) *Brucella abortus*,

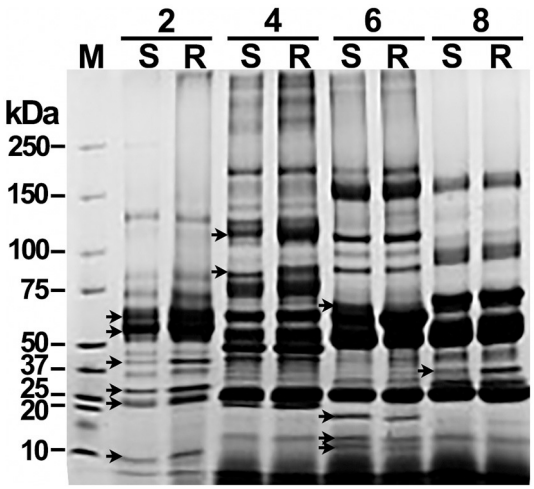

FIGURE 1 | Offgel separation of serum proteins by pI and matching by molecular weight reveal significant differences between susceptible and resistant cattle. Samples of pooled sera depleted of high-abundance proteins were fractionated by pH by offgel electrophoresis. Single offgel fractions were separated on a 4–20% gradient SDS-PAGE, loaded with 10 μg of protein per well by pairing samples from susceptible (S) and resistant (R) cattle. Proteins were Coomasie blue stained. A paired comparative densitometry analysis was carried out to seek differences of ≥ 2 times among pairs of protein bands. A Coomassie blue-stained SDS-PAGE of serum fractions 2, 4, 6, and 8 representatives of that with significant differences in protein bands (arrows) is shown. M, protein molecular weight marker.

(3) bovine herpesvirus 1 (BoHV-1), (4) bovine viral diarrhea virus (BVDV), (5) bovine parainfluenza virus 3 (BPIV-3), (6) *Leptospira* spp. infection (*L. interrogans* serovar *hardjo* type hardjoprajitno or *L. borgpetersenii* serovar *hardjo* type hardjobovis), (7) hypocalcemia, (8) ketosis, and (9) hypomagnesemia. Serum samples from 18 active clinical cases of cattle diagnosed with different infectious or metabolic diseases concomitant to bTB were included as a single group (bTB+IMD). While samples of 18 cattle matching the same diseases without bTB were grouped (bTB-IMD). The comparison included the samples of 18 cattle with bTB without any other clinical disease (bTB+) and 18 samples from cattle resistant to bTB (bTB–), used to standardize ELISAs. Additionally, blood samples of 18 healthy cattle (HS) from a bTB-free, paratuberculosis-free, and brucellosis-free herd (controls; no clinical or laboratory tests and negative to IGRA) were also included.

A Three-Step Serum Proteome Analysis

Highly Abundant Proteins Removal

To reduce individual variation and increase the chance to identify valid biomarkers, all 96 serum samples from resistant cows were pooled. Similarly, serum samples from 138 susceptible cows were pooled. Samples were therefore compared on a one-to-one basis as resistant and susceptible. Proteomic analysis to identify potentially rare biomarkers from serum samples requires depletion of over-abundant proteins and further fractionation (47). To deplete highly abundant proteins (such as albumin), we used precipitation with 10% w/v TCA in cold acetone (48). Four volumes of ice-cold acetone containing 10% w/v TCA were rapidly added to serum pooled samples and immediately mixed by gentle vortexing. The mixtures were incubated at −20°C overnight and centrifuged at 15,000 x *g* for 20 min, 4°C. The precipitate was washed twice with 1 ml of ice-cold acetone on ice for 15 min and centrifuged as above. The precipitated proteins were lyophilized, resuspended in 1× PBS (120 mM sodium chloride, 1.2 mM sodium phosphate monobasic, 2.8 mM potassium chloride, 8.8 mM sodium phosphate dibasic, pH 7.4), and quantified using the Pierce bicinchoninic acid microplate assay (Thermo Fisher Scientific, CA, USA), following the manufacturer's instructions. For qualitative analysis, 10 μg of protein per well of crude serum, depleted serum (precipitate), and the albumin-rich fraction (supernatant) were resolved by 1.5 mm, 10% SDS-PAGE, carried out at a constant voltage set to 100 V for 1 h, and Coomassie blue stained (**Supplementary Figure 1**).

Serum Proteome Fractionation and Comparative Analysis

Each of the pools of abundant-proteins-depleted sera was fractionated using OFFGEL electrophoresis. The 3100 OFFGEL electrophoresis fractionator and an OFFGEL pH 3–10 kit (Agilent Technologies, CA, USA) were used with a 12-well configuration following the supplier's protocol. The IPG gel strips (13 cm length, pH 3–10) were rehydrated with IPG Strip Rehydration Solution in the assembled device with 40 μl per well for 15 min. Two hundred μg of serum proteins were diluted in protein OFFGEL solution [8 M urea, 2 M thiourea, 40 mM 1,4-dithio-DL-threitol (DTT), and 2% v/v ampholytes pH 3–10] to a final volume of 1.8 ml and 150 μl of sample was loaded into each well. Proteins were submitted to isoelectric focusing (IEF) until reaching 50 kVh with a maximum voltage of 4000 V, 50 μA 200 mW, and a hold setting of 500 V. Twelve individual fractions were recovered and further resolved by molecular weight (kDa) through 1.5 mm, 4–20% gradient SDS-PAGE, under reducing conditions. To facilitate comparisons, fractions were loaded in SDS-PAGE (10 μg/well) organized by pairs matching resistant (R) with susceptible (S). SDS-PAGE was carried out at a constant voltage set to 100 V for 1 h. Gels were stained with Coomassie brilliant blue; images were recorded with a Gel Doc™ XR+ system (Bio-Rad Laboratories, USA) and analyzed by densitometry with Quantity One software (Bio-Rad Laboratories, USA). Single serum fractions that presented differences of two or more times the quantity of a band among paired lanes (**Figure 1**) were subsequently analyzed by two-dimensional gel electrophoresis (2D-GE).

Two-Dimensional Gel Electrophoresis

Individual sera fractions 2, 4, 6, 8, and 10 were respectively pooled (R or S) and processed to remove OFFGEL solution components (through HiTrap desalting columns) before 2D-GE. Pooled sera fractions containing 150 μg of protein each was diluted in a final volume of 350 μl of IPG rehydration buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 0.2% DTT, 0.5% IPG buffer, 0.2% w/v Bio-Lyte 3/10 ampholytes, and 0.002% bromophenol blue). The IPG gel strips 7 cm, pH 3-10 (ReadyStrip, Bio-Rad Laboratories, USA) were rehydrated

TABLE 1 | Identification of serum proteins differentially expressed in bTB-resistant/bTB-susceptible cattle by 2D-GE.

Spots no.[a]	Average ratio of abundance	*P* value[b]	Peptides matched/ sequence coverage	Mascot score[c]	Protein name	Accession entry[d]	Theoretical mass (kDa)/pI
1	1.6	< 0.0001	5/4%	87	Complement component 5	A0A0F6QMJ3/ F1MY85	188.677/6.20
2	8.5	< 0.0001	8/12%	209	Amine oxidase	E1BJN3	76.896/5.69
3	2.1	< 0.0001	17/24%	250	Serotransferrin	Q29443	77.689/6.75
4	−1.8	< 0.0001	4/12%	124	Haptoglobin	G3X6K8	41.954/7.10

[a] *Numbers correspond to spots circled in* ***Figure 2****.*
[b] *Student's t-test.*
[c] *Individual ions scores > 35 indicate identity or extensive homology ($p < 0.05$).*
[d] *UniProtKB.*

with this solution for 20 h using a DryStrip reswelling tray (GE Healthcare) covered with 1.6 ml of mineral oil. After rehydration, IEF was carried out on an IPGphor (Amersham Biosciences) to reach 52 kVh. Following IEF, gel strips were equilibrated in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% v/v glycerol, 2% w/v SDS and 1% DTT) for 15 min. The strips were further transferred to a buffer containing 2.5% iodoacetamide for 15 min. After equilibration, the second dimension was performed on 1.5 mm, 12% non-gradient polyacrylamide gels in a Mini-Protean Tetra Cell chamber (Bio-Rad Laboratories, USA). SDS-PAGE was carried out at a constant voltage set to 120 V for 1.5 h. After protein fixation for 1 h with 10% methanol and 7% acetic acid, the gel was stained using colloidal Coomassie Blue (G-250, Bio-Rad Laboratories, USA). Gel images were taken with a CCD high-resolution camera using the Gel Doc™ XR+ system (Bio-Rad Laboratories, USA). To increase confidence in the results obtained from the 2D gel electrophoresis, gels were run in triplicates with samples from each pooled fractionated sera from R and S cattle, respectively, for quantitative analysis. Gel image analysis included image alignment, spot detection, background subtraction, spot measurement, and spot matching using PDQuest 2-D analysis software (Bio-Rad Laboratories, USA). Coomassie Blue-stained spots were quantified based on their relative volumes (the spot volume divided by the total volume over the whole set of gel spots, according to the instructions provided by the manufacturer). Protein profiles were matched within each R and S gels, and then relative volumes of matched spots were compared between R and S samples for statistical analysis using the Student's *t*-test ($p < 0.05$). A factor greater than a 1.6-fold increase in the spot's average density in one group compared to the other was reported as overexpression.

In-Gel Digestion and Mass Spectrometric Analysis

Differentially expressed protein bands were excised from the Coomassie-stained gels, distained, and digested with sequencing-grade trypsin (Promega, Madison, WI, USA). Nanoscale liquid chromatography separation of tryptic peptides was performed with a nanoAcquity ultra-performance liquid chromatography (UPLC) system (Waters Corp., MA, USA), equipped with a Symmetry C18 Trap Column (5 μm, 20 mm ×180 μm, 100 A, 2G, Waters) and a PST BEH C18 (1.7 μm, 100 mm×75 μm, 130 A, 10K psi, Waters) analytical column. The lock mass compound human [Glu1]-Fibrinopeptide B (Sigma-Aldrich) (load mass 1 of 571.6852 m/z, and load mass 2 of 785.8426 m/z) was delivered at 0.5 μL/min at a concentration of 200 fmol/ml to the mass spectrometer. Mass spectrometric analysis (LC-MS/MS) was carried out in a Synapt-HDMS Q-TOF (Waters). The spectrometer was operated in V-mode, and analyses were performed in positive mode ESI. The acquisition window in MS mode was 400–2000 (m/z), and an MS/MS mode was 50–2000 (m/z). The charges of acquired ions were 2+, 3+, and 4+. The data acquisition was set at Data Dependent Acquisition (DDA). MS and MS/MS spectra were acquired at a fragmentation energy ramp of Low Mass Collision Energy (LM CE) of 15–45 volts and High Mass Collision Energy (HM CE) of 15–55 Volts. The Lock Mass reference sprayer was sampled every 30 s.

Data Analysis and Protein Identification

MS/MS spectra data sets were used to generate PKL files using Protein Lynx Global Server v2.4 (PLGS, Waters). Proteins were then identified using the Mascot search engine algorithm (Matrix Science, London; http://www.matrixscience.com). Searches were conducted using the NCBIprot (128,624,863 sequences), the *Bos taurus* protein database (UP9136; 37,880 sequences), and the contaminants database (262 sequences). Trypsin was used as the specific protease, while one missed cleavage was allowed with a 0.6 Da tolerance set for the precursor and the fragment ion masses, while carbamidomethyl (C) and oxidation (M) were selected as variable modifications. Proteins were identified based on a minimum of two unmodified highly scoring unique peptides per protein at a false discovery rate (FDR) of 1%. Proteins identified were: (1) amine oxidase (EC 1.4.3.21), (2) complement component 5 (C5), (3) serotransferrin and (4) haptoglobin (**Table 1**). The former three were over-expressed in R cattle sera while the latter in sera of S cattle. Only overexpressed proteins in the resistant group were assessed by immunodiagnostic tests (ELISAs).

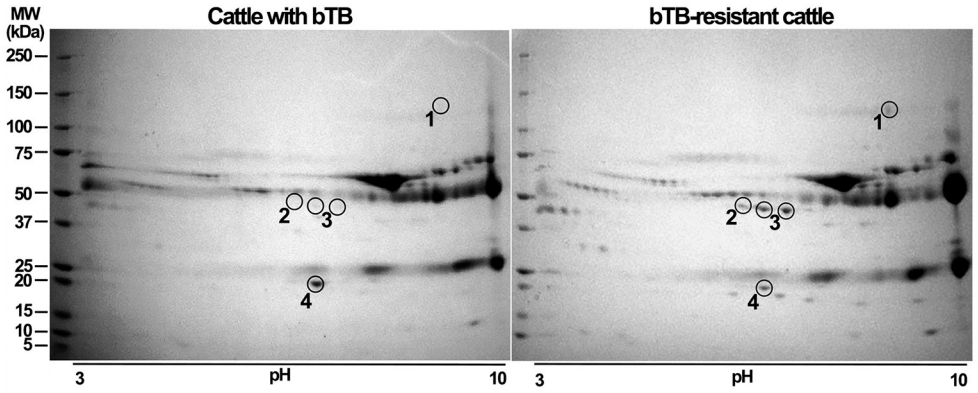

FIGURE 2 | Resolving fractions of sera by two-dimensional gel electrophoresis (2D-GE) enhances detection and precise excision of overexpressed proteins of susceptible and resistant cattle. Offgel fractions 2, 4, 6, 8, and 10 of sera from bTB-susceptible and bTB-resistant cattle were independently pooled, cleaned, separated by 2D-GE and stained with Coomassie blue. A comparative analysis of protein amount was implemented using PDQuest 2-D software. Proteins with significant differences ($p < 0.05$, Student's t-test) were excised and processed for LC/ESI-MS/MS analysis. We show a representative two-dimensional gel of proteins from bTB-susceptible cattle and one of that of bTB-resistant cattle. Proteins differentially expressed among gels are enclosed in circles and numbered. Encircled protein spots close to number 3 correspond to the same protein, but with a different isoelectric point. Protein molecular weight markers (M) were ran along the samples to determine the relative molecular weight of proteins.

ELISAs

Production of Rabbit Polyclonal Antibodies Against Candidate Biomarkers

Hyperimmune antisera were produced from 2 female New Zealand white rabbits for each protein. Each pair of rabbits was injected subcutaneously at multiple sites with 50 μg antigen (0.5 mg/ml PBS) emulsified in an equal volume of TiterMax Gold adjuvant (T2684, Sigma-Aldrich, MO, USA); rabbits were inoculated thrice at 2 weeks intervals. Antigens for injection were obtained as follows: complement C5 was isolated from bovine serum as previously described (49); bovine plasma amine oxidase (M4636) and bovine serum transferrin (T1428) were obtained from Sigma-Aldrich (Sigma-Aldrich, MO, USA). Serum from each pair of rabbits was collected 2 weeks after the last immunization by cardiac puncture under terminal anesthesia and pooled. Immunoglobulins were precipitated with 50% ammonium sulfate pH 6.8, followed by dialysis against 10 mM PBS pH 7.6 (100× v/v) and chromatography purification on DEAE Sepharose equilibrated with the same buffer (50, 51). Rabbit IgGs were quantified by UV absorbance at 280 nm and adjusted to 1 mg/ml, based on a calculated extinction coefficient of $O.D._{280} = 1.0 \approx 1.4$ mg/ml IgG. Purity was analyzed by 12.5% SDS-PAGE and Coomassie blue staining and immunoblotting (**Supplementary Figure 2**). Detection antibodies were obtained by conjugating purified rabbit IgGs with HRP (P8125, Sigma-Aldrich, MO, USA) by the periodate method (52). The IgG-HRP products were dialyzed overnight against 1X PBS at 4°C. Capture and detection (HRP-conjugated) rabbit antibodies were preserved with BioStab (55514, Sigma-Aldrich, MO, USA) and used to implement indirect sandwich ELISAs. The purified proteins used to generate the antibodies were also used as standards.

Performance Evaluation

Capture and detection antibodies were titrated in a range of standard concentrations as well as protein standards (capture antibodies: 4, 2, 1 and 0.5 μg/ml; detection antibodies: 0.4, 0.2, 0.1, 0.05 and 0.025 μg/ml; protein standards for TF 10, 5, and 1 mg/ml and for AO and C5 100, 10, 1, and 0.1 ng/ml). All parameters were analyzed in triplicate. Other dose-response curves for the concentration of each standard in the sample matrix (bovine serum: 0, 10, 25, 50, and 100%) were performed. Two standard dilution buffers were assayed: 10% blocking buffer with 0.05% Tween 20 in 1X PBS; and 2% (v/v) horse serum (H1270, Sigma-Aldrich, MO, USA) 0.05% Tween 20 in 1X PBS. Three blocking buffers were evaluated: 1% casein in 1X PBS (1610783, Bio-Rad Laboratories, USA); 0.5% enzyme immunoassay-grade fish skin gelatin (G7041, Sigma-Aldrich, MO, USA) in 1X TBS (20 mM Tris and 150 mM NaCl, pH 7.6); and 5% nonfat dry milk (1706404, Bio-Rad Laboratories, USA) in 1X TBS. The wash buffer was 0.05% Tween 20 in 1X PBS. The HRP substrate was 1% tetramethylbenzidine (TMB) dissolved in Dimethylsulfoxide (DMSO), diluted 1:100 in 0.1 M sodium acetate (pH 6.0) and 0.005% hydrogen peroxide (added just before using the substrate). The stopping solution was 1.5 N sulfuric acid. ELISAs were performed in 96-well clear flat bottom polystyrene High Binding microplates (Costar 9018, Corning, MA, USA). The optimal concentration for each antibody pair was determined by the lowest signal-to-noise ratio of the mid and low standards compared to a top standard. The signal-to-noise ratio was obtained by dividing the average optical density (OD) of each standard by the average OD of its corresponding zero standards. The optimal blocking buffer was 0.5% fish skin gelatin, which produced the lowest OD reading difference between 0, 10, and 50% of the sample matrix plus standards for the entire tested interval.

Lowest Limit of Detection, Precision, Linear Range, and Recovery Rate

The precision of the assay was determined through the construction, modeling, and evaluation of the concentration curve of protein standards using all the combinations of reagents described above. Each detection was repeated ten times for three different days by two independent people. The four-parameter logistic (4PL) fit was performed as the reference model for the standard curves (53). The linear range of the effective concentration range of each candidate biomarker that each ELISA can detect was determined with eight serially diluted concentrations of each protein performed in triplicate. The linear range was confirmed when the regression coefficient $R \geq 0.90$ and the minimum of the linear range were higher than the LLOD. The LLOD was calculated by the mean plus two standard deviations (M+2SD) of the diluent OD measured 20 times.

The recovery rate was used to evaluate the accuracy of the ELISA tests. A sample of high-concentration standard (A) was added to a sample of low-concentration standard (B) with a volume ratio (A: B) $\leq$ 1:9. The recovery rate R was calculated as follows, $R = C \times (V0+V) - (C0 \times V0)/(V \times CS) \times 100\%$, where V is the volume of sample A, V0 is the volume of sample B, C is the concentration of the mixture of A and B, C0 is the concentration of sample B, and CS is the concentration of sample A.

Validation Analysis

The concentration of AO, C5, and TF in cattle sera was measured using in-house-built and standardized indirect sandwich ELISAs. Equal numbers of serum samples (n=18 in each group) from cattle with or without bTB identified by skin test, IFN-gamma test, lesions, and *M. bovis* culture, with or without another infectious or metabolic disease, plus control healthy cattle, were included for the analysis. Flat bottom 96-well plates were coated overnight at 4°C with 50 µl of 2 µg/ml polyclonal rabbit capture antibodies against bovine AO, C5, or TF diluted in 50 mM sodium carbonate, pH 9.6. The ELISA components and serum samples were equilibrated at room temperature (RT) for 30 min before use. The detection procedure was as follows. Coated plates were washed with 0.05% Tween 20 in PBS, three times (250 µl/wash/well, 15-s soaks between washes; each washing step) using an automatic plate washer (1575, Bio-Rad Laboratories, USA). Blocking buffer (150 µl/well, 0.5% fish skin gelatin in TBS) was immediately added and incubated for 1 h, at RT shaking on an orbital shaker (2314Q, Thermo Scientific, CA, USA). After washing, 100 µl of standard sample dilutions and serum samples diluted 1:10, both in 0.05% gelatin-0.05% Tween 20 in 1X TBS, were added in triplicates. The plates were sealed with film and incubated for 2 h at RT, shaking. The plates were then washed, and 100 µl of 0.1 µg/ml HRP-conjugated detection antibodies diluted in 0.05% gelatin-0.05% Tween 20 in 1X TBS were added to wells. The sealed plates were left shaking for 1 h at RT, after which wells were washed once more. TMB substrate solution was added to each well (100 µl) and incubated in the dark without shaking for 15 min at RT. Finally, 100 µl of 1.5 N sulfuric acid was added per well, and the plate was gently mixed by hand. Plates were scanned with the iMark plate reader (Bio-Rad Laboratories, USA) at 450 and 595 nm, and the delta OD (OD450–OD595) was used as the OD data point.

The standard curves were provided from 7 dilutions of each purified standard and zero (AO: 400, 200, 100, 50.0, 25.0, 12.5, 6.25 and 0 ng/ml; C5: 300, 150, 75, 37.5, 18.75, 9.38, 4.69 and 0 ng/ml and TF: 8.67, 5.78, 3.85, 2.57, 1.71, 1.14, 0.57 and 0 mg/ml). The cut-off values were chosen by a percentile method at which the highest sensitivity and specificity are reached and ROC (receiver operating characteristic) evaluating the discriminative capacity of the diagnostic tests. The detection effect was evaluated according to the area under the ROC curve (AUC-ROC).

Statistical Analysis

Comparisons between groups were conducted using the Student's *t*-test (resistant vs. susceptible) in the abundance of protein spots in 2D-GE determined by PDQuest 2-D analysis software and concentration of candidate biomarkers in individual serum samples quantified by the in-house ELISAs. Assessment of specificity, sensitivity, and area under the curve (AUC) was carried out using Receiver Operating Characteristic (ROC) curves. Kruskal-Wallis nonparametric test with Dunn's multiple comparison *post hoc* test was carried out to compare the mean rank serum levels of candidate biomarkers in each group of cattle (bTB+, bTB+IMD, bTB-IMD, HS) against the mean rank serum levels of the bTB-resistant cattle (bTB-). Nonparametric tests were selected after data were subjected to the D'Agostino-Pearson omnibus K2 normality test that showed that the data were not normally distributed. All statistical analyses were performed with Prism 6.0 (GraphPad Software Inc., CA, USA). $p < 0.05$ was statistically significant.

RESULTS

Identification of Potential Biomarkers

To enhance the resolution of the comparative analysis, depleted sera were further fractionated by IEF with OFFGEL electrophoresis. Individual OFFGEL fractions of R and S sera were further paired and resolved by one-dimension SDS-PAGE. Afterward, densitometry of bands was performed with Quantity One software (Bio-Rad Laboratories). Protein patterns showing significant differences amongst bands of R and S fractionated sera are shown in **Figure 1** (indicated with arrows). OFFGEL fractions 2, 4, 6, 8, and 10 showed a $\geq$ 2-fold difference in density of some protein bands. Those serum fractions were subsequently combined by R or S origin and resolved by 2D-GE in triplicates for accurate excision and protein identification. OFFGEL fractions 1, 3, 5, 7, 9, and 11 did not show significant differences, and fraction 12 had not defined protein bands (not shown), and thus all they were excluded from further analysis.

Representative images of 2D-GE of processed sera from R and S cattle are presented in **Figure 2**. Potential differences of abundance in protein spots were evaluated using PDQuest 2-D software (Bio-Rad Laboratories). The software detected 173 matched spots common to the two groups of cattle. Four spots were consistently differentially expressed between groups ($p < 0.0001$) (**Figure 2**). Three protein spots (encircled and numbered 1, 2, 3 in 2D-GE) were differentially upregulated in

TABLE 2 | Performance parameters of each in-house ELISA.

	Amine oxidase	Complement C5	Serotransferrin
Linear ranges	6.25–400 ng/ml	4.69–300 ng/ml	0.76–8.67 mg/ml
LLOD	3.13 ng/ml	2.34 ng/ml	0.51 mg/ml
Recovery rates	80–120%	80–120%	80–120%
AUC-ROC (95% CI)	0.8935 (0.7906–0.9964)	0.9290 (0.8484–1.010)	0.8580 (0.7291–0.9869)
Cut-off values	192.0 ng/ml	176.5 ng/ml	2.1 mg/ml
Sensitivity %	72.2%	77.8%	77.8%
Specificity %	94.4%	94.4%	83.3%
Odds ratio	13.00	14.00	4.67
Positive predictive value	93.8%	94.3%	84.6%
Negative predictive value	74.3%	78.4%	76.2%

bTB-resistant cows compared to cows with bTB ($\geq$ 1.6). In contrast, one protein spot (number 4) was upregulated in cattle with bTB compared to cattle resistant to bTB (> 1.8). Proteins identified overexpressed were excised from gels, in-gel digested with trypsin, and processed for peptide mass-spectrometric identification by LC/ESI-MS/MS. Results are shown in **Table 1**. Two of these protein spots were identified as the same protein (number 3) with different pI. Proteins overexpressed in bTB-resistant cattle were AO, C5, and TF, while the upregulated protein in cattle with bTB was haptoglobin. Protein identification was unambiguous in all cases as judged by peptide mass accuracy and sequence coverage (**Supplementary Table 1**).

