RECENT ADVANCES IN BOVINE TUBERCULOSIS

EDITED BY: Federico Blanco, Flabio R. Araujo, Jacobus Henri De Waard and Christophe J. Queval

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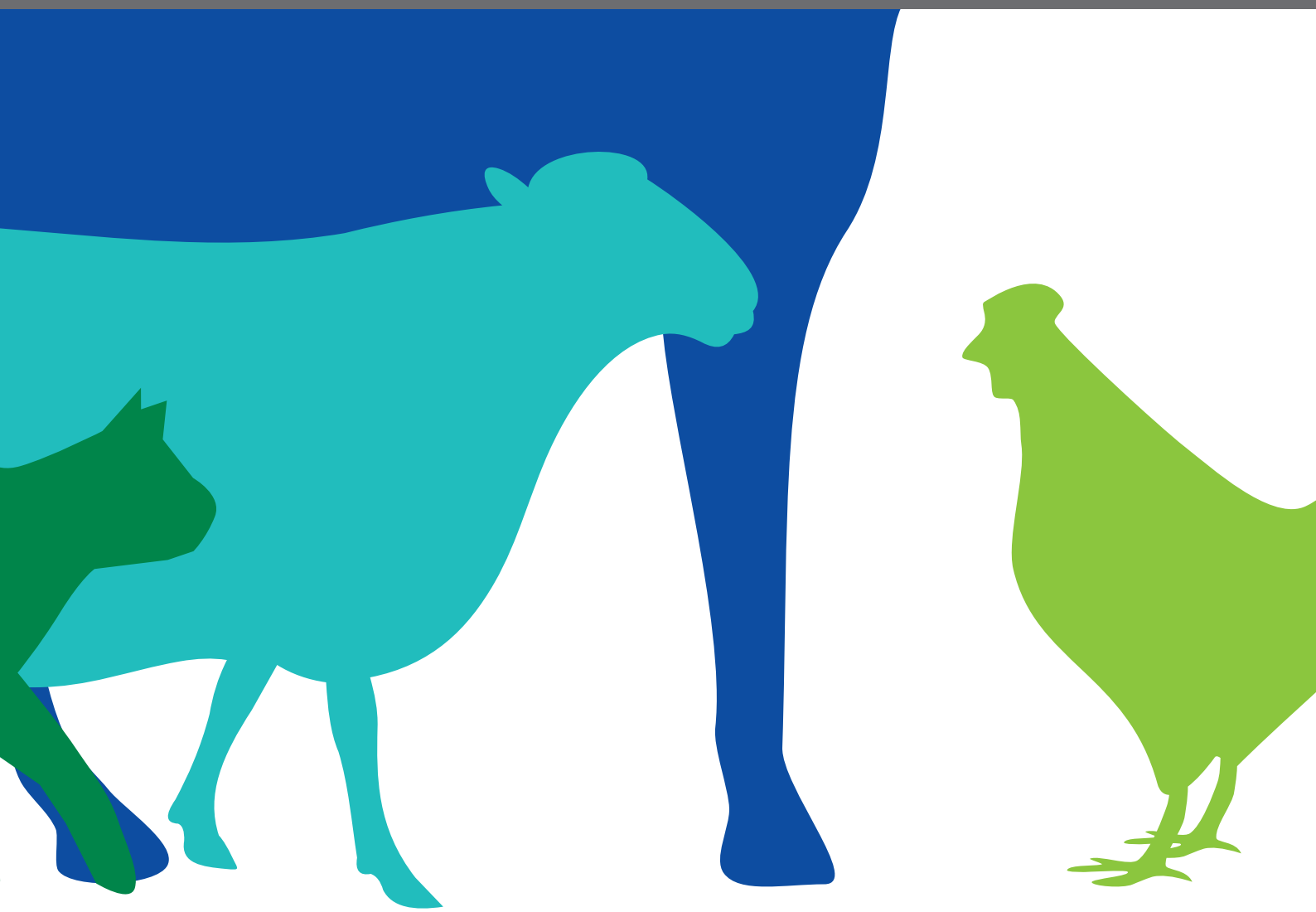

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RECENT ADVANCES IN BOVINE TUBERCULOSIS

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Table of Contents

frontiers | Frontiers in Veterinary Science

EDITORIAL
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Editorial: Recent Advances in Bovine Tuberculosis

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Keywords: ***Mycobacterium bovis***, **bovine tuberculosis, one health, diagnostic, immunology, vaccination, epidemiology**

Editorial on the Research Topic

Recent Advances in Bovine Tuberculosis

According to the World Organization for Animal Health (OIE), bovine tuberculosis (bTB) is a chronic infectious disease in animals caused by members of the *Mycobacterium tuberculosis* complex, and in most cases by *Mycobacterium bovis* (Mb) (1). It is a zoonotic disease, where cattle are the main source of infections for humans. Mb also affects other animals such as goats, sheep, cats, and dogs, as well as wild and zoo animals (2). Moreover, other species are recognized as Mb maintenance hosts in several countries: wild boar in Iberia, the European badger in Great Britain and Ireland, the African buffalo and Marsh antelope in Africa, brushtail possum in New Zealand, and white-tailed deer in the USA (3). The eradication of the disease is a major problem and policies of surveillance and control have been applied in several countries using screening and stamp-out strategies. As part of the One Health concept, bTB is a priority disease and all efforts to eradicate bTB should be compulsory (4). However, the policies enacted to eliminate this disease can have serious financial impacts on farmers. Therefore, one of the most challenging tasks in eliminating bTB is to provide accessible solutions at an affordable cost that can easily be implemented in developing countries where, in general, the prevalence of bTB in animals and humans is high.

This special issue of Frontiers in Veterinary Science is centered on the latest discoveries made in the field. It comprises 16 articles reporting on epidemiology, diagnosis, vaccination, pathology, and host-pathogen interactions and Immunology. The majority of the articles are focused on cattle. However, there are three major studies involving other hosts of Mb; goats, buffaloes, and deer. Concerning goats, the implementation and advances of the diagnosis with serology in oral fluid are discussed (Ortega et al.). The use of overlapping peptides representing the ESAT-6, CFP-10, and Rv3615c antigens in single intradermal skin test (SIT) has been used for diagnosis in buffaloes by Kumar et al. In Ireland, sika deer have been identified as a reservoir for bTB and should be considered an integral part of the bTB-control program (Kelly et al.). The zoonotic transmission of Mb from cattle to humans has been recognized for a long time, but transmission from humans to cattle is less often recognized. In this special issue, there is a report of two human/cattle transmission cases in North Dakota, USA (Lombard et al.).

Relating to the diagnosis, the oldest and most used method for bTB diagnosis is the intradermal skin test (Good et al.). Its main disadvantages are the variable sensitivity and specificity, as shown in several publications (5). Moreover, tuberculosis vaccination strategies interfere with this test as animals get sensitized, causing false positives (6). The interferon-gamma release assay (IGRA), based on the quantification of this cytokine in the supernatant of whole blood cultures, stimulated *in vitro* with Mb antigens, has been approved in several countries as a complementary diagnostic

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method (7). Different antigens can be used for the *in vitro* stimulation, wherein purified protein derivate (PPD) and peptide cocktails to acquire a higher specificity are the most common ones. In this special issue, investigators from Switzerland evaluated the specificity of three commercial IGRAs in different cattle breeds from bTB-free herds (Ghielmetti et al.).

Molecular techniques for detecting the genetic material of Mb have been developed and are used for diagnosis but also for the epidemiology of bTB. These techniques can be applied in order to detect Mb in blood, tissue, or milk and its derivatives. In one of the articles presented in this collection, researchers from Spain applied Real-time PCR (qPCR) for the direct detection of Mb in lymph nodes from carcasses obtained at the slaughterhouse. The agreement between microbiological culture and this qPCR was almost perfect (Sánchez-Carbajal et al.). Another article in this collection used whole-genome sequencing (WGS) and characterized the diversity of Mb genotypes circulating in Baja California, Mexico, with Mb isolates recovered from humans, cheese, and cattle, providing strong evidence of human-to-cattle-to-cheese transmission (Ortiz et al.). WGS, together with Single Nucleotide Polymorphism (SNP)-analysis, was also applied in the Brazilian Amazon region to analyze Mb inter-species transmission between cattle and buffaloes. Additionally, a new Mb lineage (Lb1) for South America was detected (Carneiro et al.).