Development of ELISAs for the Evaluation of Potential Biomarkers

Before evaluating AO, C5, and TF, as potential biomarkers of resistance to bTB, there was a need to develop an assay that would allow the evaluation for the presence of these proteins in cattle samples. Accordingly, we developed and standardized capture and detection rabbit antibodies and individual ELISA tests to determine the concentration of these proteins in serum samples derived from 18 cattle resistant to bTB and 18 cattle with bTB.

The performance of each ELISA against these novel biomarkers is summarized in **Table 2**. Overall, the three ELISAs showed a series of acceptable performance parameters: linear ranges of 6.25–400, 4.69–300 ng/ml, and 0.76–8.67 mg/ml, LLOD of 3.13, 2.34 ng/ml, and 0.51 mg/ml, for AO, C5, and TF. The positive detection rate for AO, C5, and TF was 93.8, 94.3, 84.6%, respectively. Linear ranges showed correlation coefficients higher than 0.90, and LLOD could be separated from the background (**Figure 3**). The areas under the ROC curves (AUC-ROC) of AO, TF, and C5 were 0.8935 (0.7906–0.9964, 95% CI), 0.8580 (0.7291–0.9869, 95% CI), and 0.9290 (0.8484–1.010, 95% CI), respectively (**Figure 4**). As observed, the mean values of AUC-ROC are greater than 85% in all in-house ELISAs, indicating a very high probability of accurate and true results with these assays. The correct diagnosis index, defined as sensitivity % - (100% – specificity %), reached its maximum at a concentration of 190.0 ng/ml for AO, 2.0 mg/ml for TF, and 176.0 ng/ml for C5. Thus, for AO, TF, and C5, the cut-off values were chosen as 192.0 ng/ml, 2.1 mg/ml, and 176.5 ng/ml. These cut-off values provided the highest sensitivities and specificities for each ELISA. The sensitivities were 72.2, 77.8, 77.8%, and the specificities were 94.4%, 94.4%, and 83.3%, for AO, TF, and C5, respectively (**Table 2**). In summary, the ELISAs were highly predictive of real results with a high capacity to detect significant differences between cattle with bTB and cattle resistant to bTB.

Evaluation of Biomarkers as Indicators of Resistance or Susceptibility to bTB

In order to validate 2D-GE and LC/ESI-MS/MS data, we performed ELISA analyses for AO, C5, and TF in naturally exposed cattle to *M. bovis*, infected (bTB+) and not infected (bTB-) and with and without a concomitant infectious or metabolic disease (IMD), as well as in healthy cattle (HS) not exposed to *M. bovis*. The mean serum concentration values ($\pm$ interquartile rank) of AO, TF, and C5, in cattle resistant to bTB and cattle with bTB are summarized in **Table 3**. Mean serum levels of these novel candidate biomarkers in different groups of cattle are presented in **Figure 5**. The serum concentration of these three proteins measured in cattle without any concomitant disease was significantly higher in cattle resistant to bTB than in cattle with bTB ($\geq$1.4 times; $p < 0.001$). At the same time, the mean serum concentration of AO was significantly higher in cattle resistant to bTB without any simultaneous disease (bTB-) than in all other groups ($p < 0.01$). C5 mean serum values of the bTB- group were not distinguishable from C5 mean serum values of the healthy group (HS), although they were significantly higher than the rest of the animals in the other groups ($p < 0.01$). TF values in serum of bTB-resistant cattle, with or without an infectious or metabolic disease (IMD), were significantly higher than in sera of cattle with bTB ($p < 0.001$), but like those of HS ($p > 0.05$).

DISCUSSION

In the present study, we have identified that the level of expression of some bovine serum proteins in Holstein-Friesian dairy cows that appear to be resistant to *M. bovis* infection is higher than the expression of the same proteins in cattle that have succumbed to bTB. These serum proteins are AO, C5, and TF and would appear to be promising biomarkers of resistance to bTB.

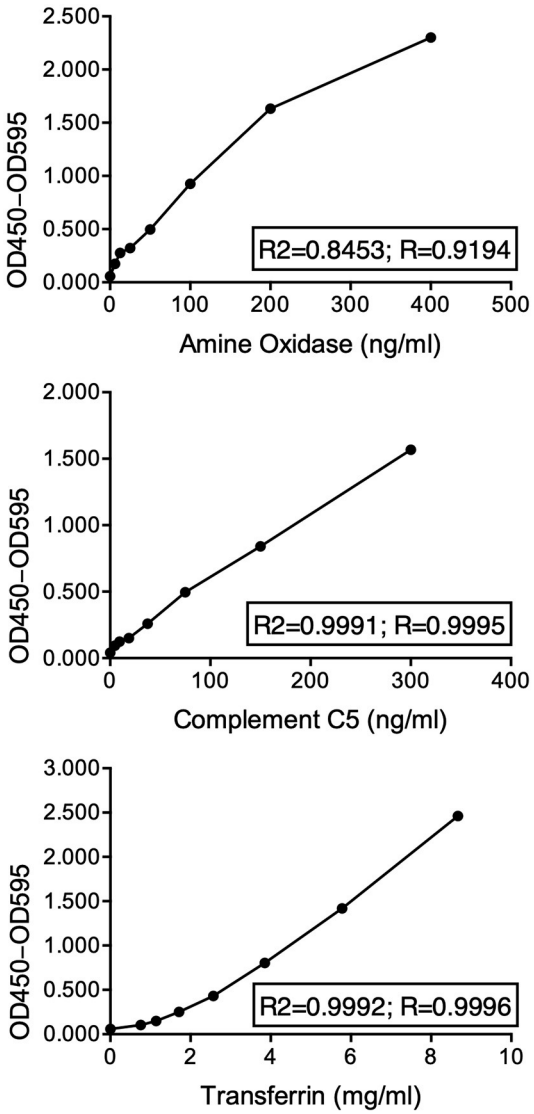

FIGURE 3 | Nonlinear regression of OD and concentration of amine oxidase, complement component 5, and transferrin confirmed precision of each in-house sandwich ELISA in their linear range. Triplicates of eight serially diluted concentrations of each purified protein were quantified by their respective in-house sandwich ELISAs. ODs (OD 450 nm – OD 595 nm) vs. concentrations were modeled by a sigmoidal, four-parameter logistic (4PL) fit regression, and the coefficient of determination (R^2) and the coefficient of correlation (R) were estimated.

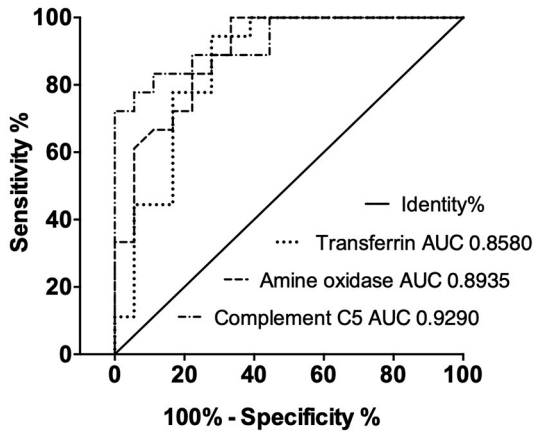

FIGURE 4 | Receiver-operating characteristic (ROC) curves of candidate biomarkers show good correct diagnosis indexes of ELISAs in clinical samples. The areas under the ROC curves (AUCs) are above 85%.

Many efforts have been directed toward identifying diagnostic protein biomarkers capable of distinguishing between cattle with subclinical *M. bovis* infection (early experimental infection−1 month) compared to cattle with *M. bovis* clinical infection (late experimental infection−4 months) or not infected. Serum levels of fetuin, alpha-1-microglobulin/bikunin precursor protein (AMBP), alpha-1 acid glycoprotein, and alpha-1B glycoprotein were elevated exclusively in *M. bovis* infected animals compared to contemporary controls and *M. paratuberculosis* infected animals. Whereas transthyretin, retinol-binding proteins, and cathelicidin were identified exclusively in *M. paratuberculosis* infection (30). These previous observations suggest that specific mycobacterial infections may evoke particular non-specific responses. However, to our knowledge, none of these proteins correlates with resistance to *M. bovis* infection. Natural resistance to infection in this work is the host's capacity to remain uninfected in spite of prolonged exposure to *M. bovis* challenge (8, 54).

While many efforts seeking to distinguish between cattle resistant and susceptible to *M. bovis* have been made, those efforts have focused mainly on the use of nucleic acid analysis, including single nucleotide polymorphisms (10, 22–25). To the best of our knowledge, this is the first study seeking to characterize serum proteins that may distinguish cattle that are resistant from those which are susceptible to bTB. The characterization of such biomarker proteins provided the opportunity to develop an ELISA test that could be used as a pen test to determine whether cattle are susceptible or resistant to bTB. We evaluated the performance of this test by correctly identifying samples from cattle known to be susceptible to bTB (i.e., showed a sign consistent with bTB infection as described in materials and methods) from those known to be resistant to bTB (i.e., animals that were long term close contacts of animals that were infected with *M. bovis* as described in materials and methods). Our ELISA systems showed linear range of 6.25–400, 4.69–300 ng/ml, and 0.76–8.67 mg/ml, LLOD of 3.13, 2.34 ng/ml, and 0.51 mg/ml, as well as sensitivities of 72.2, 77.8, and 77.8% and specificities of 94.4, 94.4, and 83.3%, respectively, for AO, C5, and TF. Linear ranges showed correlation coefficients higher than 0.90, and LLOD could be separated from the background. The positive detection rate for AO, C5, and TF was 93.8, 94.3, 84.6%, respectively, showing the high sensitivity of these detection systems.

To determine the extent to which these proteins may vary in their concentration in the plasma due to the presence of

TABLE 3 | Concentration values of candidate biomarkers in serum samples of bTB-resistant cattle and bTB-susceptible cattle determined by in-house ELISAs.

Protein	Cattle resistant to bTB (Mean ± interquartile rank, $n = 18$)	Cattle with bTB (Mean ± interquartile rank, $n = 18$)	Average ratio[a]	p value[b]
Complement component 5 (ng/ml)	193.8 ± 8.129	114.8 ± 9.225	1.7	0.0001
Amine oxidase (ng/ml)	202.3 ± 13.05	118.6 ± 9.892	1.7	0.0001
Serotransferrin (mg/ml)	2.43 ± 0.108	1.74 ± 0.113	1.4	0.0001

[a] *bTB-resistant/bTB-susceptible.*
[b] *Student's t-test.*

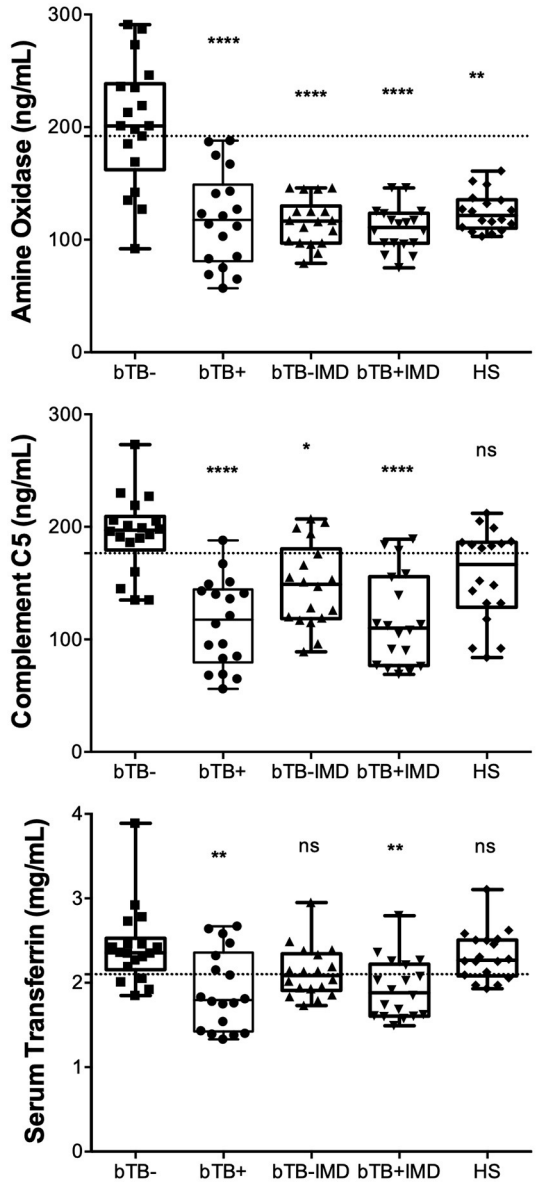

FIGURE 5 | Concentrations of AO, C5, and TF measured in clinical samples by ELISA tests exhibit significant efficacy in diagnosing bTB-resistant cattle. AO, C5, and TF were quantified by the in-house developed and standardized indirect sandwich ELISAs in serum samples (n = 18) of cattle from herds with endemic bTB, but without bTB (bTB-), cattle with bTB (bTB+), cattle without bTB, and with any infectious or metabolic disease (bTB-IMD), cattle with bTB and with IMD, and healthy cattle (HS). Statistical analysis was performed by comparing the mean rank of each group of cattle against the mean rank of the bTB-resistant cattle through the Kruskal-Wallis test. $p < 0.0001$****, $p < 0.001$**, $p < 0.01$*, ns not statistically significant. Gridlines indicate the cut-off values.

other infectious diseases, we measured AO, C5, and TF in individual serum samples of cattle exposed to *M. bovis* with a negative or positive diagnosis of bTB that were positive or negative to any unrelated infectious or metabolic disease. In addition, samples from healthy cattle without exposure to *M. bovis* (from a bTB-free herd) were included as controls. Overall, the OD values in our ELISA test of AO, TF, and C5 were significantly higher in cattle resistant to bTB than in those with bTB (≥1.4x, ≥1.7x, ≥1.7x, respectively), regardless they were suffering or not from other unrelated infectious or metabolic diseases ($p < 0.001$). However, unlike TF levels, AO and C5 serum levels were depressed in cattle resistant to bTB with a simultaneous disease (bTB-IMD). Our observations suggest that AO, C5, and TF are reliable markers of a protective host response against bTB and that AO and C5 levels are negatively affected by other pathologies present in cattle resistant to *M. bovis*, like *M. paratuberculosis* infection, and other unrelated bacterial and viral infections or metabolic diseases. Seth et al. (30) reported that the high levels of apolipoproteins in sera of contemporary unexposed controls were depressed in sera of animals after 10 months of infection with *M. paratuberculosis*. Gao et al. (31) reported that the high levels of serum amyloid A and alpha-1-acid glycoprotein in sera of uninfected controls decreased significantly in *M. bovis*-infected cattle. They also observed that serum levels of TF and C-Reactive protein (CRP), an activator of the classical complement pathway, had no significant differences between *M. bovis*-infected cattle and uninfected controls. However, CRP levels in the PPD-B-stimulated blood of *M. bovis*-infected cattle were significantly higher than those in uninfected controls, while TF levels were lower than the unstimulated blood. These authors suggested that PPD-B may activate acute-phase proteins, increasing CRP and decreasing TF (31).

Interestingly, in our study, C5 and TF serum values were similar ($p > 0.05$) between cattle resistant to bTB (bTB-) and the healthy group (HS), while AO levels in sera of these healthy animals were significantly lower compared to those of the bTB- group. Using the cutoff values of our ELISA tests for C5 and TF, more than half of HS animals would be resistant, despite not having been exposed to *M. bovis*. On the opposite, using the AO cutoff values, all HS animals would be susceptible. It is hard to interpret the variation in AO, C5, and TF levels in these not exposed cattle. Although HS cattle were randomly selected from bTB-free, paratuberculosis-free, and brucellosis-free dairy herd, the possibility of exposure to other viruses or

bacteria, like environmental mycobacteria, may lead to these variations in the levels of AO, C5, and TF, cannot be excluded. As discussed above, non-specific responses may be related to particular mycobacterial infections or other pathogens. Previous studies have shown that the levels of specific serum proteins are dynamic along with the *M. bovis* infection (30). Our results suggest that the combinational use of these potential biomarkers rather than alone may better determine the resistance phenotype in cattle. Whether the level of these proteins are only markers of the animal status (resistance to *M. bovis* infection) or are indicative of the intensity of the effector response, those are singularly related to the protective response against *M. bovis* infection, and their effect on bTB requires further investigation.

Bovine plasma amine oxidase (AO), also known as bovine serum amine oxidase (BSAO), is a liver-expressed Cu^{2+} containing enzyme, member of the class of copper-containing amine oxidases (AOC) (55). Plasma AOs are considered key enzymes in cell growth and differentiation processes. AO occurs circulating in plasma apparently due to the proteolytic cleavage of the membrane-bound form (55). The highly structural similarities between bovine AO (E1BJN3) and bovine primary amine oxidase—liver isozyme (AOCX, Q29437; 90.81% identity), bovine primary amine oxidase—lung isozyme (O46406; 82.1% identity), and bovine membrane primary amine oxidase, copper containing 3 (AOC3, Q9TTK6; 80.3% identity), strongly support this theory. Indeed, the copper amine oxidase gene encoding BSAO expressed in the bovine liver is different from, but closely related to, the copper amine oxidase gene expressed in the bovine lung, kidney, spleen, and heart (56). AO preferentially catalyzes the oxidative deamination of primary amines; thus, it is referred to as primary-amine oxidase (EC 1.4.3.21) (57). BSAO also catabolizes polyamines (putrescine, spermidine, and spermine) to produce hydrogen peroxide, aldehyde, acrolein, and ammonia (58). These, in turn, can act as antimicrobial agents and signaling molecules that contribute to leukocyte adhesion and cytotoxicity of drug-resistant cancer cell lines (58). Oxidative degradation of polyamines by bovine AO might restore immune response by avoiding M2 polarization and enhancing inducible nitric oxide synthase (iNOS), NO production, and NO-mediated bacterial killing in activated macrophages (59); however, further studies need to be addressed. AOC3 is also known as vascular adhesion protein-1 (VAP-1), a membrane-bound protein that mediates the slow-rolling and adhesion of lymphocytes to endothelial cells (60). AOC3/VAP-1 also contributes to the extravasation of neutrophils, macrophages, and lymphocytes to sites of inflammation and transiently contributes to the antigen-specific CD4+ T-cell traffic to secondary lymphatic tissues (61). AOC3/VAP-1 participates in developing pulmonary inflammation and fibrosis by regulating the accumulation of pathogenic leukocyte subtypes (62, 63). However, the dispersal of effector CD4+ T cells to lung parenchyma or airway mucosa is AOC3/VAP-1 independent (61). Bovine AOC3 has been identified as being upregulated at least two-fold in MAP-immunoreacting cows (64). However, its role in bTB is yet to be determined.

The complement system is a complex enzymatic cascade (consisting of more than 30 proteins in blood plasma) that functions in stepwise activation of several proteases produced by the liver, adipose tissue, leukocytes, and vascular cells (65). C5a and C5b products generated upon the cleavage of C5 by C3/C5 convertases have essential biological activities. C5b, the larger cleavage product of C5, initiates the formation of the cytolytic complex (C5b-9) that causes lysis of bacteria and pathogens, whereas C5a, the smaller product, is a strong chemotactic and spasmogenic anaphylatoxin that mediates inflammatory responses by stimulating neutrophils and phagocytes to the site of injury or infection (66). Downregulation of the complement factor C5 has been detected in dairy cows over-conditioned around calving during the transition period and in dairy cows exposed to heat stress, suggesting that immune function is impaired in these cattle (65, 67). To the best of our knowledge, evaluations of the protein expression of the complement component C5 of dairy cows with bTB are mainly unexplored. The role of complement factor C5 and its association with *M. bovis* infection in dairy cows represents an attractive possibility to be investigated in the future.

Bovine TF is a single-chain ß-globulin that is important for iron (Fe) transport in blood plasma and is mainly produced by hepatocytes, macrophages, and other cells (68). Intraphagosomal *M. tuberculosis* can acquire iron from both extracellular TF and endogenous macrophage sources, except that iron acquisition from macrophage cytoplasmic iron pools may be critical for the intracellular growth *of M. tuberculosis* (69). TF in the circulating plasma and leukocytes play essential roles in reducing iron availability to the pathogen by their high affinity for Fe3+ (70). Iron requires to be complexed to TF for delivery into cells; iron is known to play a role in the immune response to pathogens. It is known that low intracellular iron availability enhances iNOS transcription; Nramp1 can enhance NO formation and other pro-inflammatory immune pathways via modulation of iron homeostasis (71). Such is supported by published data showing a decrease in the TF serum levels of patients with active TB compared to patients with latent TB or healthy controls (72). However, some studies have indicated that overexpression of TF could be a specific biomarker for the diagnosis of Johne's disease (64). Plasma TF concentrations likely vary in cows with Johne's disease depending on the stage of MAP infection, and elevated TF levels may be compensating for impaired iron uptake across the damaged intestinal epithelium in chronic paratuberculosis (64).

In ruminants, serotransferrin (TF) is typically classified as negative acute-phase protein (APP) during acute infections and acute metabolic diseases (73, 74). Chronic infectious diseases of cattle, such as Johne's disease and bovine viral diarrhea, and acute infections by *Haemophilus somnus* or *Trypanosoma vivax*, were characterized by the relatively low TF serum levels in affected individuals compared to healthy controls (68, 75, 76). In contrast, administration of endotoxin, or during ketosis, did not induce changes in TF concentrations (68). In agreement with our study, levels of TF decreased significantly in bovines susceptible to ticks (Holstein, *Bos taurus taurus*) during heavier infestations of *Rhipicephalus microplus*. In contrast, there was no significant decrease in the serum concentration of TF in tick-resistant cattle (Nelore, *B. taurus indicus*) compared to susceptible cattle (77). Low basal levels of TF in tick-resistant

animals may reflect that less free iron is available in them than tick-susceptible animals (77). Increased levels of TF in serum of active human TB cases compared to healthy controls appears to be a compensatory mechanism influenced by nutritional deficiencies (78). TF overexpression in human TB and other infectious diseases is more likely linked to protective immune responses, while its downregulation is more often related to immune deficiencies (79). Blood plasma TF levels were higher in patients with drug treatable HIV than patients with drug-resistant HIV; this also correlated with virological status and immune parameters such as CD4 counts (80).

In conclusion, we have identified three significantly overexpressed serum proteins which could be used as potential biomarkers to determine resistance to bTB in diary Holstein-Friesian Mexican cattle. These proteins are involved in inflammatory/immunomodulatory responses to infections and may provide a novel avenue of research to determine the mechanisms of protection against bTB. Using validated serum biomarkers to identify and further enhance the resistance of cattle to bTB implemented as part of bTB control strategies could help eventually eradicate bTB from herds by reducing the susceptibility to infection. However, due to the low number of individuals gathered for validation, our results require further investigation to determine the extent to which these results could apply to cattle of different breeds and in different circumstances. In addition, it is clear that the ELISA requires further development and that, ideally, these markers could be evaluated in a multiplex system using perhaps monoclonal antibodies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXChange MassIVE MSV000087778.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Ethics and Biosafety Committee. Written informed consent for participation was not obtained from the owners because owners gave their verbal consent for sampling of animals previous to slaughter at the abattoir facilities.

AUTHOR CONTRIBUTIONS

JM-C, OA-L, BV-R, JG-P, and HE-S contributed to the conception and design of the study. HE-S acquired funding, coordinated the work, performed the statistical analysis, and prepared the final draft of the manuscript. OA-L and HE-S organized the database and conducted laboratory examinations. GL-R and HE-S collected clinical samples and conducted gross examinations. JM-C, OA-L, and HE-S performed the experiments. BV-R, JG-P, GL-R, and HE-S wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version, except OA-L and JM-C who lamentably departed before the final draft was achieved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.734087/full#supplementary-material

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Utility of the Intradermal Skin Test in a Test-and-Cull Approach to Control Bovine Tuberculosis: A Pilot Study in Ethiopia

Matios Lakew[1,2†], Sreenidhi Srinivasan[3†], Beruhtesfa Mesele[4], Abebe Olani[1], Tafesse Koran[1], Biniam Tadesse[1], Getnet Abie Mekonnen[1], Gizat Almaw[1], Temertu Sahlu[4], Bekele Seyoum[4], Kebede Beyecha[4], Balako Gumi[2], Gobena Ameni[2,5], Hagos Ashenafi[2], Douwe Bakker[6], Vivek Kapur[3,7] and Solomon Gebre[1]*

[1] National Animal Health Diagnostic and Investigation Center, Sebeta, Ethiopia, [2] Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia, [3] Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA, United States, [4] Alage Agricultural Technical and Vocational Education Training (ATVET) College, Alage, Ethiopia, [5] Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, United Arab Emirates University, Al Ain, United Arab Emirates, [6] Independent Researcher and Technical Consultant, Lelystad, Netherlands, [7] Department of Animal Science, The Pennsylvania State University, University Park, PA, United States

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***Correspondence:**
Matios Lakew
matioslakew@gmail.com

[†]These authors have contributed equally to this work

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Bovine tuberculosis (bTB) is one of the top three, high-priority, livestock diseases in Ethiopia and hence, the need for evaluation of potential control strategies is critical. Here, we applied the test-and-segregate followed by cull strategy for the control of bTB in the intensive Alage dairy farm in Ethiopia. All cattle reared on this farm were repeatedly skin tested using the Comparative Cervical Tuberculin (CCT) test for a total of five times between 2015 and 2021. During the first (October 2015) and second (March 2017) rounds of testing, all reactor animals (>4 mm) were culled, while those that were deemed as inconclusive (1–4 mm) were segregated and retested. At retest, animals with CCT >2 mm were removed from the herd. In the third (December 2017) and fourth (June 2018) rounds of tuberculin testing, a more stringent approach was taken wherein all reactors per the severe mode of CCT test interpretation (>2 mm) were culled. A final herd status check was performed in May 2021. In summary, the number of CCT positives (>4 mm) in the farm dropped from 23.1% (31/134) in October 2015 to 0% in December 2017 and remained 0% until May 2021. In contrast, the number of Single Cervical Tuberculin (SCT) test positives (≥4 mm) increased from 1.8 to 9.5% (from 2017 to 2021), indicating that CCT test might not be sufficient to effectively clear the herd of bTB. However, a more stringent approach would result in a drastic increase in the number of false positives. The total cost of the bTB control effort in this farm holding 134–200 cattle at any given time was conservatively estimated to be ~US$48,000. This, together with the need for culling an unacceptably high number of animals based on skin test status, makes the test-and-cull strategy impractical for nationwide implementation in Ethiopia and other low- and middle-income countries (LMICs) where the infection is endemic. Hence, there is an increased emphasis on the need to explore alternate, affordable measures such as vaccination alongside accurate diagnostics to help control bTB in endemic settings.

Keywords: bovine tuberculosis, control program, Ethiopia, prevalence, test and cull

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic, progressive, granulomatous, inflammatory disease of cattle caused by members of the *Mycobacterium tuberculosis* complex (MTBC) (1). Most high-income countries have successfully controlled bTB based on test-and-slaughter of skin test-positive animals, alongside slaughterhouse surveillance, and trade and movement restrictions of affected herds. However, complete eradication is a challenge due to potential for spillover from wildlife reservoir hosts and imperfect performance of the currently available diagnostic tests (1–3). Conversely, in most low- and middle-income countries (LMICs), the bTB prevalence is high, and implementation of a test-and-slaughter program is not affordable for both social and economic reasons and, as a result, the disease has continued to cause significant economic and public health impacts (4). Moreover, the World Health Organization (WHO) estimates that of ∼10 million new cases of human TB reported globally in 2018, ∼71,000 to 240,000 were of zoonotic origin, meaning caused by *Mycobacterium bovis* alone (5). It is important to note that this is likely an underestimate due to the lack of cross-sectional, national-level surveillance programs, under-reporting and lack of laboratory confirmation of causative agents in LMICs where the infection is endemic in both human and bovine populations (5). Hence, it is being increasingly recognized that eliminating TB in humans cannot be accomplished without first controlling bTB in cattle.

A systematic review and meta-analysis conducted on the prevalence of bTB in Ethiopia reported a pooled prevalence estimate of 5.8% (95% CI: 4.5–7.5%) (6). The report indicated that bTB is highly prevalent in intensive and semi-intensive dairy farms in the country. However, so far there is no national bTB control strategy in Ethiopia, hence, this situation favors the unregulated movement of bTB-infected cattle and further spread of infection (7). Moreover, given the increased emphasis placed on expansion of intensive dairy farms with genetically improved dairy cattle to meet economic demands and nutritional requirements, the prevalence of bTB is only likely to worsen in the coming years. Hence, there is an urgent need for the implementation of a control program for bTB to reduce its prevalence in livestock and its zoonotic impact in Ethiopia (8, 9).

The tuberculin skin test is the current standard method for diagnosis of bTB recommended by the World Organization for Animal Health (OIE) (3). This test probes the cell-mediated immune response upon intradermal injection of the stimulating antigens such as purified protein derivatives (PPD) of tuberculin. In Ethiopia, the standard test for the diagnosis of bTB is the Comparative Cervical Tuberculin (CCT) test, which involves injection of two stimulating antigens, bovine PPD (PPD-B) derived from the extract of the *M. bovis* AN5 strain and avian PPD (PPD-A) derived from the extract of *M. avium* subsp. *avium* D4ER. The CCT is used to improve test specificity, although at the cost of sensitivity when compared with the use of the Single Cervical Tuberculin (SCT) test applying PPD-B only.

Here, we describe efforts involved in controlling the prevalence of bTB in a high burden voluntary dairy farm, owned by Alage Agricultural Technical and Vocational Education Training (ATVET) College in Ethiopia. An iterative strategy of repeated test-and-segregation followed by culling of reactors was implemented and followed for a period of 6 years. We also conservatively estimated the costs associated with this program.

Abbreviations: ATVET, Agricultural Technical and Vocational Education Training; BCG, Bacille Calmette-Guerin; bTB, bovine tuberculosis; IGRA, interferon gamma release assay; LMICs, low- and middle-income countries; OIE, World Organization for Animal Health; CCT, Comparative Cervical Tuberculin; MTBC, *Mycobacterium tuberculosis* complex; NAHDIC, National Animal Health Diagnostic and Investigation Center; SCT, Single Cervical Tuberculin; WHO, World Health Organization.

MATERIALS AND METHODS

Study Location

The study was conducted from 2015 to 2021 at the dairy farm of Alage ATVET College. This college occupies a total area of 4,200 ha, located at longitude of 38°30′ east and latitude of 07°30′ north. The college is situated about 217 km southwest of the capital city, Addis Ababa. It is part of the dry plateau agro-ecology of the southwestern part of the Ethiopian Rift Valley system at an altitude of 1,590 m above sea level. This dairy farm was established in 1979 with a starting population of 300 Holstein Friesian (HF) heifers from different sources and all animals have been exclusively stall-fed under a closed intensive management system. As part of farm biosecurity measures, new exotic breed animals have not been included since establishment. However, local Boran breed cattle are introduced for cross breeding purposes after screening for bTB. The farm is not in close proximity with any other dairy farms or wildlife populations. During the final round herd test (May 2021), the farm had a total of 175 cattle, of which 136 were HF-zebu cross, 33 were local Boran breed and 6 were calves <3 months of age. The dairy farm uses artificial insemination system for breeding and has its own veterinary clinic for animal health issues.

Study Design

As part of our efforts to clear bTB from the Alage dairy farm, a repeated cross-sectional study was conducted whereby all animals (with the exception of calves <3 months of age, third trimester pregnancy (>8 months) and sick animals not suggestive of bTB), were skin tested with PPD-B and PPD-A for a total of five times between 2015 and 2021. The first round of tests was conducted in October 2015, the second in March 2017, the third in December 2017, and the fourth in June 2018. Finally, the current status of the farm was checked more recently in May 2021 using both CCT test and interferon-gamma release assay (IGRA). The testing interval between each round was not uniform but at least a 6 month gap was considered between rounds for logistical reasons. In the first and second rounds of testing, all animals testing positive (PPD-B–PPD-A > 4 mm) were slaughtered, while those in the inconclusive range (PPD-B–PPD-A = 1–4 mm) were segregated to a different location (∼1 km away from the main herd with independent management) and retested 42 days later. At retest, animals with skin thickness >2 mm were slaughtered, and non-reactors were reintroduced into the main

herd. Animals with skin thickness ranging from 1 to 2 mm at retest were kept segregated (until the next round of testing) instead of slaughter. Here, we note that this deviates from the OIE guidelines for retesting that recommends culling of all non-negative animals ($\geq$1 mm) (3). However, including animals with skin thickness ranging from 1 to 2 mm dramatically increased the total number of animals to be culled and was not practically implementable in this dairy farm. In the third and fourth rounds of CCT testing, a more stringent approach was followed wherein all the tested animals with increase in skin thickness of >2 mm by CCT test were slaughtered and no retesting was conducted. Animals that showed 1–2 mm increase in skin thickness during the third round of test were segregated until fourth round test, while those from the fourth round remained in the herd. Three years later, the farm was tested again (May 2021) with CCT test and IGRA. It was recommended to the farm to segregate all animals that were inconclusive (1–4 mm) per CCT test, reactor ($\geq$ 4 mm) per SCT test, and positive per IGRA (optical density $\geq$ 0.1).

Skin Test Procedures

The CCT test was performed following the OIE guidelines (3, 10). Bovine PPD (3000IU/dose) and avian PPD (2500IU/dose) were administered intradermally with the bevel edge of needle faced outwards (0.1 ml final volume; Prionics/ThermoFisher Scientific, Lelystad B.V., The Netherlands). The same operator measured the skin thickness just before and 72 $\pm$ 4 h after the PPD injection. The difference in the increase of skin thickness at the PPD-B and PPD-A sites before and after injection was calculated. A reaction was considered positive if the increase in skin thickness at PPD-B site of injection was more than 4 mm greater than the reaction shown at the site of the PPD-A injection. The reaction was considered inconclusive if the PPD-B minus PPD-A reaction was from 1 to 4 mm, and negative if the PPD-B minus PPD-A reaction was <1 mm (3). During the third and fourth rounds of testing, all animals with skin thickness >2 mm by CCT test were considered positive per the severe mode of interpretation (11). In the SCT test (which involves PPD-B only), the reaction was considered positive if there is an increase of 4 mm or above in skin-fold thickness and inconclusive if the increase in skin-fold thickness is more than 2 mm and <4 mm. However, SCT results were not used for decisions on culling of animals.

Interferon Gamma Release Assay

The IGRA test was conducted using the commercially available Gamma interferon test kit for Cattle (BOVIGAM^{TM}, Prionics). Whole blood samples were collected using lithium heparin vacutainer tubes. The blood samples were stimulated with PPD-B and PPD-A, at a final concentration of 300 and 250 IU/ml, respectively, and were incubated in a humidified atmosphere at 37°C and 5% CO2 for 16–24 h. In addition, RPMI1640 media with L-Glutamine (BioWhittaker, Lonza, USA) and Pokeweed Mitogen (PWM) (Sigma-Aldrich) at 10 ug/ml final concentration were also used to stimulate the whole blood as negative and positive controls, respectively. Following overnight incubation, plates were centrifuged at 300 g for 10 min at room temperature and the supernatant was harvested. The harvested plasma was tested using the BOVIGAM ELISA kit to detect the secretion of interferon gamma (IFN-γ) from the stimulated *T* cells (12–14). Procedure was carried out as per kit instructions and the absorbance was measured at 450 nm with an ELISA reader. A reaction was considered positive if the optical density (OD) value of avian PPD subtracted from bovine PPD is $\geq$0.1.

Cost Estimation

The costs incurred for implementation of the test-and-slaughter program at the Alage dairy farm was calculated in order to gain some insights into the overall expenses should such a program be implemented at national level in Ethiopia. This included the expenses associated with repeated testing of the animals for five rounds, reagents and relevant consumables, loss to the farm associated with culling (price of culled animals estimated per market price), personnel per diem and staff transportation costs. The meat of carcasses was officially inspected and those approved for consumption were sold at a price of 40 ETB (US$0.9) / kg and hence the income gained from selling the meat was considered as salvage value and deducted from the total cost.

Data Analysis

All statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA) and Statistical Product and Service Solutions (SPSS) version 20. The chi-square (χ^2) test was used for comparing prevalence estimates and $P < 0.05$ was considered for significance.

RESULTS

Repeated Testing of the Alage Dairy Farm

The first round of testing ($n = 134$; all HF-Zebu cross breeds) was carried out in October of 2015 and the bTB within-herd prevalence was 23.1% (31/134) per the CCT test (skin thickness > 4 mm). All thirty-one reactor animals were culled, while animals presenting as inconclusive (1–4 mm; $n = 30$) were segregated in a different barn located 1 km away from the main Alage herd. Twenty-six (of the thirty) animals with inconclusive results were retested after 42-days of the first test, while the remaining four animals with inconclusive test results were exempted from the retesting as they were either under medical care or at late stages of pregnancy (**Table 1**).

The second-round test ($n = 173$: 109 HF-Zebu cross and 64 Boran breed cattle) was conducted in March of 2017. The 64 local Boran cattle were not tested in the previous round and a decision was made to include them in this round of testing as they were managed in close proximity from the main Alage herd and to avoid any resulting spillover of infection. The prevalence of bTB in this round was found to be 4.6% (8/173), which was significantly lower than the prevalence observed in the first round ($\chi^2 = 23.3$; $P < 0.0001$). It is important to note that all the skin test-positive animals were among the newly included local Boran cattle. Seventeen animals (10 HF cross and 7 local Boran breed) showed inconclusive test results; 15 of them were retested and one (local Boran breed) showed positive skin reaction to the CCT

test (**Table 1**). The reactor animal was culled, while the other animals were returned to the main Alage farm.

During the third-round test conducted in December 2017 ($n = 169$), there were no reactor animals per the CCT test (>4 mm) but 7 (4.1%) animals were inconclusive (1–4 mm). When severe mode of interpretation (>2 mm) was employed, one (HF cross breed) animal tested positive to the CCT test and it was slaughtered. The inconclusive animals were segregated from the main herd until the next round of testing. Compared to the prevalence in the second round of testing, the prevalence in the third round decreased significantly ($\chi^2 = 7.936$; $P = 0.0048$) as well.

The fourth-round test was carried out in June of 2018 ($n = 181$) and the CCT test result showed no bTB reactor animal in the herd per the > 4 mm interpretation. There were, however, 16 (8.8%) animals (5 HF cross and 11 local Boran cattle) that were classified as inconclusive per the CCT test (**Table 1**), of which four animals were slaughtered as they were positive by the severe mode of interpretation.

In order to evaluate the success of the control program implemented on the Alage farm from 2015 to 2018, the National Animal Health Diagnostic and Investigation Center (NAHDIC) team returned to the farm 3 years later in May of 2021 to conduct a fifth round of testing ($n = 169$). Both skin test and IGRA were performed. Encouragingly, there were no reactors identified per the CCT test interpretation of > 4 mm. However, 8 (4.7%) animals (7HF and 1 local Boran breed cattle) were inconclusive (1–4 mm). Moreover, 2 animals were categorized as reactors by the severe mode of interpretation (>2 mm). Per the SCT test, 16 animals were test-positive (≥ 4 mm).

In the IGRA, three animals had crossed the cut-off (optical density ≥0.1) (**Figure 1**). One of these three IGRA-positive animals was also positive per the severe mode of interpretation of CCT, while the other two are either positive or inconclusive per the SCT test. All animals that were inconclusive on CCT (1–4 mm), reactor on CCT per severe interpretation (>2 mm), reactor per SCT (≥ 4 mm) test and IGRA (optical density ≥ 0.1) were segregated from the herd ($n = 21$).

Overall, following the repeated test-and-slaughter approach, the number of CCT positives (>4 mm) in the farm dropped from 23.1% (31/134) in October 2015 to 0% in December 2017 and remained 0% until May 2021. From **Table 1**, one can note that at all time points the number of test-positive animals per SCT (≥4 mm) was greater than that of CCT test, per both standard and severe interpretations (>4 mm and >2 mm). At the first testing round, the number of SCT test positives was 40.3%, which then dropped to 10.4% during the second round of testing. The lowest prevalence per SCT test was recorded during the third round (1.8%), after which it increased to 2.2% during the fourth round and then to 9.5% at the fifth-round test (**Table 1** and **Figure 2**).

Cost Analysis

The total cost of this program was conservatively estimated at ∼US$ 48,000; the breakdown of each included expense item is provided in **Table 2**. The cost associated with skin testing alone, which includes cost of reagents and logistics for personnel was estimated to contribute ∼18% to the total cost, while the major cost of the program (∼82%) was associated with culling/slaughtering of reactor animals, i.e., estimated selling price of the culled/slaughtered animals. In total, 46 bTB reactor animals were slaughtered, of which 39 were conditionally approved for human consumption, and the salvage value of these animals was deducted from the total costs. However, the carcasses of the remaining seven animals were totally condemned with no salvage value (**Table 2**). All the expenditure of the fifth-round skin and IGRA test is also summarized and included to estimate the total cost.

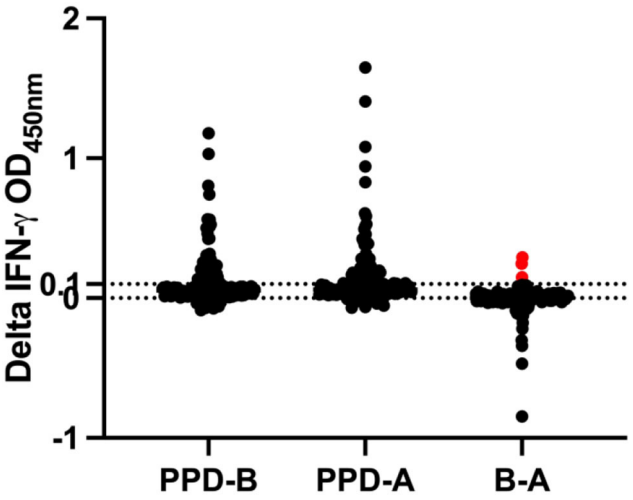

FIGURE 1 | IGRA result during fifth-round test. Samples are positive if the OD value of PPD-A deducted from PPD-B is ≥0.1. The horizontal line at 0.1 denotes the cut-off. The red data points on B-A cross the cut-off of 0.1 and hence are reactors by IGRA.

DISCUSSION

Despite being a disease of economic and zoonotic importance, control measures for bTB are non-existent in Ethiopia and most other LMICs (4, 7). Earlier efforts to control bTB in a government farm in Ethiopia showed that upon repeated skin testing and segregation of skin test positive animals, the prevalence of bTB could be dramatically reduced from 48 to 1% in a dairy cattle herd (15). In the current study, NAHDIC in collaboration with Alage ATVET College implemented a bTB control strategy in the dairy farm affiliated with the college. The approach involved repeated tuberculin testing of the dairy herd with subsequent segregation and slaughter of test-positive animals from the farm. Accordingly, the entire cattle herd was tested four times between the years 2015 and 2018, with the exception of animals <3 months of age, animals >8 months pregnant, and those that were sick but not suggestive of bTB. All test-positive animals to the CCT test were removed, while animals categorized as inconclusive in the first and second rounds of testing were segregated and retested after 42 days. All positive animals in the retest were slaughtered. This test and segregate followed by

TABLE 1 | Summary of all skin tests conducted in the Alage dairy farm between 2015 and 2021.

Test Date	Oct, 2015	March, 2017	December, 2017	June, 2018	May, 2021
Test round	**1st**	**2nd**	**3rd**	**4th**	**5th**
Number of animals tested	134	173	169	181	169
Test interpretation			**Number of test positives (%)**		
CCT (PPDb-PPDa > 4)	31 (23.1)	8 (4.6)	0 (0.0)	0 (0.0)	0 (0.0)
CCT (PPDb-PPDa > 2)	46 (34.3)	15 (8.7)	1 (0.6)	4 (2.2)	2 (1.2)
CCT (PPDb-PPDa ≥ 1)	61 (45.5)	25 (14.45)	7 (4.1)	16 (8.8)	8 (4.7)
SCT (PPDb ≥ 4)	54 (40.3)	18 (10.4)	3 (1.8)	4 (2.2)	16 (9.5)
SCT (PPDb > 2)	93 (69.4)	36 (20.8)	8 (4.7)	25 (13.8)	23 (13.6)
Number culled	31	8	1	4	–
Number inconclusive (CCT, 1–4 mm)	30	17	7	16	8
Number retested	26	15	–	–	–
Number culled following retest	1	1	–	–	–

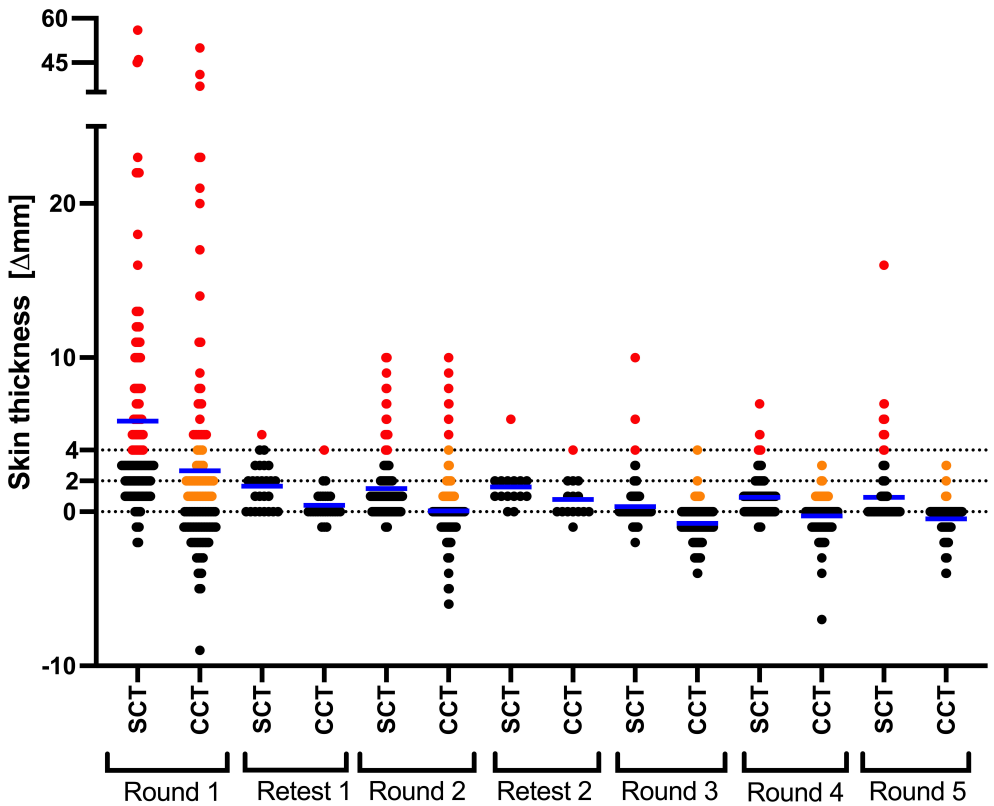

FIGURE 2 | CCT and SCT test responses in every round of testing. Results are expressed as the difference in skin thickness (in millimeters) between the pre- and post-skin test readings, with the horizontal line providing the median (shown in blue). Black, orange and red data points represent animals that tested negative, inconclusive and positive, respectively. Retests in round 1 and 2 were conducted only on the animals in the doubtful range (orange).

retesting and slaughter approach over a 3-year period drastically decreased the within-herd prevalence of bTB from 23.1 to 0%, although a few animals that were identified as inconclusive per the CCT remained in the herd.

In order to check the status of the farm, the NAHDIC team returned to Alage 3 years later in May 2021. This time both skin test and IGRA were performed. Encouragingly, no animal was positive per the > 4 mm interpretation of CCT. However, there were a total of twenty-one animals that were reactors per SCT test and IGRA, and inconclusive per CCT test, that were segregated from the main herd. This is not surprising given the fact that the Alage farm was never made completely free of bTB as inconclusive animals identified in June 2018 continued to be a part of the farm. Starting from the third round (December 2017)

TABLE 2 | Conservative estimates of the cost associated with the bTB control efforts at Alage dairy farm.

Category	Items	Details	Unit price (ETB)	Cost (ETB)	Cost (USD)
Skin testing	Reagents	PPDs for 698 animals	100	69,800	2,094
		Tuberculin syringe	140	1,960	58
		Gloves, alcohol, blade and other consumables	246	3,400	102
	Personnel cost	4 people / 7 days for each test round	600 / day	100,800	3,024
		Fuel and travel (600 km per trip)	20 / liter	20,000	600
Total				195,960	5,878
Handling of infected animals	HF cross breed cow	33 culled	40,000	1,320,000	39,600
	Local breed cow	13 culled	15,000	195,000	5,850
	Cost to manage segregated animals	No new barn constructed; no new animal attendant employed			–
Total				1,515,000	45,450
Total cost of the program (testing + culling of reactor animals)				1,710,960	51,328
Salvage value	Meat recovered from 39 animals	∼131.5 kg / animal	40 ETB / kg meat	205,140	6,154
Total cost of fifth round skin and IGRA test				122,740	2,860
Grand total				1,628,560	48,034

Exchange rate used for estimating the cost of skin test from first to fourth round was 33.33 Ethiopian Birr (ETB) for one US$, based on the exchange rate dated April 27, 2020. Exchange rate used for estimating the cost of the fifth-round test was 42.9243 Ethiopian Birr (ETB) for one US$, based on the exchange rate dated May 21, 2021.

to the fifth-round test (May 2021) all the animals were negative per the CCT > 4 mm interpretation, however the number of PPD-B reactors (SCT > 2 mm and $\geq$ 4 mm) did increase in the last testing rounds. This may indicate that CCT > 4 mm or even the severe interpretation CCT > 2 mm may not be sufficient in certifying previously bTB infected herds as bTB-free herds. Hence, further attention should be given to the development of alternative diagnostic reagents to reduce the number of false positives, uncertainties associated with the PPD skin test, and limit conflicts with herd owners. It is important to acknowledge here that bacteriological culture and confirmation of reactor animals could not be performed and is a limitation of the study. While the number of animals positive per SCT was high, it is also important to note the relative low specificity of this test, in particular in the context of regions with high environmental bacteria such as Ethiopia, further stressing the need for more accurate and specific defined diagnostic reagents (8, 10, 16). The increase in the number of reactor animals due to the interruption in routine testing between June 2018 and May 2021 indicates that expenses involved in the control of bTB are anything but short-term in nature. Despite having culled a total of 46 cattle, there were still some inconclusive animals in the final round, suggesting that extant tuberculin-based tests may not be sufficient tools for accomplishing disease eradication in endemic settings and new defined antigen tests are an urgent need (16).