Acknowledging the distribution, population structures, and transmission networks of the different Mb isolates in every region of the world is crucial for identifying the source of bTB infection, its transmission dynamics and host preference, the influence of wildlife reservoirs on transmission to cattle, and thus the implementation of effective strategies to contain it. For the genotyping of Mb, the spoligotyping technique is currently considered the gold standard. Nonetheless, this a lengthy and technically demanding method. In an article from Brazil, an alternative method for the genotyping of Mb is presented: Multispacer Sequence Typing, based on sequencing. This is more accessible for most laboratories (Bravo Sales et al.). Spoligotyping and 24 loci MIRU-VNTR typing were used in an article from the state of Pernambuco, Brazil, showing high variability of Mb strains in this region (30 strains and 15 different genetic profiles), indicating several introductions of Mb strains and consequently a failing TB control program (Melo et al.).

In this special issue, the virulence of Mb has been addressed with molecular biology techniques for a better understanding of the functional impacts of the nucleic acid differences between Mb and *M. tuberculosis* (Mt). Whole-genome transposon libraries in laboratory strains of both species were generated and the essentiality status of genes during growth under identical *in vitro* conditions was assessed (Gibson et al.). For growth, 527 genes were found to be essential in Mb, whereas 477 genes were essential in Mt and 370 essential genes were common in both species.

Concerning the vaccination of cattle, an interesting article is a meta-analysis by experts from India, the USA, the United Kingdom, the Netherlands, and Ethiopia, which evaluates the effect of BCG vaccination (Srinivasan et al.). Their analyses suggest that BCG vaccination may help to accelerate the control of bTB in endemic settings.

Last but not least, the immunology of the Mb infection has been addressed in five articles. Bovine TB pathology is characterized by pulmonary and lymph node lesions, subsequently leading to granuloma formation. Both the chronic evolution and immunopathology of bTB resemble human TB in several aspects. In this particular collection, we have two major studies that describe the evolution of bTB lesions and immune response in a tme-course manner. A group from the USA examined bacterial burden and cytokine expression in individual pulmonary granulomas from steers at 30, 90, 180, and 270 days after experimental aerosol infection with Mb (Palmer et al.), concluding that bacterial burdens did not correlate with the expression of IFN-γ, TNF-α, TGF-β, granuloma stage, or lung lesion score, although there was a modest positive correlation with IL-10 expression. In another study, the transcriptome of bovine whole peripheral blood samples collected at pre-infection and up to 12 weeks post-infection time points were analyzed for promising gene expression biomarkers for bTB. These authors identified a 19-gene transcriptional biosignature of infection consisting of genes increased in expression across the time course of 12 weeks post-infection with Mb (McLoughlin et al.).

Cell-mediated immunity plays an important role in the control of the Mb infection, and the characterization of such responses is important for understanding the immune status of the host and identifying mechanisms of protective immunity or immunopathology. Researchers from the USA analyzed the T-cell response following experimental *M. bovis* infection in cattle *via in vitro* antigenic expansion and re-stimulation to characterize antigen-specific CD4, CD8, and γδ T cells and their functional phenotype, shedding light on the variable functional ability of these cells. These data can help us better understand the cellular response to Mb infection and develop improved vaccines and diagnostic tools (Boggiatto et al.).