As has been proven time and again, test-and-cull-based programs can be hugely successful in efficiently controlling bTB (17). The rigorous application of tuberculin testing and culling of reactors has significantly reduced the prevalence and even eliminated bTB infection from farmed bovine populations in many high-income countries in the past (3, 18–20). For example, in the Unites States (US), a test-and-cull-based control program was initiated over a century ago in 1917, and together with introduction of milk pasteurization, this control program has been regarded as one of the most successful campaigns ever waged against a bacterial disease. Between 1917 and 1940, ∼232 million cattle were screened, of which, ∼3.8 million were culled. Documented reports show a 10-fold return on investment in lives saved and economic benefits to farmers in the US (21). However, can such a test and cull approach be practically implemented at a national level in Ethiopia and other LMICs? While the results of this study in terms of reduction in disease prevalence are encouraging, conservative cost estimates suggest that the total expenditure during the 3-year effort and the final fifth round test in a farm holding <200 cattle was ∼US$48,000. It is important to note that this cost estimate does not include the milk losses associated with culling of lactating cows, possible meat productivity loss, additional housing and management costs to segregate test-inconclusive animals, and the cost of social and zoonotic impacts. The national priorities for intensification of dairy production in countries like Ethiopia will only worsen the disease burden resulting in further increase in economic losses due to bTB. Ethiopia has the largest cattle population in Africa of ∼61.5 million heads (22) and applying a conservative pooled prevalence estimate of 5.8% (95% CI: 4.5 to 7.5%) as reported in a recent meta-analysis, there may be ∼3.5 million bTB-infected cattle in the country. While we acknowledge that additional studies are required to accurately predict the costs and benefits of implementing a national level test and cull-based control strategy, it is quite apparent that a program of such a scale is far from feasible in Ethiopia and other LMICs for both economic and social reasons. Moreover, there are multiple complications in adapting this approach in non-dairy herds and

pastoral production systems given extensive management styles and cultural reasons.

In this context, it is important to highlight the need for a vaccine that can reduce the burden of infection and transmission. Recent reports including natural transmission studies conducted in endemic settings have shown that Bacillus Calmette-Guérin (BCG) vaccination may have considerable utility in this regard (23). While BCG has been used for experimental vaccination of cattle against bTB since 1913, it is not yet licensed for field use as it sensitizes animals to the tuberculin-based skin tests (3, 24). This compromises the specificity of the skin test and results in an inability to differentiate infected from vaccinated animals (DIVA). Therefore, bTB control programs that use the OIE-prescribed tuberculin skin test also prohibit the use of BCG vaccination. However, safety and BCG-induced protection (although partial) of cattle against experimental challenge with *M. bovis* has been known since the promising early reports published by Calmette and Guérin. A recent meta-analysis that attempted to synthesize data from published studies across the globe estimated a direct BCG vaccine efficacy of 25% (95% CI: 18, 32) (25). While no study has yet rigorously assessed indirect vaccine efficacy, a significant reduction in lesion severity observed following BCG vaccination likely contributes to reduction in risk of transmission from vaccinates to susceptible cattle (indirect efficacy). It was also emphasized through scenario analyses with transmission dynamic models incorporating direct and indirect vaccinal effects ("herd-immunity"), that BCG vaccination alongside a DIVA diagnostic test appears to be the most promising option in endemic settings in the near future. However, this warrants detailed cost-benefit analyses prior to rolling out BCG vaccination in endemic settings. In this context, progress has been made in the field of bTB diagnosis in the development and validation of peptide-based skin test reagents comprising of antigens that are present in pathogenic strains of MTBC and absent in the vaccine strain, BCG (16, 26). The DIVA capability of these antigens has also been successfully demonstrated in both experimental and naturally infected animals, hence enabling the use of BCG vaccination as part of future bTB control programs (27, 28). It is important to note here the ease of chemical synthesis, quality control and cost-effectiveness of these peptide-based reagents.

Taken together, while the repeated test and cull approach is now being implemented at some voluntary government and private dairy farms in Ethiopia, it may not be a viable option at the national level for reasons discussed here. Hence, feasible alternatives need to be sought urgently to control bTB, an important step towards the WHO goal of ending the global tuberculosis epidemic by 2035 (29). Vaccination against bTB with BCG has been researched for over a century and provides strong evidence for the consideration of implementation of vaccine-based bTB control strategies, particularly in LMICs and other high burden settings. While the reported direct efficacy estimates are modest, it is important to note that indirect efficacy of BCG remains largely unexplored and that further investigations are needed to address this critical knowledge gap. Given the predicted intensification of cattle herds in endemic settings, these observations will have major implications for informing and implementing practical disease control policies in Ethiopia and other LMICs.

CONCLUSION

This study showed that the test and cull program significantly reduced the prevalence of bTB from Alage ATVET dairy farm within 3 years. However, the cost estimation indicates that it may not be practical to scale-up and implement nationwide in Ethiopia and other LMICs. Hence, exploring feasible and economically affordable alternative bTB control options such as test-and-segregate approaches, and vaccination need priority.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by National Research Ethics Review Committee-NRERC No. 3.10/800/07.

AUTHOR CONTRIBUTIONS

ML, BM, AO, TK, GAl, SG, TS, and KB conceived and designed the study. ML, BM, AO, TK, BS, and GAm conducted the research. ML, AO, BT, BG, GAl, HA, DB, VK, and SS analyzed the data, drafted the paper, and contributed to writing. All authors read and approved the final manuscript.

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Bovine Tuberculosis Antemortem Diagnostic Test Agreement and Disagreement in a Naturally Infected African Cattle Population

Robert F. Kelly[1], Lina Gonzaléz Gordon[1], Nkongho F. Egbe[2], Emily J. Freeman[1], Stella Mazeri[1], Victor N. Ngwa[3], Vincent Tanya[4], Melissa Sander[5], Lucy Ndip[6], Adrian Muwonge[1], Kenton L. Morgan[7], Ian G. Handel[1] and Barend M. de C. Bronsvoort[1]*

[1] The Royal (Dick) School of Veterinary Studies and Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom, [2] School of Life Sciences, University of Lincoln, Lincoln, United Kingdom, [3] School of Veterinary Sciences, University of Ngaoundere, Ngaoundere, Cameroon, [4] Cameroon Academy of Sciences, Yaoundé, Cameroon, [5] Tuberculosis Reference Laboratory Bamenda, Hospital Roundabout, Bamenda, Cameroon, [6] Laboratory of Emerging Infectious Diseases, University of Buea, Buea, Cameroon, [7] Institute of Ageing and Chronic Disease and School of Veterinary Science, University of Liverpool, Neston, United Kingdom

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Edited by:
Julio Alvarez, VISAVET Health Surveillance Centre (UCM), Spain

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Anita Luise Michel, University of Pretoria, South Africa
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***Correspondence:**
Robert F. Kelly
robert.kelly@roslin.ed.ac.uk

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The interferon-gamma (IFN-γ) assay and single comparative cervical skin test (SCITT) are used to estimate bovine tuberculosis (bTB) prevalence globally. Prevalence estimates of bTB, caused by *Mycobacterium bovis*, are poorly quantified in many Sub-Saharan African (SSA) cattle populations. Furthermore, antemortem diagnostic performance can vary at different stages of bTB pathogenesis and in different cattle populations. In this study, we aim to explore the level of agreement and disagreement between the IFN-γ assay and SCITT test, along with the drivers for disagreement, in a naturally infected African cattle population. In, 2013, a pastoral cattle population was sampled using a stratified clustered cross-sectional study in Cameroon. A total of 100 pastoral cattle herds in the North West Region (NWR) and the Vina Division (VIN) were sampled totalling 1,448 cattle. Individual animal data and herd-level data were collected, and animals were screened using both the IFN-γ assay and SCITT. Serological ELISAs were used to detect exposure to immunosuppressing co-infections. Agreement analyses were used to compare the performance between the two bTB diagnostic tests, and multivariable mixed-effects logistic regression models (MLR) were developed to investigate the two forms of IFN-γ assay and SCITT binary disagreement. Best agreement using the Cohen's κ statistic, between the SCITT (>2 mm) and the IFN-γ assay implied a 'fair-moderate' agreement for the NWR [κ = 0.42 (95%CI: 0.31–0.53)] and 'poor-moderate' for the VIN [κ = 0.33 (95% CI: 0.18–0.47)]. The main test disagreement was the animals testing positive on the IFN-γ assay and negative by the SCITT. From MLR modeling, adults (adults OR: 7.57; older adults OR = 7.21), females (OR = 0.50), bovine leucosis (OR = 2.30), and paratuberculosis positivity (OR = 6.54) were associated with IFN-γ-positive/SCITT-negative disagreement. Subsets to investigate diagnostic test disagreement for being SCITT-positive and IFN-γ-negative also identified that adults (adults OR = 15.74; older adults OR = 9.18) were associated with IFN-γ-negative/SCITT-positive disagreement. We demonstrate that individual or combined use of the IFN-γ assay and SCITT can lead

to a large variation in bTB prevalence estimates. Considering that animal level factors were associated with disagreement between the IFN-γ assay and SCITT in this study, future work should further investigate their impact on diagnostic test performance to develop the approaches to improve SSA prevalence estimates.

Keywords: bovine tuberculosis, diagnostic test performance, cattle, epidemiology, Africa, *Mycobacterium bovis*

INTRODUCTION

Bovine tuberculosis is a chronic disease of cattle, caused by *Mycobacterium bovis*, and is an important zoonosis associated with close interaction with cattle or consumption of raw dairy products (1). Close contact between cattle and people is commonplace in livestock rearing communities in Sub-Saharan Africa (SSA). Consequently, it is vital to understand the potential direct impacts of bTB on livestock health as well as its public health risks in such communities. A key metric in understanding bTB epidemiology is to quantify the burden of disease in cattle by estimating bTB prevalence. However, the prevalence estimates in the live animals rely on the use of diagnostic tests that are imperfect. Identifying characteristic TB lesion pathology has been used to describe the epidemiology of bTB in abattoirs in many African countries (2). Specificity of lesion detection is high (>95%) and can be improved further with culture from lesions or using molecular techniques such as PCR to characterise *M. bovis* bacilli (3, 4). However, identification of lesions is not a gold-standard diagnostic test, as its sensitivity can be low (28.5%), particularly in the early stages of infection (5) and can only be used in cattle postmortem.

Antemortem diagnostic tests for bTB are based upon measuring the specific aspects of the immune response to *M. bovis* antigens (2, 6, 7). *Mycobacterium bovis* infections predominately stimulate cell-mediated immunity (CMI) responses in cattle (8–10); therefore, antemortem diagnostic tests have tended to focus on detecting this CMI response in different formats, including the *in vivo* tuberculin skin tests known as the single intradermal test (SIT) and single comparative intradermal tuberculin test (SCITT) (2, 6, 11) and the *in vitro* IFN-γ assay. The SCITT is recognised by the World Organisation for Animal Health (OIE) as the primary diagnostic test for bTB diagnosis (3), with a very high specificity (median: 100%; CrI: 99–100%) (7, 12) but low sensitivity (median: 50.0%; CrI: 26–78%) reported in a recent extensive meta-analysis (13). As a result, there is an extremely high risk of infected animals testing negative (i.e., false-negative animals) resulting in gross underestimation of the bTB prevalence and, when used in control programmes, an increased risk of retaining positive animals that may continue to transmit within the herd or to naïve herds when animals are traded. The IFN-γ assay detects the predominant Th1 immune response to *M. bovis* and experimentally can detect positive animals from 2 weeks post-infection (14). Consequently, the IFN-γ assay is reported to have a higher sensitivity (median: 67%; CrI: 49–82%) than the SCITT with a slightly lower specificity (median: 98%; CrI: 96–99%) (13). In addition to diagnostic performance, the logistics involved in the use of either test is also important to appreciate when applied to a SSA context. The SCITT requires restraint of the animal and injection of the avian and bovine tuberculin intradermally in the neck. It has the drawbacks of requiring a repeat visit to remeasure the skin after 3 days and good handling facilities to assess skin thickness. This is particularly challenging in the SSA setting because of the absence of cattle identification systems in many SSA countries and the dangers when handling African cattle breeds that are predominately horned. The IFN-γ assay on the other hand requires a single blood sample that can be done from the tail vein. The disadvantage compared to the SCITT is that the IFN-γ assay does require local laboratory facilities to conduct the time-limited cell stimulation and incubation stages followed by the collection of the plasma and an ELISA.

The probability of a diagnostic test correctly classifying animals depends on its sensitivity, specificity, and the underlying true prevalence of the disease in the population. The SCITT and IFN-γ assays measure different aspects of the CMI response (15, 16) and therefore may not detect the same population of bTB-positive cattle. In addition, the performance of the two tests may vary between different cattle populations and, depending on the performance requirements for testing, can be adjusted through changing the diagnostic cut-off value for a positive of either test (16–19). Another approach is the so-called parallel testing, where the combination of being positive on one or both tests has been used to improve overall sensitivity of the testing system (20–22). It is important, given that the SCITT and IFN-γ assays detect the different aspects of the CMI response, to understand why these two tests disagree to better interpret their results in the field. Factors that may lead to test disagreement can be classified into those associated with laboratory method, operator error, or host-related factors. Understanding the impact of host-related factors is of particular interest to understand variation in test performance depending on animal's characteristics. For example, diagnostic tests could be selected, or their interpretation adjusted based on the signalment of individual animals. Host-related factors previously identified include animal age, breed, pregnancy status, and causes of immunosuppression such as co-infections (7). Results from a previous study in Cameroon found that co-infection with *Fasciola gigantica* reduced the sensitivity of the IFN-γ assay (23), and studies elsewhere highlight a similar impact on SCITT sensitivity with other *Fasciola* species (24). Other immunosuppressive co-infections may also impact bTB antemortem diagnostic test performance (25–27), such as immunosuppressing viruses or *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which have been shown to lead to false-negative results (28). Identifying factors that influence IFN-γ assay and SCITT performance and where disagreement is

occurring between tests will highlight the potential challenges using these tests and what adjustments may be needed to improve interpretation of the results.

In this study, we investigate the level of agreement between the IFN-γ assay and the SCITT and host-related factors associated with their diagnostic disagreement. We then compare the impact of their different performances on the prevalence estimates of bTB using the IFN-γ assay, the SCITT and the "parallel" test combination for pastoral cattle populations in Cameroon.

MATERIALS AND METHODS

Study Design

The dataset used for this analysis was a part of a larger study investigating the epidemiology of bTB in Cameroonian cattle populations (29). The study sites were the Northwest Region (NWR) and the Vina Division (VIN) of the Adamawa Region (AR) of Cameroon (**Figure 1**), previously highlighted as important cattle keeping areas of Cameroon (30). A cross-sectional study was conducted, sampling two pastoralist populations (NWR & VIN) where *M. bovis* has been confirmed present (30, 31). Sampling was conducted between January–May 2013 in the NWR and September–November 2013 in the VIN, respectively. Pastoralist cattle populations in the NWR and VIN were estimated to be 506,548 and 176,257, respectively, from the Ministry of Livestock, Fisheries and Industrial Agriculture /Ministere de l'Elevage des Peches et Industries Animales (MINEPIA) vaccination records (32). The pastoralist cattle population eligible for sampling were herds listed in vaccination records at 81 ZVSCCs in the NWR and 31 ZVSCCs in the VIN in 2012. There were 5,053 cattle herds in the NWR and 1,927 in the VIN, with a range of 1–215 animals per herd. A population weighted stratified random sample of registered herds was sampled in each of the two study sites. The sample was stratified by sublocation within each administrative area; seven divisions in the NWR and eight subdivisions in the VIN. The number of herds sampled from each sublocation was proportional to the total number of herds within that sublocation. The sample size for this bTB focused project was based on an clustered random sample with an estimated animal level bTB prevalence of ∼10%, a within herd variance of 0.15 and between herd variance of 0.01, an average herd size of 70, a relative cost of 12:1 for herd/animal and relative error of ± 15% (Survey Toolbox; AusVet) (33). This gave a target sample size of 15 cattle per herd and 88 herds under the simplifying assumption of perfect test performance. The final sample was 100 herds with 50 at each site (NWR and VIN). Within each herd, the 15 samples were stratified to each of three age classes: young (≥0.5 years and <2 years), adult (≥2 and <5 years), and old (≥5 years). If there were insufficient animals of one age group, additional animals of any age were sampled.

Data Collection

Pastoral herds were visited and a local translator explained the project to the herdsman in either Foulfulde, Pidgin English, or French. Individuals were asked, in the language in which they were most comfortable, to give verbal consent to participate in the study. The same local translator, who was unaware of animal disease-status, selected 15 animals per herd at random. The animals were identified used disposable numbered Tyvek® wrist bands on the forelimb and horn on the side the SCITT would be conducted on. All sampled animals were then examined by the same veterinarian, with data recorded at an individual animal level to accompany samples. These included signalment [sex, age by dentition score (DS), breed, and body condition score (BCS)]. The method for ageing by DS and BCS was carried out on 5-point scales (34). "Improved" breed cattle were defined as cattle that had the phenotypic appearance of mixed *Bos indicus* breeding. "Exotic" cattle defined as those cattle that had the phenotypic appearance or which were reported by the farmer to be fully or mixed *Bos taurus*. Plain and heparinised blood samples were collected from the jugular or tail vein. All information was initially recorded on paper forms, which were later transferred into a relational Access database (Microsoft Access®). Unless otherwise stated, all animals sampled were tested using all infectious disease diagnostic tests.

Diagnostic Tests for Bovine Tuberculosis and Other Infectious Diseases

IFN-γ Assay

For the IFN-γ assay (20), three aliquots of 1.5 ml of heparinised blood per animal were stimulated with either 15 μl of avian PPD, bovine PPD (Prionics® Lelystad Tuberculin PPD), or PBS and then incubated for 24 h at 37°C within 8 h of sampling (usually <3 h). Incubated blood was centrifuged at 300 g for 10 min, and then, three plasma aliquots per animal were stored at −20°C in a portable travel freezer. Electrical supplies were maintained by mains electricity, portable generators, or from vehicle batteries where necessary in the field. Plasma samples were transported at −20°C to Laboratory of Emerging Infectious Diseases (LEID), University of Buea, Cameroon, where the IFN-γ assay ELISA was conducted. Prior to starting the protocol, reagents were reconstituted and samples were allowed to reach room temperature (22 +/-5°C). The avian PPD, bovine PPD, and PBS-stimulated plasma samples were diluted 1:1 with dilution buffer. Diluted plasma samples were added to the pre-coated 96 well plate along with duplicates of kit positive, negative, and PBS controls. The 96 well plate was incubated on a microplate shaker, at 600 rpm for 60 min at 22 +/-5°C, and once complete, the plate washed 6 times with wash buffer. About 100 μl of conjugate was added to the 96 well plate incubated for 60 min and washed as previous. About 100 μl of enzyme substrate was added to the 96 well plate incubated for 30 min as previous in the dark. Finally, 50 μl of stopping solution was then added to the 96 well plate and read at 450 nm using an automated microplate reader (Thermoscientific® Multiskan Go). For interpretation, PBS control ODs were subtracted from corresponding sample ODs. The acceptable averaged negative bovine was <0.13 and positive bovine control was >0.70. For each sample, the difference in OD of the sample stimulated with bovine PPD minus the mean OD of the avian PPD was calculated for interpretation. At standard interpretation, as per commercial kit instructions, animals with a

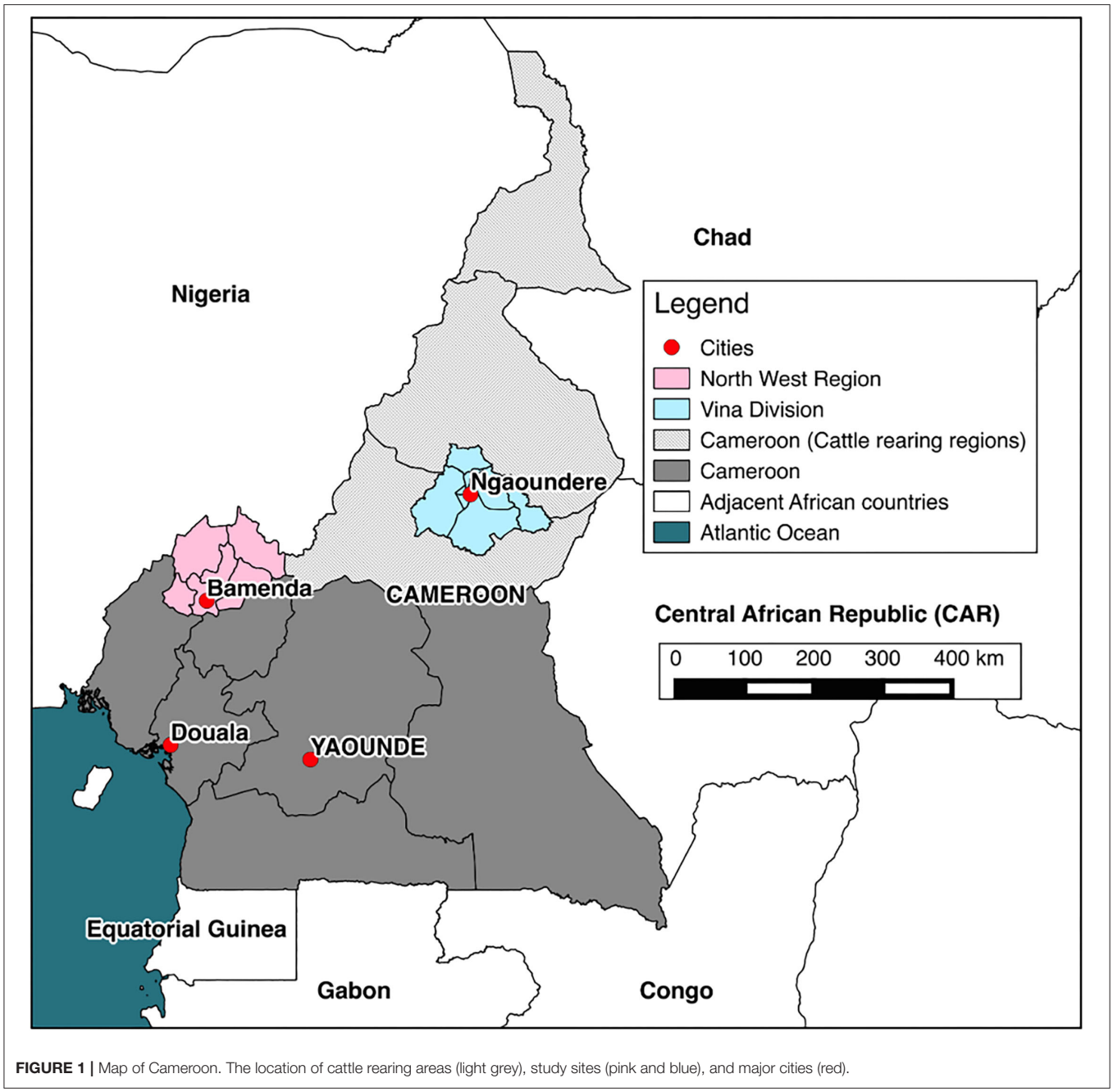

FIGURE 1 | Map of Cameroon. The location of cattle rearing areas (light grey), study sites (pink and blue), and major cities (red).

bovine PPD plasma sample of ≥0.1 that of avian PPD and PBS were classed as *M. bovis* infected.

Single Cervical Intradermal Tuberculin Test (SCITT)

All SCITTs were performed and interpreted by the same experienced veterinarian (RK) following the standard protocol used in the United Kingdom. At the time of sampling, each animal was appropriately restrained by either casting on the ground or tied up to trees using ropes (Day 0). Left or right side of the cervical neck was used, depending upon accessibility, and ID bands were placed on the leg and/or horn on the same side of the animal. Two areas, approximately 12 cm apart, in the mid-cervical neck had the hair clipped (using scissors) to mark the injection sites. The skin thickness was measured using skin callipers and recorded for each site prior to injection. A total of two multidose automatic syringes (McLintock®) were used to inject 0.1 ml of avian and bovine PPD (Prionics® Lelystad Tuberculin PPD) intradermally in the dorsal and ventral sites, respectively. The injection site was palpated to confirm whether PPDs were injected intradermally. Multidose automatic syringe needles were swabbed with surgical spirit between individual cattle. On a return visit, approximately 72 h later (Day 3),

the skin thickness of the two injection sites was remeasured using skin callipers. The difference in bovine and avian PPD skin measurements was calculated for each bovine to determine whether the animal is infected with *M. bovis*. First, the difference between the skin measurement of PPD injection sites on days 0 and 3 was calculated. Then, the difference between bovine and avian was calculated:

- Avian skin reaction difference (A) = skin thickness day 3 – skin thickness day 0 (mm)
- Bovine skin reaction difference (B) = skin thickness day 3 – skin thickness day 0 (mm)
- PPD skin reaction difference = B–A

Second, clinical signs at the injection site (e.g., oedema) were recorded. The results for each animal were then interpreted at two cut-off values. At severe interpretation, animals with a PPD skin reaction difference of >2.0 mm +/- clinical signs at the injection site indicated the presence of *M. bovis* infection. At standard interpretation, a PPD skin reaction difference of >4.0 mm +/- clinical signs at the injection site indicated the presence of *M. bovis* infection. These two cut-off values were then evaluated in subsequent agreement analysis.

Serum Antibody ELISAs

After collection, all serum samples were heat treated at 56°C for 120 min and stored at −20 °C until tested. Serum samples were then tested for exposure to various infections using serological ELISAs. For the *F. gigantica* antibody ELISA (35), immulon-2 ELISA 96-well plates were coated with 100 µl of 1 µg/ml *F. gigantica* E/S antigen in 0.1 M carbonate buffer (pH 9.6). Plates were incubated for 1 h at room temperature and then refrigerated at 2–4°C overnight. Plates were then washed six times (two short washes and one 5-min wash repeated for two times) with pH 7.2 PBS containing 0.05% Tween-20 (PBS-Tween). Each well was blocked with 200 µl of blocking buffer for 1 h at 37°C [4% skimmed powder (Marvel, Premier International Foods®, Spalding UK)] in PBS-Tween. Plates were washed six times, and 100 µl of sera diluted 1:200 in blocking buffer was added to each well. Positive and negative serum controls were added to the plate in duplicate, at the same concentration as the test sera, and incubated at 37°C for 1 h. The plates were again washed, 100 µl of 1:1500 mouse anti-bovine IgG HRP conjugate (Serotec®, UK) in blocking buffer was added, and then, the plates were incubated at 37°C for 1 h. After washing, 100 µl of TMB substrate (acetate buffer pH 5 and tetramethylbenzidine in a methanol-based solution, MAST Diagnostics, Bootle, Merseyside, UK) was added and incubated at room temperature for 20 min in the dark. Finally, 100 µl of stopping solution (10% hydrochloric acid) was added and the colour change measured at 450 nm using an automated microplate reader (Thermoscientific® Multiskan Go). The results were obtained as an optical density (OD) and expressed as a percent positivity value (PPV). For this study, samples PPV <23.4% are considered negative and PPV ≥23.4% are considered positive (35).