Despite the high degree of identity that Mt and Mb share both at the genetic level as well as during the infection process, the two pathogens display distinct tropism and virulence depending on the host. While Mb is highly virulent and pathogenic for cattle and a range of other mammals, Mt is mostly restricted to humans. From France and Ireland, there is a study in this special issue that investigated the bovine innate response following Mb or Mt infection in a lung slice model of two local cattle breeds. A striking difference in the early lung response to Mb infection was found between these two breeds. The transcriptomic signature induced by infection was very low for the Charolaise breed, whichever mycobacterial strain was used, pointing to the possible control of Mb infection at the genetic or epigenetic level. In the other breed, the type 1 interferon pathway was only induced by Mb infection but not Mt infection (Remot et al.).

In conclusion, in this special issue of Frontiers in Veterinary Science, you can find articles summarizing the recent advances in tackling bTB. This special issue

presented by leading researchers in the field is a great opportunity to update your knowledge concerning the control, diagnosis, immunology, and epidemiology of this infectious disease.

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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ORIGINAL RESEARCH
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Genetic Diversity and Potential Paths of Transmission of *Mycobacterium bovis* in the Amazon: The Discovery of *M. bovis* Lineage Lb1 Circulating in South America

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Bovine tuberculosis (bTB) has yet to be eradicated in Brazil. Herds of cattle and buffalo are important sources of revenue to people living in the banks of the Amazon River basin. A better understanding of *Mycobacterium bovis (M. bovis)* populational structure and transmission dynamics affecting these animals can significantly contribute in efforts to improve their sanitary status. Herein, we sequenced the whole genome of 22 *M. bovis* isolates (15 from buffalo and 7 from cattle) from 10 municipalities in the region of the Lower Amazon River Basin in Brazil and performed phylogenomic analysis and Single Nucleotide Polymorphism (SNP)-based transmission inference to evaluate population structure and transmission networks. Additionally, we compared these genomes to others obtained in unrelated studies in the Marajó Island ($n = 15$) and worldwide ($n = 128$) to understand strain diversity in the Amazon and to infer *M. bovis* lineages. Our results show a higher genomic diversity of *M. bovis* genomes obtained in the Lower Amazon River region when compared to the Marajó Island, while no significant difference was observed between *M. bovis* genomes obtained from cattle and buffalo ($p \geq 0.05$). This high genetic diversity is reflected by the weak phylogenetic clustering of *M. bovis* from the Lower Amazon River region based on geographic proximity and in the detection of only two putative transmission clusters in the region. One of these clusters is the first description of inter-species transmission between cattle and buffalo in the Amazon, bringing implications to the bTB control program. Surprisingly, two *M. bovis* lineages were detected in our dataset, namely Lb1 and Lb3, constituting the first description of Lb1 in South America.

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Most of the strains of this study (13/22) and all 15 strains of the Marajó Island carried no clonal complex marker, suggesting that the recent lineage classification better describe the diversity of *M. bovis* in the Amazon.

Keywords: tuberculosis, bovine TB transmission, clonal complex, whole genome sequencing, spoligotype, *Mycobacterium bovis*, bovine tuberculosis

INTRODUCTION

Mycobacterium bovis (M. bovis) is a member of the *Mycobacterium tuberculosis* complex (MTBC) and is the leading causative agent of bovine tuberculosis (bTB), an OIE (World Organization for Animal Health) notifiable disease that affects mainly cattle, buffalo, and other domesticated and wild animals, but can also be transmitted to humans (zoonotic TB) (1, 2). bTB is distributed worldwide but has very low prevalence in most industrialized countries and has even been eradicated in few nations. However, the disease remains a major problem in developed countries with wildlife reservoirs that end up transmitting the pathogen to domestic livestock and vice-versa, and in developing countries where inefficient bTB control programs result in high disease endemicity and spread (1, 2). In the Brazilian Amazon, few studies aiming to better understand bTB's epidemiology were performed (3–6). The prevalence within animals in the area ranged from 0.1% (4) to 5.4% (6) and the major risk factors associated with bTB were the introduction of new animals into the herds (4), the buffalo species, herds with more than 100 animals, and the presence of cattle and buffalo in the same farm (6).

MTBC members evolved from a most recent common ancestor with *M. canettii*, and are characterized as clonal species demonstrating high genomic similarity (7). Currently, the use of whole-genome sequencing (WGS) to understand tuberculous mycobacteria populational structure is widespread and provided the basis for outbreak tracing and phylogenetic analysis resulting in the classification of human-adapted MTBC into 8 lineages, with *M. tuberculosis* accounting for L1 to L4 and L7-L8, and *Mycobacterium africanum* comprising of L5 and L6 (8, 9). On the other hand, *M. bovis* has been historically classified by Clonal Complexes (CCs), which are identified by genomic deletions, few Single Nucleotide Polymorphism's (SNPs), and/or spoligotypes patterns (10). Accordingly, four different *M. bovis* CCs have been described presenting distinct geographical distribution patterns: African 1 and 2 restricted to Africa, European 2 commonly found in the Iberian Peninsula, and European 1 distributed globally (11–14). With the advent of WGS, recent studies at a global scale provided insights into the population structure and evolution of *M. bovis* lineages (9), showing that CCs do not represent the whole genomic diversity of the isolates (9, 15, 16) and suggesting the existence of at least four *M. bovis* lineages, named Lb1 through Lb4, and three "unknown groups" (9). With the populational structure of *M. bovis* based on WGS starting to be unveiled, additional studies covering different geographic locations are needed to better comprehend worldwide disease spread and to provide new insights regarding the use of genomes to understand disease transmission at the herd and farm levels.

Molecular epidemiological investigation has proved to be a useful tool for TB control and surveillance, which allows us to better understand the dynamics of disease transmission and precisely identify the infectious agent (17). In addition, the knowledge regarding strain diversity within host species has special contribution in areas under risk of zoonotic TB occurrence, thereby providing new insights in strain distribution that may help establishing strategic measures for TB control and prevention (18, 19). In Brazil, few and dispersed molecular epidemiologic studies of *M. bovis* have been reported (20–24), and the WGS characterization of the pathogen is just starting to be unraveled (24–26). While few studies focused on areas of high dairy herd productivity (25, 26), a recent study evaluating *M. bovis* genomes obtained from buffalo and cattle of the Marajó Island, Northern Brazil, was performed and showed the existence of a monophyletic group without CC classification (24). Therefore, the aim of this study was to apply whole-genome and SNP-based phylogenomic analyses to obtain novel information regarding the genetic diversity of *M. bovis* strains circulating in buffalo and cattle from the region of the Lower Amazon River Basin. We believe that such information will guide policy development and strategies to contain the disease in livestock, and thus reduce the risk associated with transmission to humans.

MATERIALS AND METHODS

M. bovis Isolate Selection

A total of 24 *M. bovis* isolates were selected, representing one herd of each municipality involved in two previous studies (6, 20) that obtained a total of 63 *M. bovis* isolates from Amazonas State (12 from cattle and 45 from buffalo) and from Pará State (5 from cattle and 1 from buffalo). The selection of *M. bovis* isolates maintained the proportionality according to species (cattle and buffalo) and the local prevalence from the previous studies (6, 20). Accordingly, these isolates were from tissue samples of 8 cattle and 16 buffalos collected at the slaughterhouse from herds with or without known tuberculin skin test (TST) status originating from 12 different municipalities: Alenquer ($n = 1$), Apui ($n = 1$), Autazes ($n = 1$), Careiro da Varzea ($n = 1$), Itacoatiara ($n = 2$), Manacapuru ($n = 1$), Novo Ceu ($n = 8$), Parintins ($n = 3$), Prainha ($n = 2$), Presidente Figueiredo ($n = 1$), and Urucara ($n = 3$). Samples were collected from June 2016 to October 2017.