Screening for MAP exposure was conducted using the ID.vet Screen® Paratuberculosis Serum Indirect Multi-species ELISA (36). In brief, 10 µl of each serum sample was diluted to 1:10 with the provided dilution buffer. The diluted sample was added to a well on the purified MAP antigen coated microplate and incubated at 21°C for 45 min. The microplate was washed three times with 300 µl of wash solution, and 100 µl of supplied conjugate was added for 30 min at 21°C. Using the same method, the microplate was washed again, and 100 µl of substrate solution was added to each well for 15 min at 21°C. To stop the reaction, 100 µl of stop solution was added to each well and the microplate was read at 450 nm. For a microplate to be considered valid, the mean of the duplicate positive (PC) and negative (NC) controls was calculated. For a microplate to pass, mean PC optical density (OD) needed to be > 0.35 and the mean positive and negative OD ratio (ODPC/ ODNC) needed to be >3. For each sample, the sample to positive ratio (S/P%) was calculated by (ODsample–ODNC) / (ODPC -ODNC) X 100. The manufacturers suggest that samples S/P% ≤60% are considered negative; S/P% >60% and <70% are considered doubtful; and S/P% ≥70% are considered positive. For this study, samples S/P% <70% are considered negative and S/P% ≥70% are considered positive.

Screening for bovine leukaemia virus (BLV) exposure was conducted using the ID Screen® BLV Serum Competitive ELISA (37). In brief, 25 µl of each serum sample was diluted to 1:4 with the provided dilution buffer. The diluted sample was added to a well on the BLV gP51 antigen-coated microplate and incubated at 21°C for 45 min. The microplate was washed three times with 300 µl of wash solution, and 100 µl of supplied conjugate was added for 30 min at 21°C. Using the same method, the microplate was washed again, and 100 µl of substrate solution was added to each well for 15 min at 21°C. To stop the reaction, 100 µl of stop solution was added to each well, and the microplate was read at 450 nm. For a microplate to be considered valid, the mean of the duplicate PC and NC was calculated. For a microplate to pass, mean NC OD needed to be > 0.7 and the mean positive and negative OD ratio (ODPC/ ODNC) needed to be >0.3. For each sample, competition percentage (S/N%) was calculated by (ODsample / ODNC) x 100. The manufacturers suggest that samples S/N% ≤50% are considered positive; S/N% >50% and <60% are considered doubtful; and S/N% ≥60% are considered negative. For this study, samples S/N% ≤50% are considered positive and S/N% >50% are considered negative.

Screening for bovine viral diarrhoea virus (BVDV) exposure was conducted using the ID Screen® BVDV Serum Competitive ELISA (38). In brief, 15 µl of each serum sample was diluted to 1:9 with the provided dilution buffer. The diluted sample was added to a well on the BVDV p 80 antigen-coated microplate and incubated at 37°C for 45 min. The microplate was washed three times with 300 µl of wash solution, and 100 µl of supplied conjugate was added for 30 min at 21°C. Using the same method, the microplate was washed again and 100 µl of substrate solution was added to each well for 15 min at 21°C. To stop the reaction, 100 µl of stop solution was added to each well and the microplate was read at 450 nm. For a microplate to be considered valid, the mean of the duplicate PC and NC was calculated. For a microplate to pass, mean NC OD needed to be > 0.7 and the mean positive and negative OD ratio (ODPC/ ODNC) needed to be >0.3. For each sample, competition percentage (S/N%) was

calculated by (ODsample / ODNC) x 100. The manufacturers suggest that samples S/N% ≤40% are considered positive; S/N% >40% and ≥50% are considered doubtful; and S/N% >50% are considered negative. For this study, samples S/N% ≤40% are considered positive and S/N% >40% are considered negative.

Statistical Analyses

All statistical analyses were performed using packages and functions in R version 3.6.1 (39). Graphics were produced using the *ggplot2* package (40). Spatial data were displayed using QGIS 2.2® (41) or *tidyverse* collection of R packages (42) and shapefiles obtained from the GADM database of Global Administrative Areas (www.gadm.org).

Bovine Tuberculosis Diagnostic Test Agreement

Agreement analyses were in this study to compare the performance between diagnostic as no gold-standard diagnostic tests were available. Agreement was defined as how much two diagnostic tests, which measure the same response, agree with each other (43). In the absence of gold-standard diagnostics, agreement analysis is used in this study to explore the positive cut-off values for the SCITT with continuous results for both tests to be converted to categorical results (e.g., positive and negative). For the IFN-γ assay, ≥0.1 was used as the positive cut-off value compared to the SCITT at >2 mm and >4 mm cut-off by study site. Percentage agreement and Cohen's κ statistic were used to quantify binary agreement between two tests. The agree, *kappa2,* and *rater.bias* functions in the *irr* package (44) were used to calculate percentage agreement and Cohen's κ statistic. Percentage agreement was used as a provisional measure of agreement between two diagnostic tests and was calculated as follows (43):

$$\text{Percentage agreement or observed agreement (OP)} = (a+d)/(a+b+c+d)\times 100$$

Percentage agreement does not distinguish agreement between positive or negative diagnostic test results and does not adjust for chance. Consequently, the Cohen's κ statistic was also calculated to determine the level of agreement, beyond chance, between two categorical diagnostic tests (e.g., positive or negative) (45):

$$\text{Expected agreement (EP)} = [((a+b)(a+c))/n + ((c+d)(b+d))/n]/n$$

$$\text{Cohen'sκstatistic} = (OP - EP)/(1 - EP)$$

Cohen's κ statistic was interpreted as =1 (perfect agreement), 0.81–1 (almost perfect agreement), 0.61–0.8 (substantial agreement), 0.41–0.6 (moderate agreement), 0.21–0.4 (fair agreement), 0.01–0.2 (poor agreement), and ≤0 (no agreement) (46). The effect of very low or high prevalence is thought to have a negligible effect on the calculation (44, 47).

Prevalence Estimates

The structure of the cross-sectional study design was incorporated into analyses using the *svydesign, confint,* and *svyby* functions in the *survey* package (48). For estimating bTB prevalence using SCITT, IFN-γ, and combined tests, true prevalence (43) was estimated using the epi.prev function in the epiR package (49) for pastoral cattle population by study site. Specific differences in sample statistics were identified by non-overlapping confidence intervals (CI) at 95% level (50).

Risk Factors Associated With Diagnostic Test Disagreement

Multivariable mixed-effects logistic regression (MLR) models were built to investigate the two forms of IFN-γ assay and SCITT binary disagreement using a combined dataset from both study sites, subsetted by the two forms of diagnostic disagreement:

1. IFN-γ assay-positive and SCITT-negative (**Figure 2**).

a. Model a): Subset of IFN-γ assay (≥0.1)-positive animals, using SCITT (≤2 mm) status as the dependent variable (IFN-γ/SCITT: +/- = 1; +/+ = 0).
b. Model b): Subset of SCITT (≤2 mm)-negative animals, using IFN-γ assay (≥0.1) status as the dependent variable (SCITT/IFN-γ: -/+ = 1; -/- = 0).

2. IFN-γ assay-negative and SCITT-positive (**Figure 2**).

c. Model c): Subset of IFN-γ assay (<0.1)-negative animals, using SCITT (>2 mm) status as the dependent variable (IFN-γ/SCITT: -/- = 0; -/+ = 1).
d. Model d): Subset of SCITT (>2 mm)-positive animals, using IFN-γ assay (<0.1) status as the dependent variable (SCITT/IFN-γ: +/- = 1; +/+ = 0).

A number of two subsets of each type of disagreement between the two diagnostic tests were used to produce four disagreement models. The outcome variable was the binary result for the alternative test with the result of interest being the contrary binary result, e.g., subset positive and outcome variable negative. Host-related factors (e.g., age, sex, breed, and evidence of co-infections) were included. Model selection was performed through model averaging; this approach allowed us to generate, rank, and weight several models supporting hypothesised associations to the outcome of interest (disagreement) (51). A set of explanatory variables were included in the global model for each disagreement, and this was the starting point to generate a model set to compare and average. Submodels were generated through the *dredge* function implemented in the MuMIn package (52). Each submodel was assessed by its AIC (53), and submodels with a Δ AIC ≤ 2 were averaged (54). For all models, herd was included as a random effect. Receiver operating characteristic (ROC) curves were used to assess the capacity of the models to discriminate between agreements and disagreements.

RESULTS

Cattle Sample

In total, 100 pastoral herds were recruited, 50 in the NWR and 50 in the VIN, with 14–15 cattle sampled per herd. Complete dataset for both tests was available for 750 cattle in NWR and 741 cattle in VIN ($n = 748$ for IFN-γ assay; $n = 741$ for SCITT).

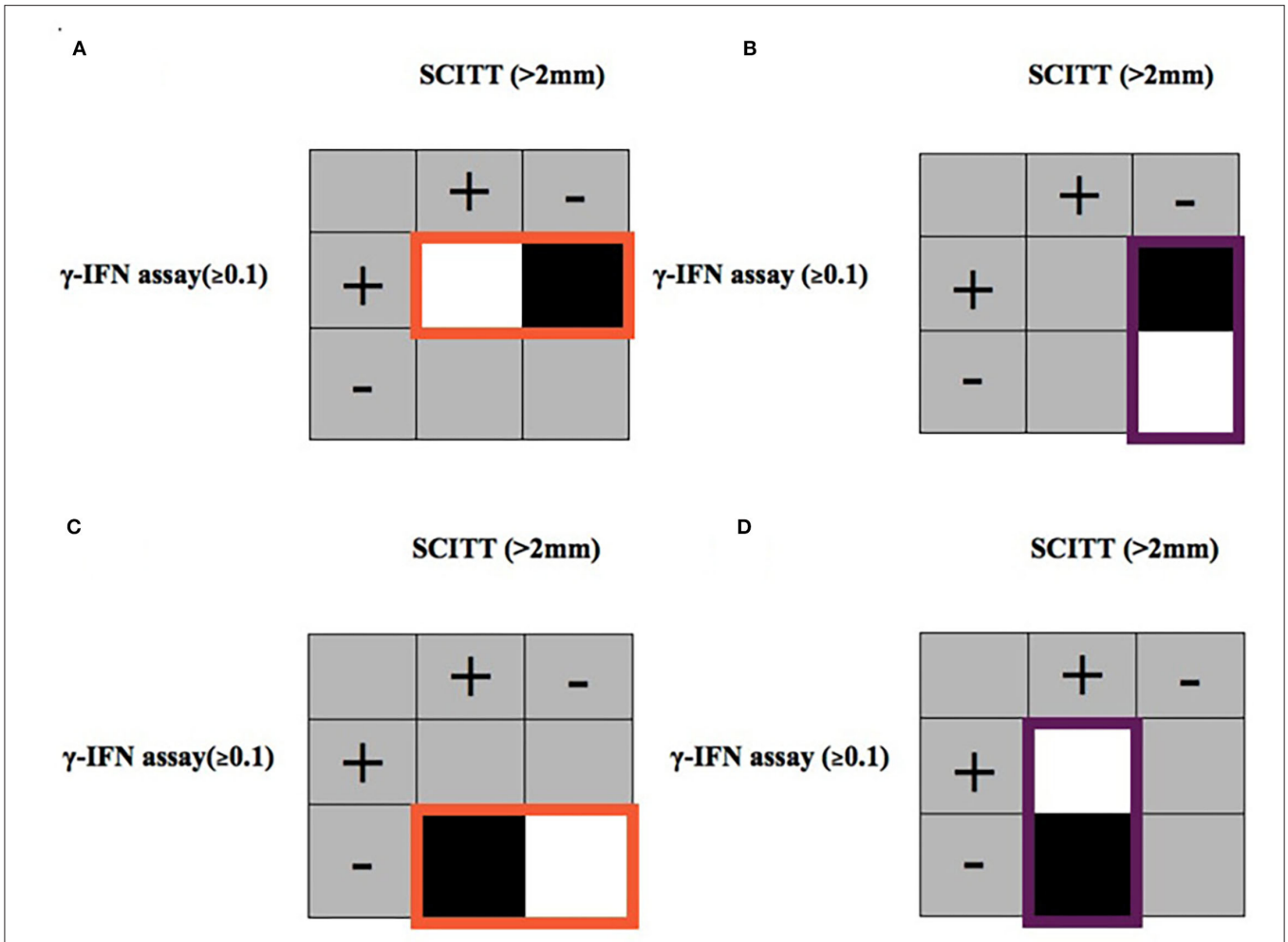

FIGURE 2 | Diagrammatic representation of data subset to investigate two types of test disagreement. 1. IFN-γ assay-positive (≥0.1) and SCITT-negative (≤2 mm) using models **(A,B)**. Model **(A)** Subset of all cattle with a IFN-γ assay-positive response highlighted in orange with SCITT as the dependent variable. Model **(B)** Subset of all cattle with a SCITT-negative response highlighted in purple with IFN-γ assay as the dependent variable. Black areas indicate "positive" (disparate results) and white areas indicates "negative" (agreeing results) of the dependent variable. 2. IFN-γ assay-negative (<0.1) and SCITT-positive (>2 mm) in pastoral cattle using models **(C,D)**. Model **(C)** Subset of all cattle with a IFN-γ assay-negative response highlighted in orange with SCITT as the dependent variable. Model **(D)** Subset of all cattle with a SCITT-positive response highlighted in purple with IFN-γ assay as the dependent variable. Black areas indicate "positive" (disparate results) and white areas indicates "negative" (agreeing results) of the dependent variable.

A detailed summary of animal (55) and herd-level data (29) has been previously published. This study utilises the animal-level dataset, accounting for herd and study site in the analysis where appropriate.

Bovine Tuberculosis Diagnostic Test Agreement

The raw continuous results for the SCITT (difference in skin thickness in mm) and IFN-γ assay (OD difference) stratified by site are presented in **Figure 3**. The frequency of avian PPD greater than bovine PPD reactions for SCITT (at >2 mm: 1.25%, 95% CI: 0.79–1.97%; at >4 mm: 0.01%, 95% CI: 0.00–0.39%) and IFN-γ (at ≥0.1: 7.11%, 95% CI: 5.90–8.55%) was low. The agreement between the binary two test results, with the SCITT at >2 mm and >4 mm positive cut-off values, is presented in **Table 1**. The percentage agreements were relatively high but do not adjust for agreement by chance and because of the low apparent prevalence can be misleading, and thus, the Cohen's κ statistic gives a better measure adjusting for chance. The agreement at >2 mm cut-off was slightly higher for the NWR compared to at >4 mm cut-off, but the different cut-offs were identical for the VIN. The scores were relatively low suggesting fair-moderate agreement for the NWR but poor-moderate agreement for the VIN. As the confidence interval for the Cohen's κ statistic overlapped for estimates between the study sites, and previous studies highlighting the use of >2 mm positive cut-off (30), the SCITT >2 mm positive cut-off value was chosen for the remainder of the analysis.

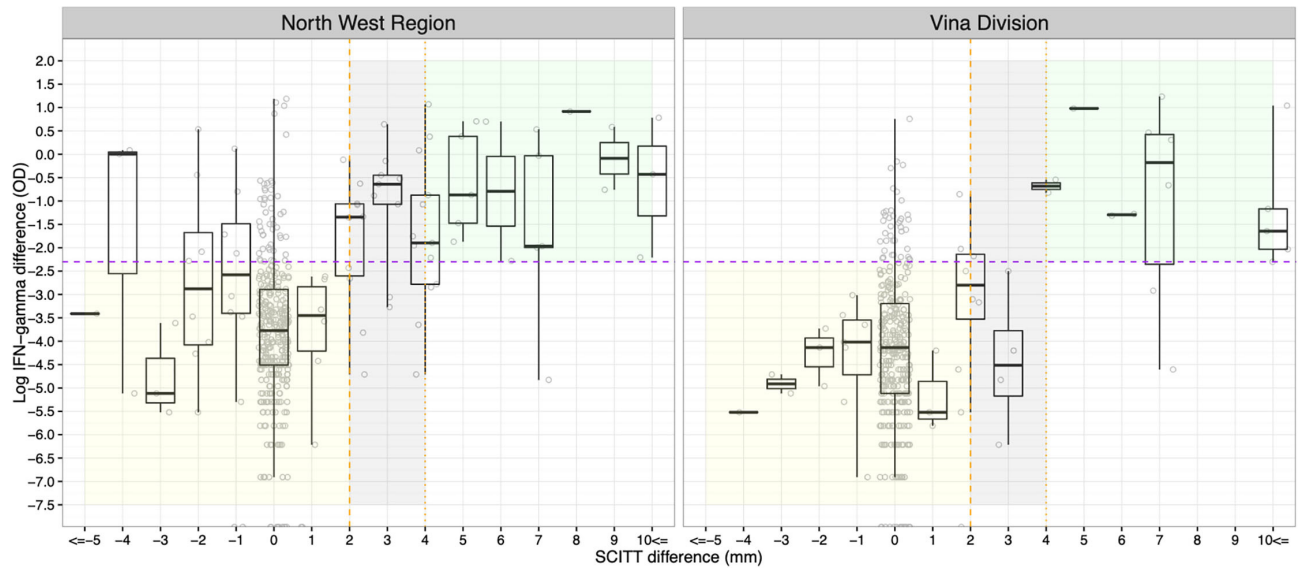

FIGURE 3 | For the IFN-γ assay, results are displayed on a log scale for clarity and ≥0.1 positive cut-off values are shown (horizontal purple dashed line; ln (0.1) = −2.3). For the SCITT >2 mm (vertical orange dashed line) and >4 mm (vertical orange dotted line), positive cut-off values are shown. The green area denotes the test positive cattle for IFN-γ assay (≥0.1) and SCITT (>4 mm). The grey area denotes proportion of additional test positive cattle for IFN-γ assay (≥0.1) and SCITT (≥2 mm). The yellow area denotes test negative cattle for IFN-γ assay (<0.1) and SCITT (≤2 mm).

TABLE 1 | Comparisons of agreement and Cohen's κ statistic between IFN-γ assay (≥0.1) and SCITT (>2 mm and >4 mm) for pastoral cattle sampled in the North West Region and Vina Division.

North West Region (*n* = 750)					
IFN-γ assay (≥0.1)		SCITT (>2 mm)		Percentage agreement	Cohen's κ statistic (95% CI)
		+	-	90.8%	0.42 (0.31–0.53)
	+	30	55		
	-	14	651		
IFN-γ assay (≥0.1)		SCITT (>4 mm)		Percentage agreement	Cohen's κ statistic (95% CI)
		+	-	90.5%	0.28 (0.17–0.39)
	+	16	69		
	-	2	663		
Vina Division (*n* = 741)					
IFN-γ assay (≥0.1)		SCITT (>2 mm)		Percentage agreement	Cohen's κ statistic (95% CI)
		+	-	93.7%	0.33 (0.18–0.47)
	+	13	36		
	-	11	681		
IFN-γ assay (≥0.1)		SCITT (>4 mm)		Percentage agreement	Cohen's κ statistic (95% CI)
		+	-	94.5%	0.33 (0.18–0.47)
	+	11	38		
	-	3	689		

Bovine Tuberculosis Apparent Prevalence Estimates

The apparent prevalence estimates between the NWR and VIN are compared in **Figures 4**, **5**; based on the IFN-γ assay (≥0.1), SCITT (>2 mm) and a parallel combination (PC) of the two tests (i.e., positive on either or both tests at the specified cut-offs). The highest estimates were generated when a parallel approach is used, followed by the IFN-γ and SCITT. No significant difference in overall animal-level apparent prevalence was noted between study sites when using any single or PC tests, although the prevalence was consistently lower in the VIN than the NWR (**Figure 4**). The different tests give quite a different impression of the magnitude in apparent prevalence across administrative areas and herds that are defined as positive (at least 1 positive animal per herd) (**Figure 5**).

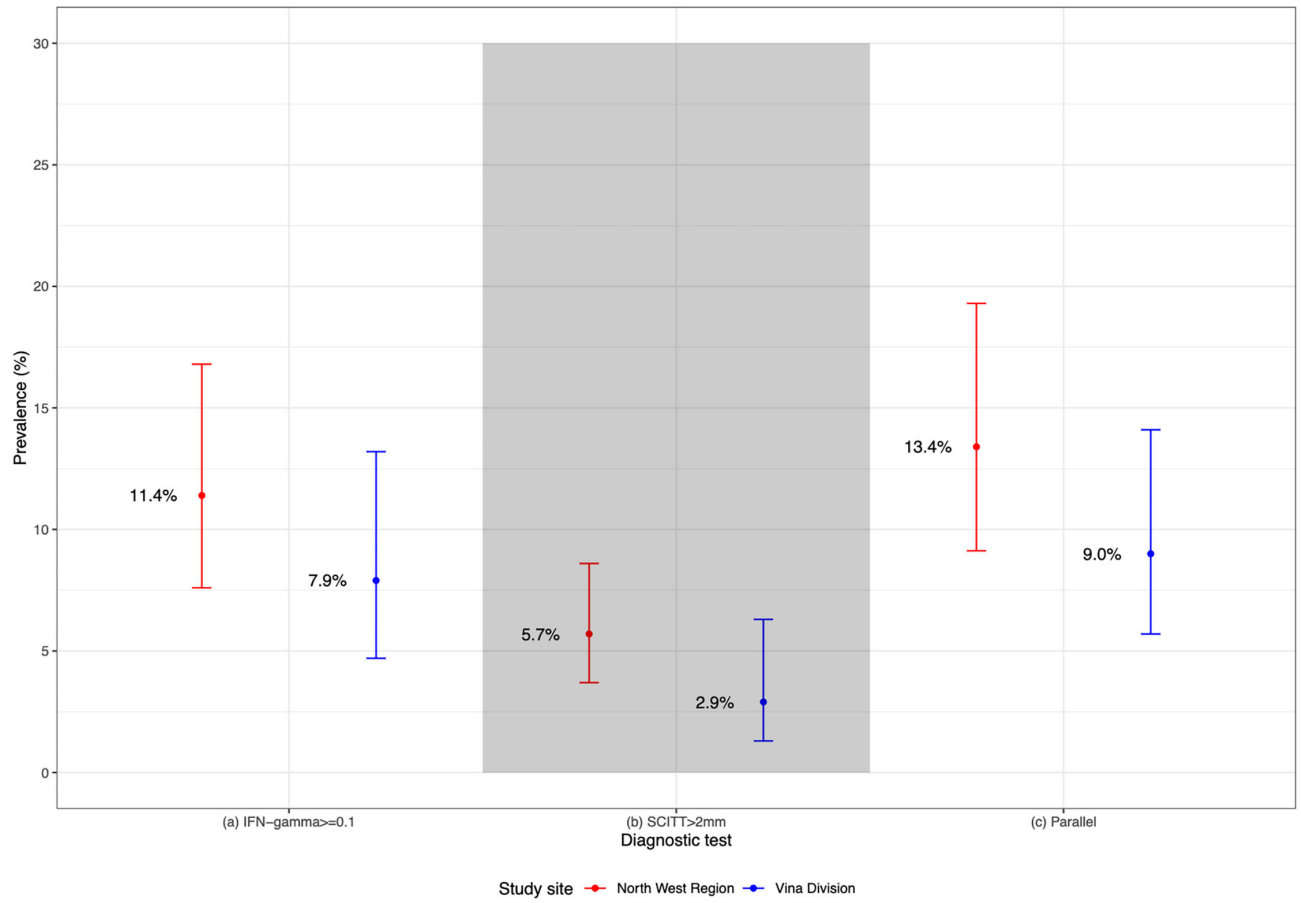

FIGURE 4 | Summary of apparent bTB prevalence, using IFN-γ assay (≥ 0.1), SCITT (>2 mm) and parallel combination testing. IFN-γ assay (≥ 0.1) (NWR $n = 750$; VIN $n = 748$); SCITT (>2 mm) and Parallel (NWR $n = 750$; VIN $n = 741$).

Risk Factors Associated With Bovine Tuberculosis Diagnostic Test Disagreement

IFN-γ Assay-Positive and SCITT-Negative

Results from sampled cattle were subsetted to investigate diagnostic disagreement for being IFN-γ assay-positive and SCITT-negative using two methods to produce two final models (**Figures 2A,B** described the disagreement investigated using the models outlined **Table 2**). The final models are presented in **Table 2**. Model a looked at animals that were IFN-γ-positive and then looked at factors associated with testing SCITT-negative ($n = 133$). Only age was strongly associated with disagreement. In other words, older animals were more likely to test negative on the SCITT test given they were IFN-γ-positive. Model b looked at the reverse and looked at SCITT-negative animals and factors associated with them being positive for the IFN-γ ($n = 1422$). Here, female animals were less likely to be IFN-γ-positive compared to males, and also interestingly, animals having had exposure to BLV or paraTB were more likely to be in disagreement. The area under the ROC curve for the final averaged models was 0.94 (95% CI: 0.90–0.97) and 0.87 (95% CI: 0.85–0.91) for models a and b, respectively. This was an indicative of good model performance in relation to classifying the disagreements.

IFN-γ Assay-Negative and SCITT-Positive

Cattle were also subsetted to investigate diagnostic test disagreement for being SCITT-positive and IFN-γ assay-negative using two methods (**Figures 2C,D** described the disagreement investigated using the models outlined **Table 3**). The final models are presented in **Table 3**; model c looked at animals that were IFN-γ-negative and then looked at factors associated with testing SCITT-positive ($n = 1{,}364$). Only age was strongly associated with disagreement, where animal that has tested negative on the IFN-γ it is more likely to be SCITT-positive if it is an older animal (adult or older adult) compared to the young animal. For model d, using the subset of animals testing SCITT-positive, there were no factors strongly associated with animals testing IFN-γ-negative (disagreement), but the sample size for this subset was very small ($n = 68$). The area under the ROC curve for the final averaged models was 0.90 (95% CI: 0.83–0.96) and 0.99 (95% CI: 0.97–1.00) and for models c and d, respectively. These results indicate that the models correctly classified the disagreements.

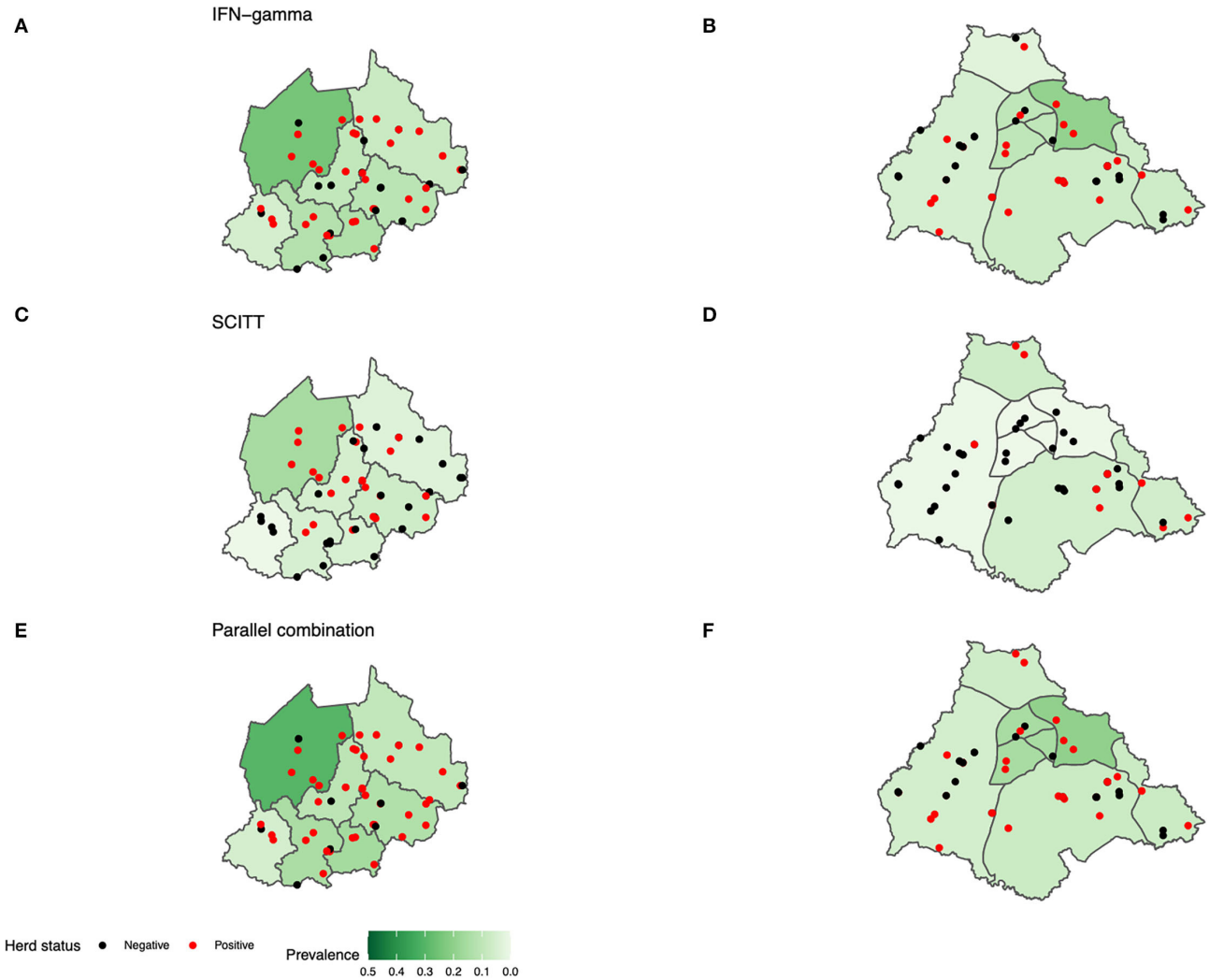

FIGURE 5 | Map study site by sublocation prevalence for pastoral cattle. **(A,B)** IFN-γ assay (≥0.1), **(C,D)** SCITT (>2 mm) and **(E,F)** Parallel combination [subfigure **(A,C,E)** NWR $n = 750$. **(B)** VIN $n = 748$. **(D,F)** VIN $n = 741$]. Positive herds have at least one infected animal per herd.

DISCUSSION

In this study, we highlight that the use of the IFN-γ assay, SCITT, and parallel combination tests can give a quite different impression of the overall magnitude of bTB prevalence in a naturally infected cattle population. Although individual or combined test prevalence estimates overlapped in the two localities, the lowest and highest estimates in each locality differed by multiples of ∼2–3. Internationally, the individual and combined use of IFN-γ assay and SCITT are used to estimate prevalence to prioritise local resources in bTB surveillance and control programmes (2, 3). Inaccurate estimates of bTB prevalence will likely have a detrimental public health impact where resources for control are limited. This is of particular concern in SSA contexts where *M. bovis* is undoubtedly a public health issue, due to a close interaction between cattle and humans, and disease surveillance is infrequent. Understanding the level of agreement and host factors associated with disagreement will improve interpretation of future bTB prevalence estimates in SSA cattle populations.

Although the antemortem tests measure the CMI response to *M. bovis* infection (56), and a degree of agreement is likely, agreement between the two tests was only "moderate" [$\kappa = 0.42$ (moderate agreement: 0.41–0.60)] at best when using positive cut-off values of ≥0.1 and >2 mm positive cut-offs for the IFN-γ assay and SCITT, respectively. Even lower agreement was noted between the IFN-γ assay and the SCITT was reported at >4 mm, defined as poor [$\kappa = 0.13$ (poor agreement: 0.01–0.2)]. Poor agreement has also been reported between the two assays in Ethiopia where the >4 mm cut-off was used for the SCITT and ≥0.1 for the IFN-γ assay (57). Other studies in SSA, including Cameroon, reported that bTB-positive *B. indicus* cattle respond differently to *M. bovis* PPD, when compared to *B. taurus* cattle, with improved agreement when using the >2 mm

TABLE 2 | Final models to investigate risk factors for pastoral cattle testing IFN-γ assay-positive and SCITT-negative.

Model a (n = 133) 11 models	**Global model: SCITTdiff2∼ AGE2 + ANISEX + ABREED2 + FgLivB + BVDAbPN + paraAbPN + strata1 + (1|HER_ID)** ***Binary outcome: SCITT-negative.***	
Variable	Level	OR (95% CI)
Age	Young	Reference
	Adult	7.57 (1.69–33.84)
	Old Adult	7.21 (1.65–31.54)
Model b (n = 1422) 9 models	**Global model: bovigam01∼ AGE2 + ANISEX + ABREED2 + FgLivB + LVAbPN + BVDAbPN + paraAbPN + strata1 + (1|HER_ID)** ***Binary outcome: IFN-γ-positive.***	
Variable	Level	OR (95% CI)
Sex	Male	Reference
	Female	0.50 (0.31–0.83)
Enzootic Bovine Leucosis	Negative	Reference
	Positive	2.30 (1.04–5.05)
Paratuberculosis	Negative	Reference
	Positive	6.54 (2.57–16.61)

Two models investigate diagnostic disagreement: (model a) Dependent variable SCITT-negative (SCITTdiff2) in IFN-γ-positive subgroup (n = 133). (model b) Dependent variable IFN-γ assay-positive (bovigam01) in SCITT-negative subgroup (1422). Explanatory variables included are AGE2 (age: young, adult or old adult), ANISEX (sex: female or male), ABREED2 (breed: improved or Fulani), FgLivB (F. gigantica serology result: negative or positive), LVAbPN (bovine leucosis virus serology: negative or positive), BVDAbPN (bovine viral diarrhoea virus serology result: negative or positive), paraAbPN (Paratuberculosis serology: negative or positive), strata1 (study site) and random effect HER_ID (herd sampled from).

TABLE 3 | Final models to investigate risk factors for pastoral cattle testing IFN-γ assay-negative and SCITT-positive.

Model c (n = 1364) 7 models	**Global model: SCITTdiff2∼ ANISEX + ABREED2 + FgLivB + LVAbPN + BVDAbPN + AGE2 + strata1 + (1|HER_ID)** ***Binary outcome: SCITT-positive.***	
Variable	Level	OR (95% CI)
Age	Young	Reference
	Adult	15.74 (2.10–120.20)
	Old Adult	9.18 (1.12–75.41)
Model d (n = 68) 8 models	**Global model: bovigam01 ∼ ANISEX + ABREED2 + FgLivB + BVDAbPN + LVAbPN + strata1 +(1|HER_ID)** ***Binary outcome: IFN-γ-negative***	
Variable	Level	OR (95% CI)
Nothing significant.		

Two models investigate diagnostic disagreement: (c) Dependent variable SCITT-positive (SCITTdiff2) in IFN-γ-negative subgroup (n = 1,364). (d) Dependent variable IFN-γ assay-negative (bovigam01) in SCITT-positive subgroup (68). Explanatory variables included are AGE2 (age: young, adult or old adult), ANISEX (sex: female or male), ABREED2 (breed: improved or Fulani), FgLivB (F. gigantica serology result: negative or positive), LVAbPN (bovine leucosis virus serology: negative or positive), BVDAbPN (bovine viral diarrhoea virus serology result: negative or positive), paraAbPN (paratuberculosis serology: negative or positive), strata1 (study site) and random effect HER_ID (herd sampled from).

cut-off for the SCITT (58–60). Suggesting that use of a lower >2 mm cut-off for the SCITT could be more appropriate in cattle populations in SSA. The IFN-γ assay and the SCITT also do not identify the same bTB positive population of cattle, possibly because the two tests are measuring different aspects of the CMI response (15, 61). Sampled cattle were from a naturally infected population, where *M. bovis* infected cattle are likely infected at different time points, likely to be at different stages in pathogenesis and consequently have marked variation in their host immune responses at the time of sampling. The IFN-γ assay is considered to be more sensitive than the SCITT and can detect *M. bovis* infection weeks–months earlier than the SCITT test (10, 62), which could explain why in our study, the IFN-γ assay consistently detecting more positives than the SCITT (at either study site). This suggests that potentially, the IFN-γ assay may be more suitable for highlighting the potential magnitude of prevalence in resource-limited settings, by detecting animals earlier post-infection. Further work is required to understand the diagnostic performance of the IFN-γ assay compared to the SCITT to improve the accuracy of prevalence estimates in naturally infected cattle populations.

Using subsets of the complete dataset, of test positive and negative animals, we were able to explore host-related factors for disagreement. Being IFN-γ assay-positive and SCITT-negative was a far more common form of disagreement and, as reported in other studies (16, 63–66), potentially associated with the low sensitivity of the SCITT, although reasons for reported lower sensitivity of the SCITT are poorly eluded. The impact of co-infections is likely to depend on pathogen or subtype exposed to, burden and timing of exposure (7, 18, 67). Bovine leucosis virus and paratuberculosis positivity were reported to increase the risk of this form of disagreement. As in our study, animals sampled from naturally infected populations may be co-infected with immunosuppressing or modulating infections and have been reported to influence CMI immune responses (7). Exposure to bovine leucosis virus can result in a persistent immunosuppression which may lead to depression in CMI responses, although its less clear why one CMI diagnostic may be affected more than another. Non-tuberculous mycobacteria have been shown to infect Cameroonian cattle (34) yet as both CMI diagnostic tests included a control avian PPD in their protocols, and the impact of non-tuberculous mycobacteria is likely to vary (68–70). Other researchers have identified that the performance of both the SCITT and the IFN-γ assay can be affected by paratuberculosis (*Mycobacterium avium* subspecies *paratuberculosis*) positivity in experimental infections (28, 71–73) specifically with increased reactivity to avian PPD. Although it is currently unclear why the IFN-γ-positive/ SCITT-negative disagreement was predominant in our study, this might be related to younger animals being less impacted by MAP immunosuppressive responses than older animals (>1 year of age) (74). Although frequency of avian PPD and paratuberculosis positives was low in this study, the addition of mitogen to IFN-γ assay may be helpful to improve diagnostic sensitivity (13, 75) when testing Cameroon or similar SSA cattle populations.

Interestingly, *F. gigantica* exposure was not associated with increased risk of disagreement between the CMI diagnostics. This is contra to an abattoir study, also conducted by authors, which demonstrated that *F. gigantica* was associated with decreased IFN-γ positivity and larger bTB lesions (23). Although it is unclear why a similar relationship was not also noted in this field study, multiple parasite and host factors are likely to affect the interaction between *M. bovis* and *F. gigantica* at any given time point. For example, experimental studies have demonstrated that a related *Fasciola* species, *Fasciola hepatica*, has been associated with decreases in diagnostic sensitivity for IFN-γ and SCITT when compared to detection of bTB lesions postmortem (76), but the extent of the effect on bTB diagnosis is determined by the order of infection of the two organisms (76). In our study, fluctuations in exposure or burden are not captured by the *F. gigantica* antibody ELISA (35) as the test detects exposure to infection at some point in an animal's lifetime. Future work should focus on investigating the variation in the bovine immune response to *M. bovis* in naturally infected cattle beyond singular time points and the dynamic impact of co-infections on bTB diagnostic test performance.

Advanced age was reported to be associated with both types of disagreement (IFN-γ-positive/ SCITT-negative and IFN-γ-negative/ SCITT-positive). In later stages of infection, IFN-γ responses can fluctuate throughout the course of *M. bovis* infection and may lead to IFN-γ assay false negatives (56, 65) when SCITT is positive. Furthermore, IFN-γ responses become anergic in chronic *M. bovis* infections (56), and advanced age of cattle could be a proxy for chronicity when investigating disagreement. IFN-γ assay-negative and SCITT-positive disagreement were less frequently reported which is potentially due to the higher specificity of the SCITT (16, 63–65). Cattle with chronic *M. bovis* infections are thought to be more likely to become SCITT-positive if they initially start out as IFN-γ-positive (12, 77), which may partly explain why this form of disagreement occurred less frequently.

In the absence of a gold-standard diagnostic for bTB, we were unable to fully explore the diagnostic performance of animals sampled using antemortem diagnostic tests. However, we were able to explore the associations with host factors that may account for diagnostic disagreement, using multivariable mixed-effects logistic regression modelling and a model averaging selection method. Compared to traditional methods of model selection, model averaging techniques allowed us to better identify the factors associated with the disagreements and the risk factors through a better estimation of the coefficients. Similar to Bayesian approaches, several models can be ranked and weighted to provide a quantitative measure of relative support for each competing hypothesis (51). By comparison, more traditional approaches such as stepwise methods, although also resulting in a final model, completely ignore model uncertainty (78). It is clear that co-infections can have complex impacts on bTB test diagnostics performance, and this may have important implications for bTB prevalence estimates along with future surveillance and control programmes in SSA. Although we did not test for every co-infection possible, our study has highlighted the need to consider animal level factors when interpretating bTB diagnostic test results to develop accurate prevalence estimates in cattle populations of interest.

CONCLUSION

Inaccuracies in local prevalence estimates hinder the progress of bTB surveillance and control programmes. In this study, we demonstrated that individual or combined use of the IFN-γ assay and SCITT can lead to a large variation in bTB prevalence estimates. Animal-level factors may impact on the agreement between CMI diagnostics and could limit our understanding of bTB epidemiology in endemic settings, where animals of various disease states exist. Quantifying the impact of factors, such as co-infections, should be prioritised to improve the accuracy of diagnosis and understanding of bTB epidemiology across cattle populations in SSA as well as other LMICs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was reviewed and approved by the University of Edinburgh Ethics Committee, UK (ERC No: OS02-13) and by the Institute of Research and Development (IRAD), Cameroon. All participants gave informed verbal consent to the translator before participating and could opt out at any stage. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BB, LN, and VT conceived the original project. BB, RK, KM, LN, VT, MS, and NE designed the field study, the databases, and the survey instrument. RK, NE, VN, VT, KM, and BB developed the field SOPs and collected the data. RK, EF, and LG conducted laboratory work. RK and BB cleaned the initial dataset. RK, LG, BB, and IH contributed to the analysis. RK was responsible for writing the initial drafts. All authors contributed comments for the final draft. All authors contributed to the article and approved the submitted version.

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Bovine Tuberculosis Epidemiology in Cameroon, Central Africa, Based on the Interferon-Gamma Assay

Robert F. Kelly[1,2], Lina Gonzaléz Gordon[2], Nkongho F. Egbe[3], Emily J. Freeman[1], Stella Mazeri[2], Victor N. Ngwa[4], Vincent Tanya[5], Melissa Sander[6], Lucy Ndip[7], Adrian Muwonge[2], Kenton L. Morgan[8], Ian G. Handel[2] and Barend M. D. C. Bronsvoort[2]*

[1] Farm Animal Services, The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom, [2] Epidemiology, Economics and Risk Assessment (EERA) Group, The Roslin Institute, The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom, [3] School of Life Sciences, University of Lincoln, Lincoln, United Kingdom, [4] School of Veterinary Medicine and Sciences, University of Ngaoundere, Ngaoundere, Cameroon, [5] Cameroon Academy of Sciences, Yaoundé, Cameroon, [6] Tuberculosis Reference Laboratory Bamenda, Hospital Roundabout, Bamenda, Cameroon, [7] Laboratory of Emerging Infectious Diseases, University of Buea, Buea, Cameroon, [8] Institute of Ageing and Chronic Disease and School of Veterinary Science, University of Liverpool, Liverpool, United Kingdom

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Anita Luise Michel, University of Pretoria, South Africa

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***Correspondence:**
Robert F. Kelly
robert.kelly@roslin.ed.ac.uk

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Despite sub-Saharan Africa (SSA) accounting for ∼20% of the global cattle population, prevalence estimates and related risk factors of bovine tuberculosis (bTB) are still poorly described. The increased sensitivity of the IFN-γ assay and its practical benefits suggest the test could be useful to investigate bTB epidemiology in SSA. This study used a population-based sample to estimate bTB prevalence, identify risk factors and estimate the effective reproductive rate in Cameroonian cattle populations. A cross-sectional study was conducted in the North West Region (NWR) and the Vina Division (VIN) of Cameroon in 2013. A regional stratified sampling frame of pastoral cattle herds produced a sample of 1,448 cattle from 100 herds. In addition, a smaller cross-sectional study sampled 60 dairy cattle from 46 small-holder co-operative dairy farmers in the NWR. Collected blood samples were stimulated with bovine and avian purified protein derivatives, with extracted plasma screened using the IFN-γ enzyme-linked immunosorbent assay (Prionics Bovigam®). Design-adjusted population prevalences were estimated, and multivariable mixed-effects logistic regression models using Bayesian inference techniques identified the risk factors for IFN-γ positivity. Using the IFN-γ assay, the prevalence of bTB in the dairy cattle was 21.7% (95% CI: 11.2–32.2). The design-adjusted prevalence of bTB in cattle kept by pastoralists was 11.4% (95% CI: 7.6–17.0) in the NWR and 8.0% (95% CI: 4.7–13.0) in the VIN. A within-herd prevalence estimate for pastoralist cattle also supported that the NWR had higher prevalence herds than the VIN. Additionally, the estimates of the effective reproductive rate R_t were 1.12 for the NWR and 1.06 for the VIN, suggesting different transmission rates within regional cattle populations in Cameroon. For pastoral cattle, an increased risk of IFN-γ assay positivity was associated with being male (OR = 1.89; 95% CI:1.15–3.09), increasing herd size (OR = 1.02; 95% CI:1.01–1.03), exposure to the bovine leucosis virus (OR = 2.45; 95% CI: 1.19–4.84) and paratuberculosis (OR = 9.01; 95% CI: 4.17–20.08). Decreased odds were associated with contacts at grazing, buffalo (OR = 0.20; 95%

CI: 0.03–0.97) and increased contact with other herds [1–5 herds: OR = 0.16 (95% CI: 0.04–0.55); 6+ herds: OR = 0.18 (95% CI: 0.05–0.64)]. Few studies have used the IFN-γ assay to describe bTB epidemiology in SSA. This study highlights the endemic situation of bTB in Cameroon and potential public health risks from dairy herds. Further work is needed to understand the IFN-γ assay performance, particularly in the presence of co-infections, and how this information can be used to develop control strategies in the SSA contexts.

Keywords: bovine tuberculosis, *Mycobacterium bovis*, interferon-gamma assay, epidemiology, cattle, Cameroon

INTRODUCTION

Mycobacterium bovis infection, the main cause of bovine tuberculosis (bTB), has been reported in many species, including wild and domesticated animals, but has particular importance in cattle (1, 2). Infection with *M. bovis* does not always result in clinical disease, as many animals live with latent infections without clinical signs (3). Subclinical infection is the most common presentation in endemic settings, which is difficult to detect. Importantly, *M. bovis* is zoonotic, with transmission through living in close contact with cattle or consumption of raw milk or other animal products (4). In humans, *M. bovis* infection is estimated to be responsible for 3.1% of human TB cases globally, often linked with immunosuppressive infections like HIV (5, 6). A small sample of human TB cases screened at a central tuberculosis reference laboratory in the NWR identified three *M. bovis* cases in a sample of 175 human TB cases (1.7%; 95%CI: 0.3–4.9) (*Personal communication*), highlighting the potential transmission from cattle to humans. Many sub-Saharan Africa (SSA) communities have close contact with cattle and consume untreated dairy products putting them at increased risk of *M. bovis* infection. The epidemiology of bTB is poorly described in SSA cattle populations, with limited local or national testing/control programmes in place. However, it is important to have a more granular understanding of bTB epidemiology within specific populations to understand the production impacts and human health risks and to target the regional control efforts (7).

The Central African country of Cameroon is an example of an SSA country where cattle are economically and culturally important for many rural and peri-urban communities (8). Historically, cattle production has been undertaken by the Fulani ethnic group, a pastoral community spanning Central and West Africa (9, 10). Cattle keeping is core to Fulani culture, not only for meat and milk production but importantly as financial capital. The Fulani extensively graze *Bos indicus* cattle breeds and many still adopt nomadic seasonal grazing practices known as transhumance. Meat and milk are sold at cattle markets for local consumption and act as a vital source of protein for urban populations. Over the past 20 years, small-scale dairy farmer co-operatives have appeared, particularly in the Northwest Region (NWR) of Cameroon (11). These dairy farmers tend to be from non-Fulani backgrounds and rear small numbers of *Bos taurus* cattle, mainly Holstein-Frisian type animals, semi-intensively in basic stalled housing. Milk is sold through their farmer co-operative to peri-urban communities at local public markets. Bovine tuberculosis has been described as endemic in the country and poses a significant public health risk due to high levels of milk consumption (11, 12).

Despite the potential zoonotic risk of bTB, in Cameroon, meat inspection for TB lesions is the only means of protecting public health, and there is no routine *ante mortem* diagnostic surveillance in cattle populations. To understand the impacts of bTB within Cameroon, it is important to improve our understanding of the local epidemiology, including the prevalence within specific cattle populations, and to highlight potential risk factors or important co-infections to target control measures where needed. Previous estimates of bTB prevalence, using post-mortem examination (PME) and *ante mortem* diagnostics, vary between 0.1 and 40% (13–19) and risk factors for bTB positivity may vary within cattle populations in Cameroon due to differences in management practices but have been minimally investigated.

Abattoir surveys are essential to highlight the presence of bTB within a population, to gather strains for typing and to assess diagnostic test performance. However, they are inevitably biased and are not representative of the cattle population as a whole, and it can be challenging to collect metadata for risk-factor analyses. Post-mortem detection of lesions is also insensitive and may lead to an underestimation of the prevalence, particularly missing early-stage infections (20). *M. bovis* infections predominatly stimulate cell-mediated immunity (CMI) responses in cattle (21–23); therefore, *ante mortem* immunological diagnostic tests are important tools for bTB epidemiological studies as well as test and cull control programmes. Diagnostic tests used to detect part of this CMI response include the *in-vitro* interferon-gamma (IFN-γ) assay and the *in-vivo* tuberculin skin tests [single intradermal test (SIT) and single comparative intradermal tuberculin test (SCITT)] (20, 24, 25). The SCITT is recognized by the World Organization for Animal Health (OIE) as the primary diagnostic test for *ante mortem* bTB diagnosis (26), with a high specificity (median: 99.5%; CI: 78.8–100%). However, the SCITT has a relatively low sensitivity (median: 50.0%; CI: 26–78%) based on a recent meta-analysis (27), resulting in potentially half the infected animals being missed and strongly suggesting its performance is much lower than previous claims (28, 29). The IFN-γ assay also detects the predominant Th1 immune response to *M. bovis*, and can experimentally detect positive animals 2 weeks post-infection (30). Consequently, the IFN-γ assay is reported to have a higher sensitivity (median: 67%; CrI: 49–82%) than the SCITT with slightly lower specificity (median:

98%; CI: 96–99%) (27). A test with a high sensitivity would be advantageous in estimating the prevalence of a disease that has zoonotic consequences in a setting with no control measures (31–33) to minimize false-negative results. Unlike the skin tests, the IFN-γ assay can be used in the field without the need for a return visit and therefore could be valuable in epidemiological surveys in low- and middle-income settings (LMICs).