DNA Extraction

M. bovis isolates were reactivated in Stonebrink media and incubated until positive growth at 37°C. DNA extractions from colonies suggestive of *M. bovis* for genomic sequencing were performed according to the protocol of van Embden

et al. (27), with modifications. Initially, for inactivation, 2-3 colonies were resuspended in 400 µl TE buffer (10 mM Tris-HCI and 1 mM ethylenediaminetetraacetic acid—EDTA, pH 8.0) and heated at 80°C for 30 min. Subsequently, 50 µl of lysozyme (10 mg/ml) were added and incubated at 37°C for 1 h. Then, 75 µl of 10% SDS (sodium dodecyl sulfate) and 10 µl of proteinase K (10 mg/ml) were added and incubated at 65°C for 10 min. Next, 100 µl of 5M NaCl (sodium chloride) and 100 µl of CTAB (cetyltrimethylammonium bromide) were added, followed by stirring and incubation at 65°C for 10 min. After that, 750 µl chloroform/isoamyl alcohol (24:1) were added, stirred and centrifuged at 12,000 g for 5 min. The aqueous phase (surface) was transferred to another tube, 450 µl of isopropanol were added and incubated at −20°C for 30 min and then centrifuged again at 12,000 g for 15 min at room temperature. The supernatant was discarded, and the pellet washed once with 1 ml of ice-cold ethanol (70%) with centrifugation at 12,000 g for 5 min. After drying the tube by evaporation at room temperature, the DNA was resuspended in 20 µl of TE buffer and stored in a freezer at −20°C. The quality and concentration of the extracted DNAs were evaluated using Nanodrop (Thermo Fisher Scientific). Procedures were performed in a Biosafety Level 3 Laboratory located at the Embrapa Gado de Corte, Campo Grande, Brazil.

Genome Sequencing

WGS was performed at the NGS multi-user platform of Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. Briefly, DNA quantification was performed using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) and the Agilent High Sensitivity DNA Kit (Agilent, California, USA). WGS of *M. bovis* isolates was carried out on a HiSeq instrument (Illumina, San Diego, CA) using HiSeq Rapid SBS Kit v2 (200 cycles) chemistry and the Nextera DNA Flex Library preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Sequencing reads were deposited in Sequence Read Archive (BioProject number PRJNA675550), NCBI and accession numbers are described in **Supplementary Data Sheet 1**.

Genome Quality Assessment and Regions of Difference Identification

Obtained reads were trimmed using a Trimmomatic version 0.38 (28) for adapters and low-quality base removal (sliding window 5:20). Trimmed reads were then evaluated for reads size, per