In this study, we estimated the prevalence of bTB using the IFN-γ assay for pastoralist and dairy cattle populations. We then identified and quantified potential risk factors for bTB in cattle kept in pastoralist herds, critical to our understanding of bTB epidemiology in Cameroonian cattle populations. Finally, we estimated the effective reproductive number using the age-stratified results for each of the two sites.

MATERIALS AND METHODS

Study Population, Sampling Design, and Methods

Pastoralist Livestock Keepers

The study sites were the Northwest (NWR) and Vina Division (VIN) of the Adamawa Region of Cameroon. Both are of similar geographical size of ∼17,000 km^2 (**Figure 1**) (34). A population-based stratified (by sub-location) random cross-sectional survey was conducted between January–May, 2013 in the NWR and September–November, 2013 in the VIN. The participants were pastoralists whose herds were listed in the Ministry of Livestock, Fisheries and Animal Industries vaccination records at 81 local veterinary centers in the NWR and 31 in the VIN in 2012. A total of 5,053 pastoralist herds in the NWR and 1,927 in the VIN, with a range of 1–215 cattle per herd, were included in the sampling frame. The list of herds in each site was stratified by the administrative area; there are seven divisions in the NWR and eight sub-divisions within the VIN giving roughly equal geographical size for logistical purposes. A random sample of 50 herds was taken from each site and sampling was proportional to the total number of herds listed in each of the divisions/sub-divisions within each of the two sites. The sample size was based on a clustered random sample of cattle assuming a cattle level prevalence of ∼10% (35), a within-herd variance of 0.15 and between herd variance of 0.01, an average herd size of 70, a relative sampling cost of 12:1 for herd:cattle and relative error of ±15% (Survey Toolbox; AusVet) (36). This gave a target sample size of 15 cattle per herd and 88 herds, assuming perfect test performance. To allow for potential losses or dropout and to have balanced samples from the two sites, we aimed for 50 herds in each of the two sites in the NWR and VIN. Within each herd, the 15 sampled animals were randomly selected and stratified into three age classes; >6 months to <2 years old (young), 2–5 years old (adult), and older than 5 years (old).

Herds kept by pastoralists were visited either at a site where the cattle grazed or were handled. A local translator explained the project to the pastoralist herdsman or dairy farmer in either Foulfulde, Pidgin English or French. Individuals were asked to give verbal consent to participate in the study in the language in which they were most comfortable. All sampled animals were then examined by the same veterinarian, with data recorded at an individual animal level to accompany samples. These included signalment [sex, age by dentition score (DS), breed and body condition score (BCS)], and if the anthelmintic treatment had been administered in the previous 12 months. The method for aging by DS and BCS was carried out on 5-point scales (35). Dentition score was collapsed into age classifications and animals were categorized as young (<2 years), adult (≥2 and <5 years), and old adult (≥5 years) for the purposes of analysis. "Improved" breed cattle were defined as cattle that had the phenotypic appearance of mixed *Bos indicus* breeding. "Exotic" cattle defined as those cattle that had the phenotypic appearance or which were reported by the farmer to be fully or mixed *Bos taurus*. Plain and heparinised blood samples were collected from the jugular or tail vein of the selected animals. Then, a herd-level questionnaire was administered through an interview in the respondent's preferred language, to collect data on husbandry practices relating to the herd, dairying practices, knowledge and awareness of infectious diseases. The total number of cattle present and location using the GPS coordinates (using Garmin eTrex® Venture) were also recorded.

Smallholder Dairies in the North West Region

The second survey was of the small-scale dairy farmers who were all registered with the Ministry of Livestock, Fisheries and Animal Industries. The address list for 2012 was obtained from their NWR office in Bamenda. Dairy co-operatives were established as part of a non-governmental organization (NGO) initiative in the 1990s to improve milk production in the area. Donated Holstein-Friesian cattle from Ireland and Kenya were imported and given to families to be reared in zero-grazing systems (37). Calves born from these original cattle were then passed on to other members joining the co-operatives. There were 229 dairy farmers grouped into 13 co-operatives with 3–52 farmers per co-operative. The co-operatives were categorized geographically into four groups, with three being sampled as they represented separate co-operatives. The fourth group consisted of four widely dispersed co-operatives and was not sampled for logistical reasons. Thus, 164 dairy farmers were included in the sample frame. A stratified (by co-operative group) random sample of dairy farmers was selected proportional to the number of dairy farmers in each group. Based on the assumption of perfect test performance, a prevalence of ∼6% in adult cattle (35) and each dairy farmer having two adult cows resulting in a sample size of 46 dairy farmers (Survey Toolbox; AusVet) (36). Dairy herds were visited at the homestead, sampled in the same way as pastoral cattle.

Interferon-γ Assay and Diagnostic Tests for Other Infectious Diseases

The IFN-γ assay (31) was carried out following the manufacturer's instructions as follows. Three aliquots of 1.5 ml heparinised blood per animal were incubated with either 15 μl of avian purified protein derivative (PPD), bovine PPD (Prionics® Lelystad Tuberculin PPD) or PBS for 24 h at 37°C within 8 h of sampling. Cultured blood was then centrifuged at 300 *g* for 10 min, plasma aliquotted and subsequently

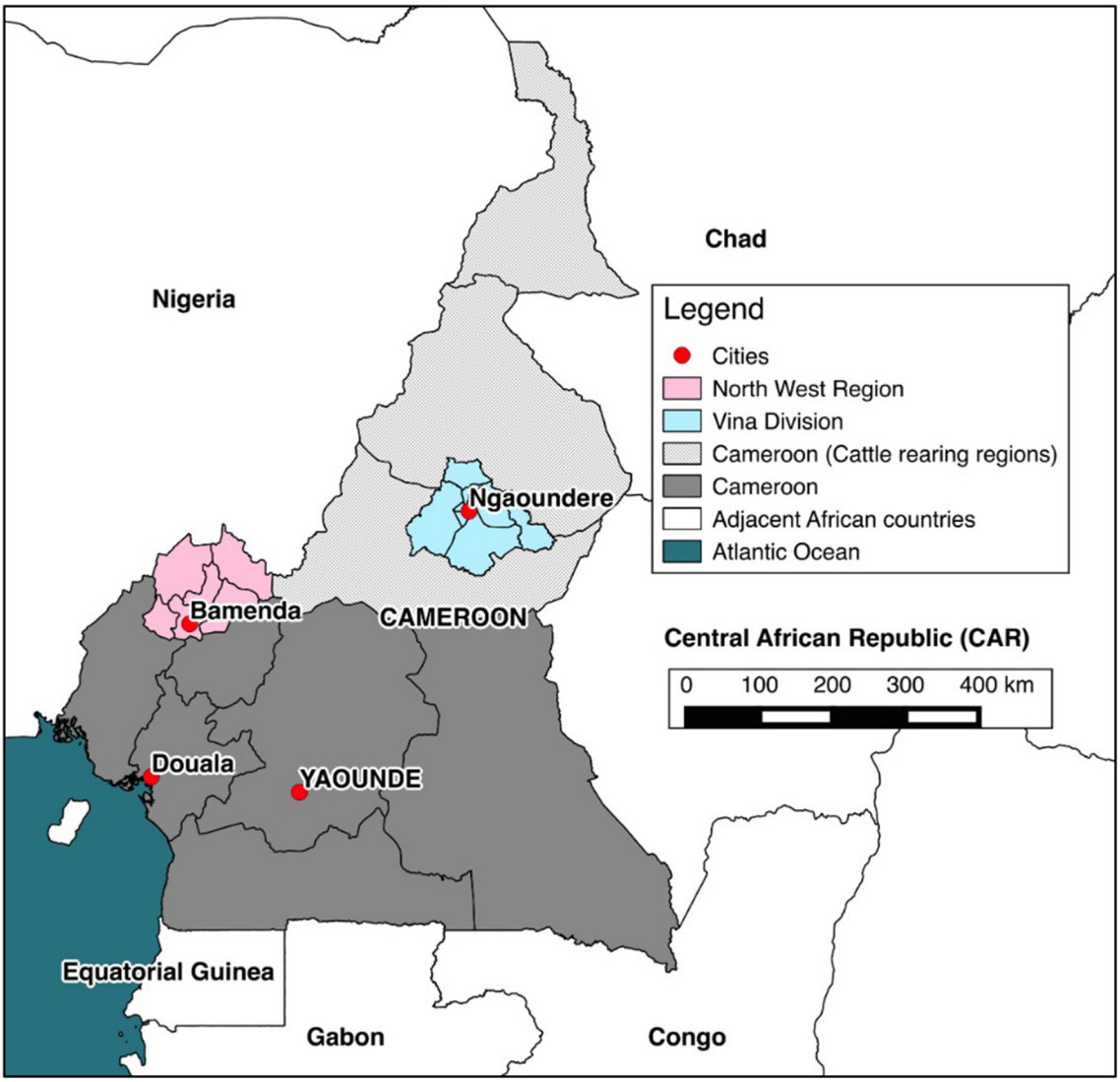

FIGURE 1 | Map of Cameroon. The location of cattle rearing areas (light gray), study sites (pink and blue) and major cities (red).

stored at −20°C in a portable travel freezer. Electrical supplies were maintained by electricity, portable generators or vehicle batteries, wherever required in the field. Plasma samples were transported at −20°C to the Laboratory of Emerging Infectious Diseases (LEID), University of Buea, Buea, Cameroon to conduct the IFN-gamma ELISA. Reagents were reconstituted where appropriate and samples were allowed to reach room temperature (22 ± 5°C). The avian PPD, bovine PPD, and PBS previously stimulated plasma samples were diluted 1:1 with dilution buffer. Diluted plasma samples were added to the pre-coated 96-well plate along with duplicates of positive, negative and PBS control kits. The ELISA procedure followed the manufacturer's instructions (38). By standard interpretation, as per commercial kit instructions, animals with a bovine PPD plasma sample of ≥0.1 than avian PPD and PBS indicate the presence of *M. bovis* infection.

As part of the risk factor analysis, we were interested in the associations with co-infections, especially infections that may be immunosuppressive [bovine viral diarrhea (BVD), bovine enzootic leukosis (BLV)] or could potentially influence the interpretation of the IFN-γ assay [*M. avium subspecies paratuberculosis* (paraTB)]. After collection, all serum samples were heat-treated at 56°C for 120 min and stored at −20°C until tested. Serum samples were then tested for exposure to various infections using serological ELISAs. Screening for antibodies to BVD Virus (BVDV) was conducted using the ID Screen® BVDV Serum Competitive ELISA (39) following the manufacturer's instructions. The competition percentage (S/N%) was calculated as the OD for the sample divided by the OD for the negative control times 100 to convert to a percentage. The manufacturers suggest samples S/N% ≤40% are considered positive; S/N% >40 and ≤50% are considered

inconclusive and S/N% >50% are considered negative. For this study, samples S/N% ≤40% were considered positive and S/N% >40% were considered negative. Screening for Bovine Leukemia Virus (BLV) exposure was conducted using the ID Screen® BLV Serum Competitive ELISA (40) following the manufacturer's instructions. The competition percentage (S/N%) was calculated as for the BVDV ELISA. The manufacturers suggest samples S/N% ≤50% are considered positive; S/N% >50 and <60% are considered inconclusive and S/N% ≥60% are considered negative. For this study, samples with an S/N% ≤50% were considered positive and those with an S/N% >50% were considered negative. Screening for antibodies to MAP was conducted using the ID Screen® Paratuberculosis Serum Indirect Multi-species ELISA (41) following the manufacturer's instructions. The sample to positive ratio (S/P%) was calculated by (ODsample—ODNC)/(ODPC—ODNC) × 100. The manufacturers suggest samples S/P% ≤60% are considered negative; S/P% >60 and <70% are considered inconclusive and S/P% ≥70% are considered positive. For this study, samples S/P% <70% were considered negative and S/P% ≥70% were considered positive.

Statistical Analysis

All information was initially recorded onto paper forms, which were later transferred into a relational Access database (Microsoft Access®). The herd-level descriptive results have been published previously (34). All statistical analyses were performed using packages and functions in R version 3.6.1 (42). Graphics were produced using the *ggplot2* package (43). Spatial data were displayed using the QGIS 2.2® (44) or *tidyverse* collection of R packages (45) and shapefiles obtained from the GADM database of Global Administrative Areas (www.gadm.org). The structure of the pastoralist survey was incorporated into analyses using the *svydesign, confint,* and *svyby* functions in the *survey* package (46). This allowed the stratified study design to be accounted for in the prevalence estimates. Confidence intervals (CI) and Bayesian credibility intervals (CrI) are reported at a 95% level throughout and statistical significance was defined at *P*-values ≤0.05. Throughout the paper, we refer to the apparent prevalence as the "prevalence" for ease of reading. The "true" prevalence after adjusting for the test imperfections can be crudely approximated by multiplying the reported prevalence estimates by 1.5.

The effective reproductive number (R_t) was estimated as follows. The average life expectancy was estimated as $\frac{1}{\mu} = \sum_{x=1}^{\infty} l_x$, where l_x is the survival rate at age x and is calculated as the ratio of the number of animals at age x divided by the number of animals in age class 1 (animals aged up to 1 year old). For an individual of age a, the standard SIR (susceptible, infectious and recovered) model predicts that the probability that an individual is still susceptible is given by $S(a) = \exp[-a\mu(R_t-1)]$, where a is the animal's age and μ is 1 over the average life expectancy. As the numbers of the susceptible and infectious are binomially distributed, the likelihood function of these numbers was obtained as a function of R_t. R_t was then inferred as the value that maximized the logarithm of this likelihood function:

$$log\, L\,(R_t) = \sum_{i=0}^{n} \log\left(\exp\left(-a_i\mu\,(R_t-1)\right)\right) + \sum_{i=0}^{m} \log(1-\exp\left(-b_i\mu\,(R_t-1)\right))$$

where n is the number of susceptible animals (sero-negative) of age $a_1, \ldots, a_n$, and m is the number of sero-positive animals (ages $b_1, \ldots, b_m$) for all animals (47). Due to the homogeneity of the dairy cattle sample, this analysis was only conducted for pastoralist cattle.

Risk factors for IFN-γ assay positivity were investigated in pastoralist cattle using multivariable mixed-effects logistic regression models run using a Bayesian framework. The outcome was the individual animal bTB IFN-γ status. A backward stepwise model-building approach was used for model specification (48). Explanatory variables of interest collected using the herdsman questionnaire were identified *a priori* by the authors and included host factors, husbandry-related features and risk contacts based on their presumed influence on the risk of exposure to *M. bovis*. Specifically, among the variables of interest were contacts with wildlife, such as warthogs, antelope and buffalo, going on the seasonal transhumance, the size of the herd and the number of other herds contacted at grazing. IFN-γ status with exposure to co-infections was also explored, including BVD, BLV and paraTB. Age, sex and site (NWR or VIN) were included as potential confounders. Non-informative prior distributions for the parameters were used as recommended by Gelman et al. (49), including the intercept and the random effect (herd). The data and the priors were combined to estimate a posterior distribution using Markov Chain Monte Carlo (MCMC) scheme. The Watanabe-Akaike Information Criteria (WAIC) and Leave-One-Out CrossValidation Criteria (LOO-IC) were used to compare the models and select the optimal model (the model that better describes the outcome balancing parsimony and fit). Model convergence and stability checks were performed through the inspection of trace and autocorrelation plots of the MCMC samples for each chain. Graphical analysis of binned residuals was used to perform a predictive posterior check (50). Finally, a receiver operating curve (ROC) was used to test the ability of the model to discriminate between IFN-γ positive and negative animals. This was built using the *pROC* package (51). The Bayesian models were implemented using functions included in the *brms* package in R; this package uses "Stan" for full Bayesian inference (52). Posterior distributions were estimated for each explanatory variable and their effects are summarized using the mean and 95% Credible Intervals (CrI). The exponential transformation was used to generate the odds ratio (OR) for epidemiological interpretation.

Ethical Statement

The study was reviewed and approved by the University of Edinburgh Ethics Committee, UK (ERC No: OS02-13) and by the Institute of Research and Development (IRAD), Cameroon. All participants gave informed verbal consent to the translator before participating and could opt-out at any stage.

RESULTS

In total, 100 pastoralist herds were recruited: 50 in the NWR and 50 in the VIN. Of these 100, 23 were replacements from the same veterinary center for herds that declined or were unable to participate in the study. All 46 selected dairy farmers participated and none were replaced. In total, 750 pastoralist kept cattle were sampled from 50 herds (15 per herd) in the NWR and 748 from 50 herds (14–15 per herd) in the VIN. In the dairy cross-sectional study, 60 cattle (1–4 per herd) were sampled from 46 dairy farmers. For pastoralist cattle, there were equal numbers of males and females in the young age group and mainly females in older age groups, as expected with the slaughter of males from 3 to 4 years of age for consumption (**Figure 2**). The dairy herds were very small, and all animals in a herd were sampled. A detailed summary of animal- (53) and herd-level data (34) have been previously published.

Prevalence of IFN-γ Positive Pastoralist and Dairy Cattle

The overall animal-level true prevalences were 11.4% (95% CI: 7.6–17.0) in the NWR, 8.0% (95% CI: 4.7–13.0) in the VIN, and the apparent prevalence in dairy animals in the NWR was 21.7% (95% CI: 11.2–32.2). Due to the homogeneity of the dairy cattle sample, the remainder of the analysis focused on pastoralist cattle (NWR and VIN). A within-herd prevalence estimate generated from the raw proportions and the distribution across the NWR and VIN pastoralist herds are presented in **Figure 3A**. The individual animal status grouped by herd and ordered by within-herd prevalence is presented in **Figure 3B**. The trend demonstrates that the NWR appears to have higher prevalence herds than the VIN, which is consistent with the overall prevalences.

The age-stratified prevalences are presented in **Figures 4A,B**. There appears to be little increase in prevalence with age in the VIN, while there is a slight increase with age in the NWR with the slope of the prevalence (linear regression coefficient = 0.009) statistically >zero ($P = 0.03$) based on a simple linear model of prevalence by age. This lack of increase with age may suggest a relatively low force of infection (FOI) meaning most cattle acquire infection in their first year of life. The FOIs (blue lines **Figure 4B**) are not consistent with a constant FOI and this supports the hypothesis that most transmission occur when the animals are very young. The estimates of the effective reproductive rate R_t were 1.12 for the NWR and 1.06 for the VIN, adding to the evidence that there are potentially different endemic patterns in the different sites and that there is a higher transmission rate in the NWR.

The design-adjusted prevalences by division/sub-division are given in **Table 1** and mapped in **Figure 5**. They show a wide range of IFN-γ prevalences, with a very low prevalence in Mbé in the north of the VIN and a high prevalence in Menchum in the northwest of the NWR.

Risk Factors for bTB in Pastoralist Cattle in Cameroon Based on the IFN-γ Assay

A multivariable logistic regression model was developed to explore host, husbandry and contact risk factors for IFN-γ positivity (**Figure 6**). The final model suggests that bulls have almost twice the odds of being IFN-γ positive compared to cows (OR = 1.89; 95% CI:1.15–3.09). Contact with buffalo was associated with a reduced risk (OR = 0.20; 95% CI: 0.03–0.97),

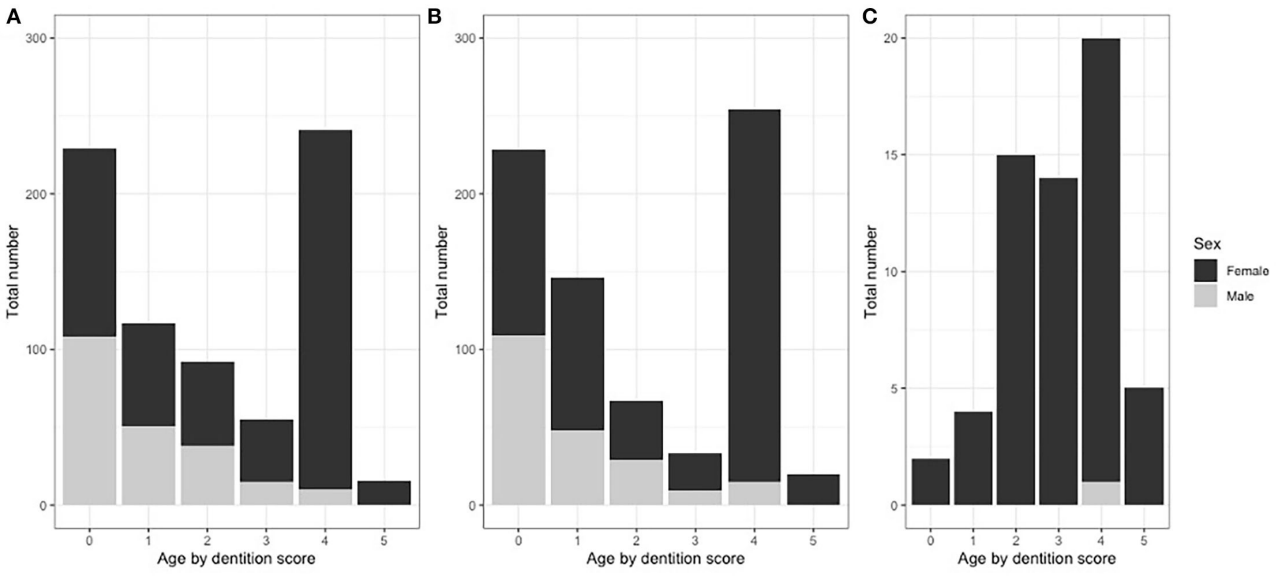

FIGURE 2 | Proportions of cattle sample by dentition score, sex and study site grouping. **(A)** NWR pastoral cattle ($n = 750$), **(B)** VIN pastoral cattle ($n = 748$), and **(C)** NWR dairy cattle ($n = 60$).

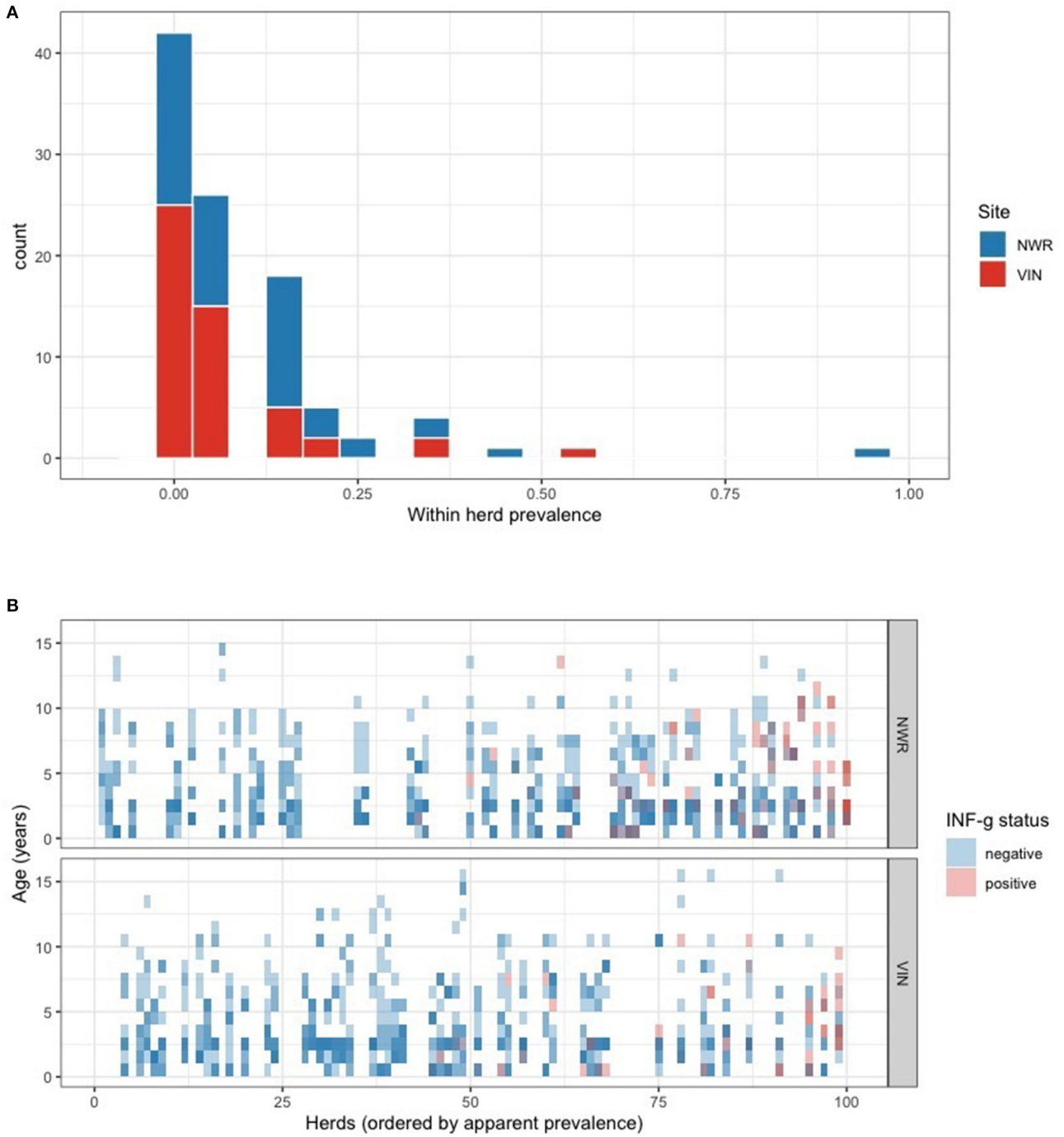

FIGURE 3 | (A) Within-herd IFN-γ positivity prevalence distribution stratified by the two pastoralist sites in the NWR and VIN. **(B)** Tile plot showing each individual animals IFN-γ positivity status (blue = negative, red = positive). Animals are grouped together in vertical columns according to their herd and the herds are ordered from right to left from highest to lowest within-herd prevalence.