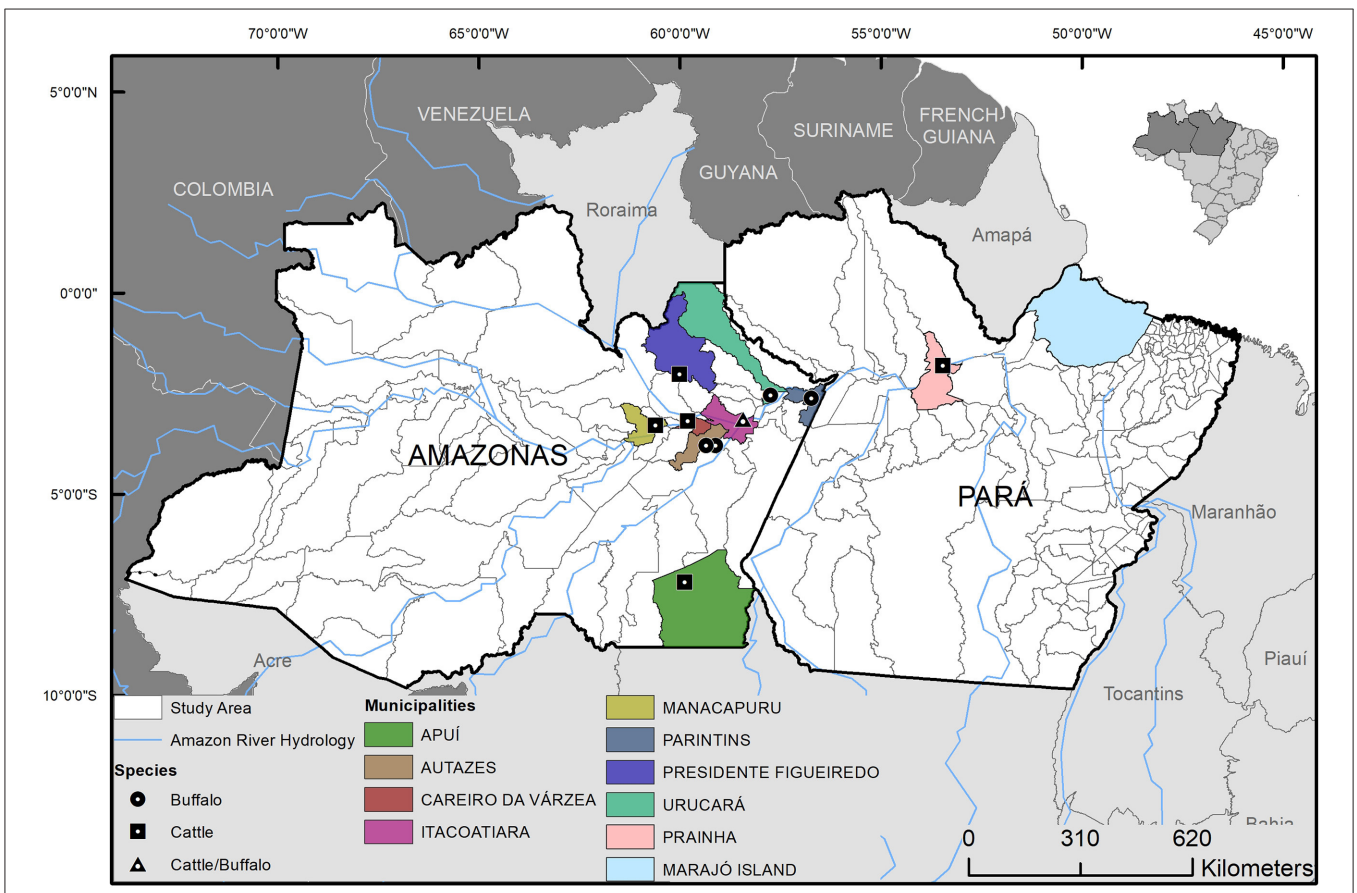

FIGURE 1 | Geographic origin and host species of the *Mycobacterium bovis* isolates from municipalities of Lower Amazon River Basin, Brazil. Isolates were selected from 63 *M. bovis* strains previously isolated from cattle and buffalo (6, 20).

base and read sequence quality, presence of adapters, and GC content using FastQC (29). The GC content had to be around 65% (which is common to mycobacterial genomes) and without multiple peaks (i.e., possible contamination with sequences of different GC content) per quality criteria.

As to confirm that genomes were from *M. bovis*, reads were mapped against *M. tuberculosis* H37Rv using Burrows-Wheeler Aligner (bwa-mem) (30) and the positions according to reference genome of RD1 (4,354,000–4,358,331 nt), RD4 (1,696,017–1,708,748 nt), and RD9 (2,330,880–2,332,100 nt) were evaluated for coverage as previously described (31). The genome was considered as *M. bovis* species when RD1 was absent (i.e. region was intact) and RD4 and RD9 were present (i.e. regions were deleted) (32). Obtained coverage against *M. tuberculosis* H37Rv was also used as quality criteria, considering 95% as a minimum mapping percentage for a genome to be included in the analysis.

Spoligotyping and Clonal Complexes

Spoligotypes were also investigated *in silico* using SpoTyping (31). Identified genetic spacers were processed in the *M. bovis* Spoligotype Database (www.mbovis.org) to retrieve a spoligotype pattern and SB number. The CCs African 1 (Af1) and 2 (Af2) and European 1 (Eu1) and 2 (Eu2) were evaluated as previously described (9). Briefly, the SNP in the *guaA* gene was investigated in the bam files generated from read mapping against *M. tuberculosis* H37Rv, by checking the position 3,813,236. For the RDs, the same bam files were used to investigate read depth using samtools depth (33) and GNU parallel 2018 (34) for the following regions: RDEu1 (1,768,074–1,768,878 nt), RDAf1 (665,042–668,394 nt), and RDAf2 (680,337–694,429 nt) also as described previously (9).