and contact with antelope was statistically significant (OR = 0.63; 95% CI: 0.31–1.26). The odds of positivity increased with herd size (OR = 1.02 for each additional herd contacted on an average day; 95% CI:1.01–1.03), and there was an unexpected protective association between the number of herds contacted at grazing and positivity [1–5 herds: OR = 0.16 (95% CI: 0.04–0.55); 6+ herds: OR = 0.18 (95% CI: 0.05–0.64)]. Finally, there was no evidence of an important association with BVD exposure, but there was increased odds of positivity if an animal was also seropositive to BLV (OR = 2.45; 95% CI: 1.19–4.84) or paraTB

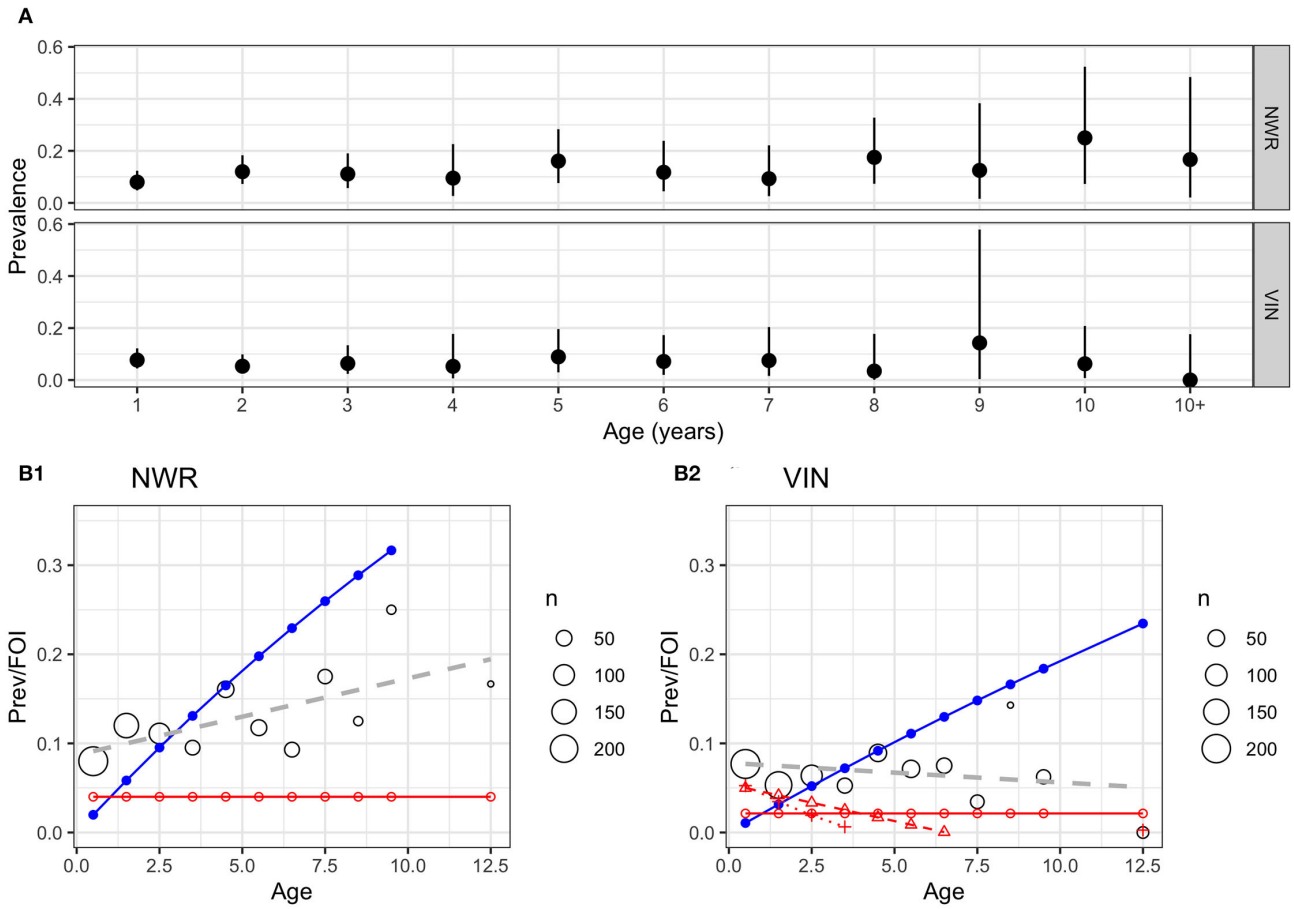

FIGURE 4 | (A) Age-stratified IFN-γ positivity prevalence by age (years) with unadjusted 95% CI for the two pastoralist cattle keeping sites in NWR and VIN. **(B1,B2)** Age-stratified prevalence of IFN-γ positivity prevalence in cattle in two sites in Cameroon in 2013. The predicted prevalence based on a simple linear age function (blue line) and a constant force of infection (λ) (red line) based on the Muench model. The black circles show the mean prevalence for that age strata with the size proportional to the number of animals in that age strata and a simple linear regression model of IFN-γ positivity with age in years (gray dashed line).

(OR = 9.01; 95% CI: 4.17–20.08). The area under the ROC curve for the final averaged models was 0.86 (95% CI: 0.82–0.89), indicating good model performance in relation to classifying IFN-γ assay positive cattle using this dataset.

DISCUSSION

This study revealed there was a high prevalence of bTB based on the IFN-γ assay, which is comparable to the previously reported estimates in other regions of SSA (54–57). This was the first time the test was used in Cameroon. There was also marked variation across the administrative regions. The assay has the advantage of requiring only a single visit to an animal that has obvious logistical advantages in these remote settings. Laboratory facilities to incubate and harvest the plasma within <8 h should not be underestimated when using the diagnostic in remote field settings.

The high level of IFN-γ assay positivity is likely to reflect a high prevalence of *M. bovis* infection across all cattle populations where there is a lack of control measures. We previously highlighted concerning levels of lesions detected at slaughter in our associated abattoir-based studies in NWR and VIN (35). Interestingly, the lesion-based studies in the abattoir showed a higher prevalence of bovine TB lesions at the Ngaoundere abattoir in the VIN (11.33%) compared to that in the Bamenda abattoir in the NWR (3.99%). The finding here shows the reverse, a lower positivity on the IFN-γ assay in the VIN. This is likely due to the difference in the age structure of cattle taken to the abattoir as discussed in our earlier work, i.e., at the Ngaoundere abattoir majority of the slaughtered animals were older female animals. Consequently, differences in prevalence estimates between abattoir studies and stratified cross-sectional studies could be influenced by differences in slaughter practices between the two study sites and catchment area of abattoirs being beyond the identified study area. Earlier reports using the SCITT (13) also showed a higher animal-level prevalence in the NWR (5.58%, 95% CI: 3.89–5.44%) compared to the VIN (2.57%, 95% CI: 1.42–3.72) (58), which is consistent with the current observations. Indeed, related cross-sectional studies show a similar relationship, with prevalence in the NWR (20.95%) being

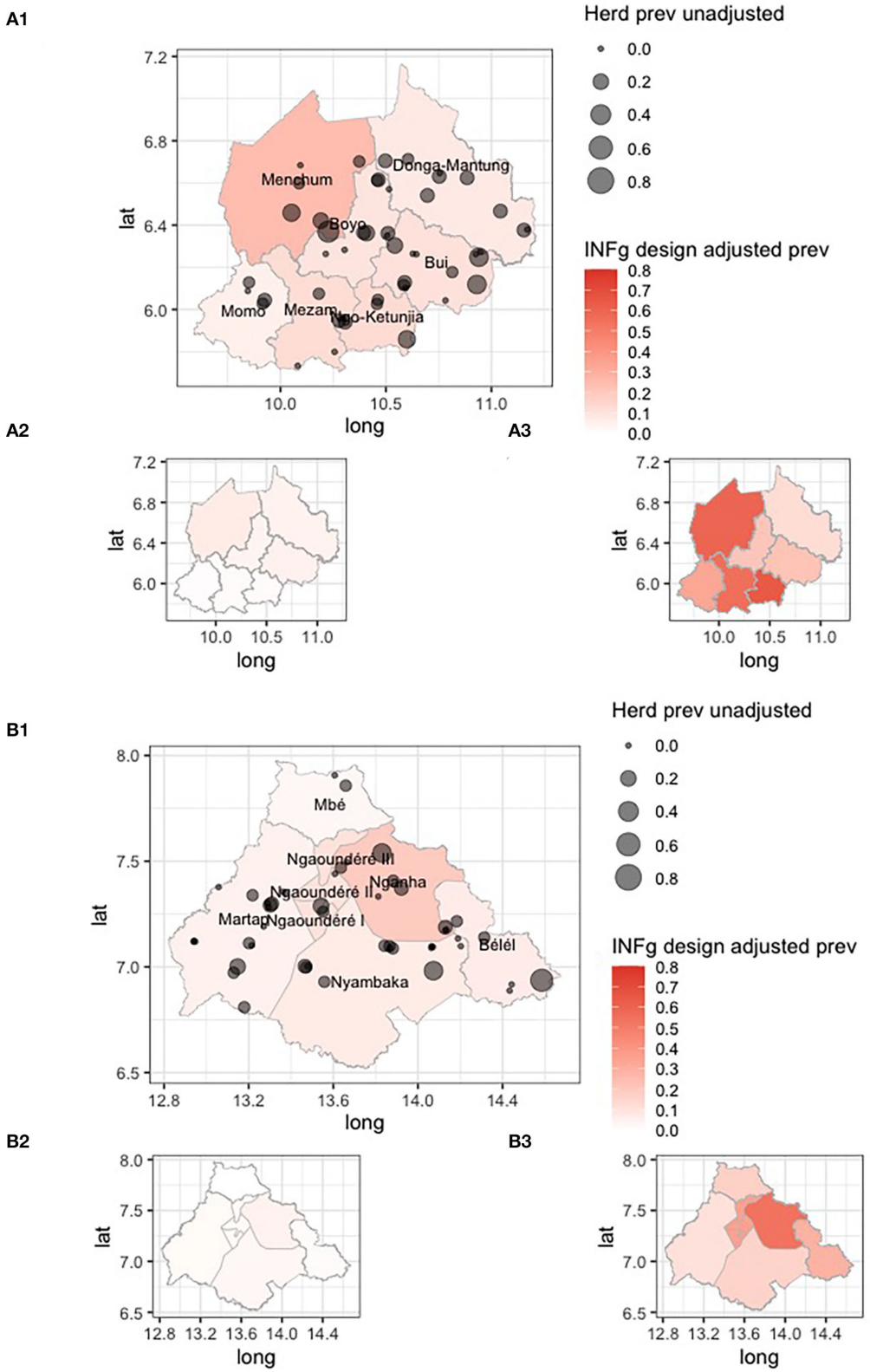

FIGURE 5 | Choropleth maps of the Northwest Region (NWR) **(A1)** and Vina Division (VIN) **(B1)** in Cameroon colored by design adjusted IFN-γ prevalence for the administrative strata, overlaid with the approximate location of individual herds sized by the raw proportion of animals positive within each herd. The smaller inset choropleth maps are for the lower **(A2,B2)** and upper **(A3,B3)** 95% confidence intervals, respectively.

TABLE 1 | Design-based animal-level seroprevalence (not adjusted for test performance) of RVF antibodies in cattle in two sites in Cameroon in 2013 stratified by Division (NWR) and sub-Division (VIN).

Division/sub-division	Raw proportions (positive/sample)	Design-based seroprevalence (%)	Design-based 95% CI (%)
Northwest region (NWR)			
Boyo	8/90	9.6	4.0–21.0
Bui	18/195	10.9	5.1–21.7
Donga-Mantung	14/180	7.8	4.8–12.4
Menchum	15/75	24.6	6.8–59.2
Mezam	19/105	12.6	1.6–55.3
Momo	4/60	5.4	0.7–33.1
Ngoketunjia	6/45	13.1	1.3–64.2
Vina division (VIN)			
Belel	12/150	6.7	1.3–28.5
Martap	11/255	5.0	2.2–11.0
Mbé	1/30	2.7	0.0–17.3
Ngan-Ha	8/73	19.2	4.7–53.4
Ngaoundere	5/60	10.1	2.3–35.2
Nyambaka	12/180	6.9	2.8–15.7

greater than in the VIN (0.55%), although this difference seems extreme compared to the other studies (53–56). Some of the differences with the current study can be explained by the study design, diagnostic test or cut-off value differenced; therefore, a direct comparison should be made with prudence and care. For example, differences in prevalence estimates between abattoir studies and stratified cross-sectional studies could be influenced by differences in slaughter practices between the two study sites and catchment area of abattoirs being beyond the identified study area.

This is the first report of bTB in the dairy cattle population from the NWR, and it indicates a potential public health problem of *M. bovis* transmission in urban populations. Particularly considering the relatively poor understanding of bTB and limited pasteurization practices in this group of livestock keepers may facilitate transmission to urban human popualtions where demand for dairy products is on the rise (34).

A key question is whether bTB has reached some sort of endemic stability or is in an epidemic phase in Cameroon. It is probably important also to view the country as a complex network of transmission, driven by local on cattle movements. In this regard, one would expect that bTB in cattle populations from NWR should be stable, given the geographical isolation from other areas of cattle trade (8). On the other hand, a highly dynamic disease pattern in VIN given the continuous cattle movement due to transhumance from the Central African Republic, Chad toward the western Sahel (59). Previous studies have analyzed the market network in Cameroon, and it was very consistent with this notion (8, 59). The molecular analysis also revealed a high *M. bovis* strain diversity mostly due to singleton strains in the VIN with limited evidence of cattle population overlap between VIN and NWR (60). The conclusion from our previous work was that lower rates of transmission were driving the locally expanding infections of bovine tuberculosis in NWR and was characterized by a lower strain diversity dominanted by SB0953 spoligitype (60). The local expansion of bovine tuberculosis in NWR could be due to a high density of cattle population in this isolated region, which increases the opportunity of contact between cattle likely infected with SB0953 spoligotype. Unlike other studies (61–63), there is no increased infection rate with age, which could be because our study did not have the statistical power to detect this if it exists, or indeed this could represent an early-in life exposure to the disease. The VIN exhibited the opposite, a lower ERN, a higher diversity, and a lower recent transmission index, which likely represents a stable endemic status of bovine tuberculosis.

The use of IFN-γ assay highlights significant infection of Cameroonian cattle with *M. bovis* and provides an opportunity to investigate potential risk factors associated with infection. This is particularly interesting because IFN-γ assay can detect *M. bovis* infection weeks or months earlier than the SCITT test (23, 64). The analysis showed that female cattle had lower odds of being identified as IFN-γ positive. This observation is in contrasts with findings from the previous abattoir study, where female cattle were twice as likely to be lesion positive (35). This could be because female cattle are kept longer for reproductive purposes, which means they can be chronically infected, i.e., with gross lesions visible at abattoir inspection but anergic to the IFN-γ assay immunologically. It is also very clear there was a marked difference in the age structure of the populations observed passing through the abattoir compared to at pasture.

Contact with wildlife was associated with lower odds of being IFN-γ positive, i.e., pastoralist cattle grazing with antelope and buffalo was protective, so such cattle were less likely to test positive of the IFN-γ. Although susceptibility of Cameroonian antelope species has not been specifically investigated, antelope species have been reported infected with *M. bovis* (65) like buffalo in South Africa (66), and in some cases implicated as part of the transmission cycle in parts of SSA (67). It is not clear why, therefore, contact with wildlife should be

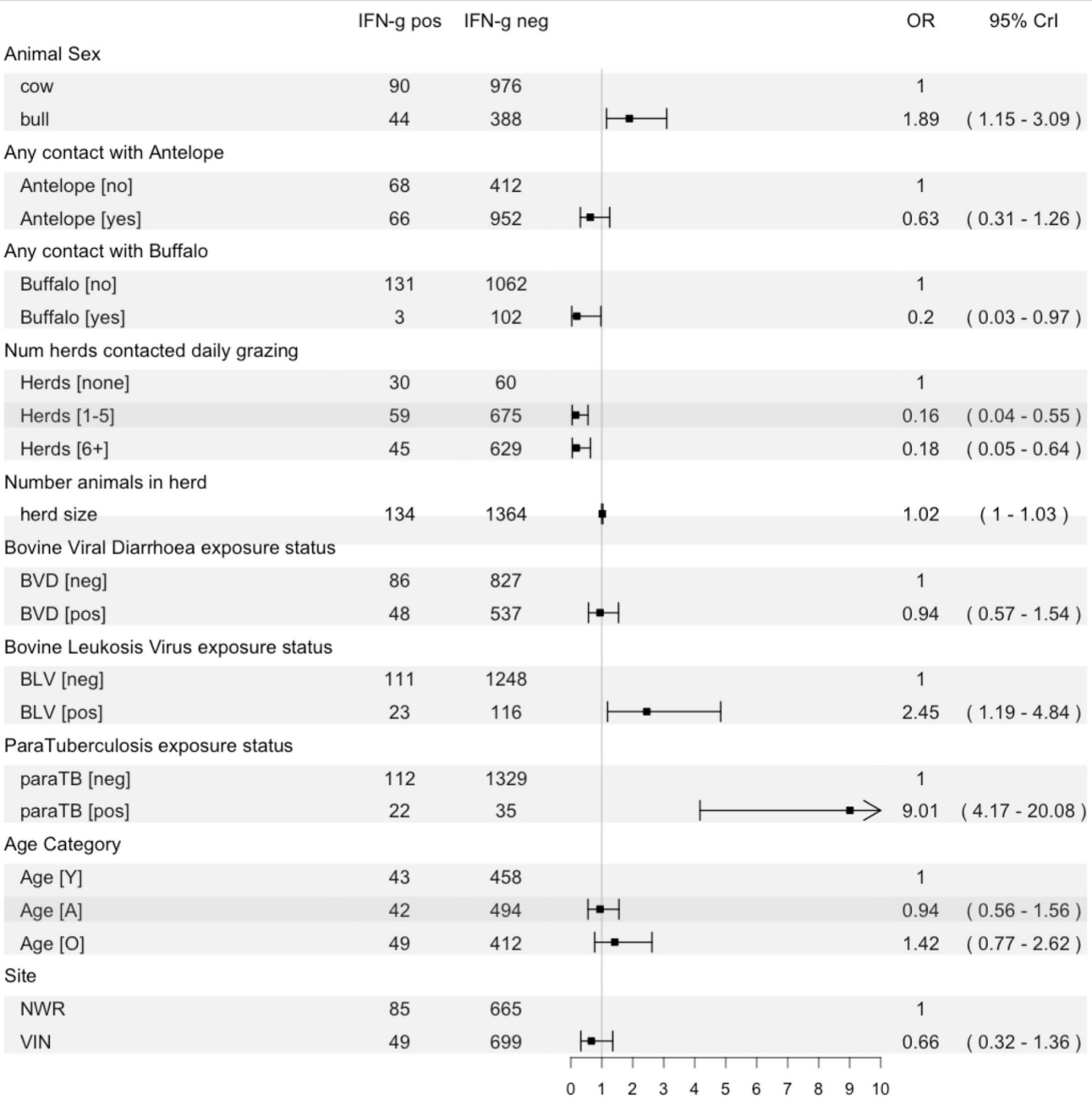

FIGURE 6 | Forest plot showing the fixed effects in the final multilevel model of risk factors for IFN-γ positivity with the raw counts for each variable, a plot of the odds ratio with 95% CrI and the numeric result and CI.

protective but could be related to cattle being grazed in remote areas where there is generally increased space and reduced stocking density. Buffalo are only found in the remote areas of Cameroon, and herds visiting these areas are at much lower densities potentially, explaining this negative association (author observation). Statistically, the associations are non-significant for antelope and, although significant for buffalo, the number of cattle in contact were extremely small, which may reflect a false association. However, risk of other zoonotic bacterial infections has been positively associated with wildlife contact, such as *Brucella species* (53, 68). Consequently, it is difficult to interpret the real importance of wildlife contact and *M. bovis* transmission, with future studies needing to focus on the multiple species to understand transmission dynamics.

Similarly, the association between increasing numbers of contacts at grazing and decreased odds of positivity is difficult to interpret as it seems counter-intuitive. Extensive management practices may reduce the transmission pressure of *M. bovis*

transmission compared to intensive cattle rearing systems (69). As most cattle in this study are grazed on communal pastures (34), contamination from *M. bovis* may be diluted by large grazing areas (56) and sunlight desiccation of *M. bovis* (70). It is possible that the increasing number of contacts at grazing may be a proxy for environmental factors or extensive management of pastoral cattle that may reduce *M. bovis* transmission not captured by the questionnaire. Supported by the result that a larger herd size was associated with increased odds of positivity, as would be expected in most infectious diseases (71) usually related to increased infection pressure if an infected animal is present. Future surveillance studies involving cattle, other livestock and free-roaming animals are required to explore the potential dynamics of infection between domestic, wildlife species and their environment.

A particularly interesting result is the association between BLV and paraTB exposure status/seropositivity and IFN-γ/bTB infection status. Given the cross-sectional nature of this study, we were not able to determine the sequence of exposures to know if, for example, BLV preceded exposure to bTB and this induced immunosuppression making the animal more susceptible. There is not much mentioned in the literature about co-infection with BLV (28), hence it is difficult to interpret the full impact of this result. More is understood about the association of paraTB [caused by *Mycobacterium avium subspecies parastuberculosis* (MAP)] and bTB, for example, it has been observed that in populations with higher rates of paraTB or related non-tuberculous mycobacteria (NTM), the IFN-γ test specificity poorly lead to a large number of false positives (72, 73). The very strong association observed here may be more to do with the impact of MAP or NTM co-infection on test performance than the actual risk of infection or disease. Overall, these results highlight that without controlling for exposure to other co-infections, when interpreting the IFN-gamma assay results, are likely to hinder the progress of future bTB surveillance in Cameroon.

Using IFN-γ assay in a representatively sampled naturally infected cattle population, we were able to explore the prevalence and factors which influence bTB positivity in Cameroonian pastoralist cattle populations. We were unable to do this for smallholder dairy cattle due to the relatively small sample and homogeneity of this cattle population. Overall, there is a need for the assessment of IFN-γ assay diagnostic test performance in all Cameroonian cattle populations using Bayesian non-gold standard methods to improve the accuracy of prevalence estimates and risk factor identification. As a follow-up to this work, we are developing a mathematical model integrating molecular and immunological parameters to further unravel the dynamics of bovine tuberculosis in Cameroon.

CONCLUSION

Bovine tuberculosis is endemic in pastoralist and dairy cattle populations in Cameroon. Its transmission may be related to a wide range of differences in cattle-keeping practices within the country. Here, we present evidence supporting our previous work in Cameroon on the difference in epidemiological dynamics at play in the two sites. The current study suggests that these two settings are associated with high and low IFN-γ assay positivity rates, respectively. This assay could be of value in LMIC settings where the need for a single visit only has substantial logistical and financial implications. However, further work is needed to understand the associations observed between co-infections with BLV and paraTB and the potential impact on test performance that might need to be accounted for in interpreting the use of the IFN-γ assay in these populations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants and animal study was reviewed and approved by the University of Edinburgh Ethics Committee, UK (ERC No: OS02-13) and by the Institute of Research and Development (IRAD), Cameroon. All participants gave informed verbal consent to the translator before participating and could opt out at any stage. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent for participation was not obtained from the owners because many of the participants were illiterate so verbal consent was granted and recorded on paper questionnaires.

AUTHOR CONTRIBUTIONS

BB, LN, and VT conceived the original project. RK, KM, LN, IH, VT, MS, and NE designed the field study, the databases, and the survey instrument. RK, NE, VN, VT, KM, and BB developed the field SOPs and collected the data. RK, EF, and LG conducted laboratory work. RK and BB cleaned the initial dataset. RK, LG, BB, IH, and SM contributed to the analysis. RK was responsible for writing the initial drafts. All authors contributed comments for the final draft.

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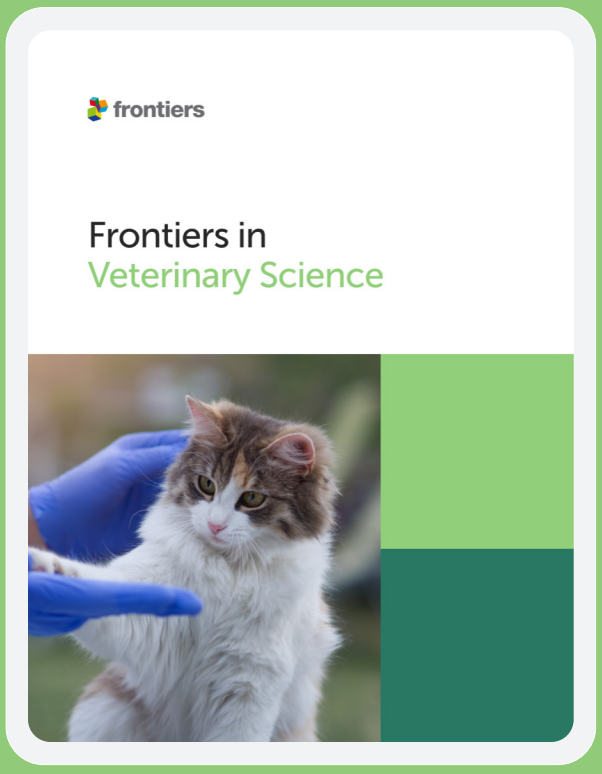

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