Variant Calling

Trimmed *M. bovis* reads were mapped against *M. bovis* AF2122/97 (NC_002945.4) using bwa-mem (30). Duplicated reads were removed using Picard v2.18.23 (https://github.com/broadinstitute/picard). SNPs were called using Samtools v1.9 mpileup (33) and VarScan v2.4.3 mpileup2cns (35), selecting read depth of 7, mapping quality and minimum base quality of 20, and strand bias filter on, followed by annotation using snpEFF (36). INDELs (insertions and deletions), as well as SNPs from repetitive regions (PE/PPE, transposases, integrases, maturase, phage and repetitive family 13E12 genes) were removed from the analysis using a previously described awk command (9). Genomes were also evaluated based on the number of heterogenous SNPs, considering 15% as a maximum amount for a genome to be included in the analysis.

Phylogenetic Reconstruction

As to evaluate genetic diversity of *M. bovis* in the geographic region, 15 quality-approved *M. bovis* genomes previously sequenced and obtained from cattle and buffalo of the Marajó Island (24) (ENA accession number ERP116404) were used to evaluate the phylogenetic relatedness with the *M. bovis* genomes sequenced in this study. The Marajó Island is geographically close to the targeted region analyzed herein (**Figure 1**), together with the Lower Amazon River Basin are the regions of major concentration of bufallo in the country, and these *M. bovis* genomes (24) were the only strains of the North of Brazil sequenced up until this study. These genomes were quality-assessed and SNPs were obtained as described above. A matrix of concatenated SNPs of the *M. bovis* genomes was constructed as described (9) and used to estimate a maximum likelihood (ML) phylogeny. RAxML version 8.2.12 (37) was used to construct this phylogenetic tree, selecting the GTRCAT model and autoMRE for best-scoring ML tree and a maximum of 1,000 bootstrap inferences. Genomes of *Mycobacterium caprae* (*M. caprae*) (ERR1462591, ERR1462625, ERR1462617, ERR1462581) were also included in the SNP matrix to serve as outgroup.

TABLE 1 | Distribution of *Mycobacterium bovis* in Amazon by municipality, host, and spoligotype.

Municipality	Species	Spoligotype
Apui	Cattle	SB0822
Autazes	Buffalo	Unknown*
Careiro da Varzea	Cattle	SB0295
Itacoatiara	Buffalo	SB0295
	Cattle	SB0295
Manacapuru	Cattle	SB0822
Novo Ceu	Buffalo	SB0822 (3)
	Buffalo	SB1190 (2)
	Buffalo	SB0295 (2)
	Buffalo	SB0121
Parintins	Buffalo	SB0822 (2)
	Buffalo	SB1800
Prainha	Cattle	SB0822
	Cattle	SB0295
Presidente Figueiredo	Cattle	SB0822
Urucara	Buffalo	SB1800
	Buffalo	SB0822
TOTAL		22

**Unknown spoligotype pattern: we have submitted the pattern to M.bovis.org database, but up until this publication, a new SB number has not been provided.*

Minimum Spanning Tree

A pairwise SNP-distance matrix and a minimum spanning tree were constructed using PHYLOViZ (38) with default parameters and using a concatenated SNP matrix of the *M. bovis* genomes as input.

Phylogenomic Analysis With Lineage Representatives of *M. bovis* Genomes

Mycobacterium bovis genomes obtained from this study and those from Marajó Island (24) were compared against a collection of 106 genomes representing the four *M. bovis* lineages (Lb1–Lb4) and unknown groups 1, 2, and 3 from a recent study that evaluated worldwide distribution and evolution of *M. bovis* genomes (9) (**Supplementary Data Sheet 1**). In addition, 21 quality-approved *M. bovis* genomes from France (39), which include representatives of the recently suggested CC called European 3 (40), were also included in the analysis

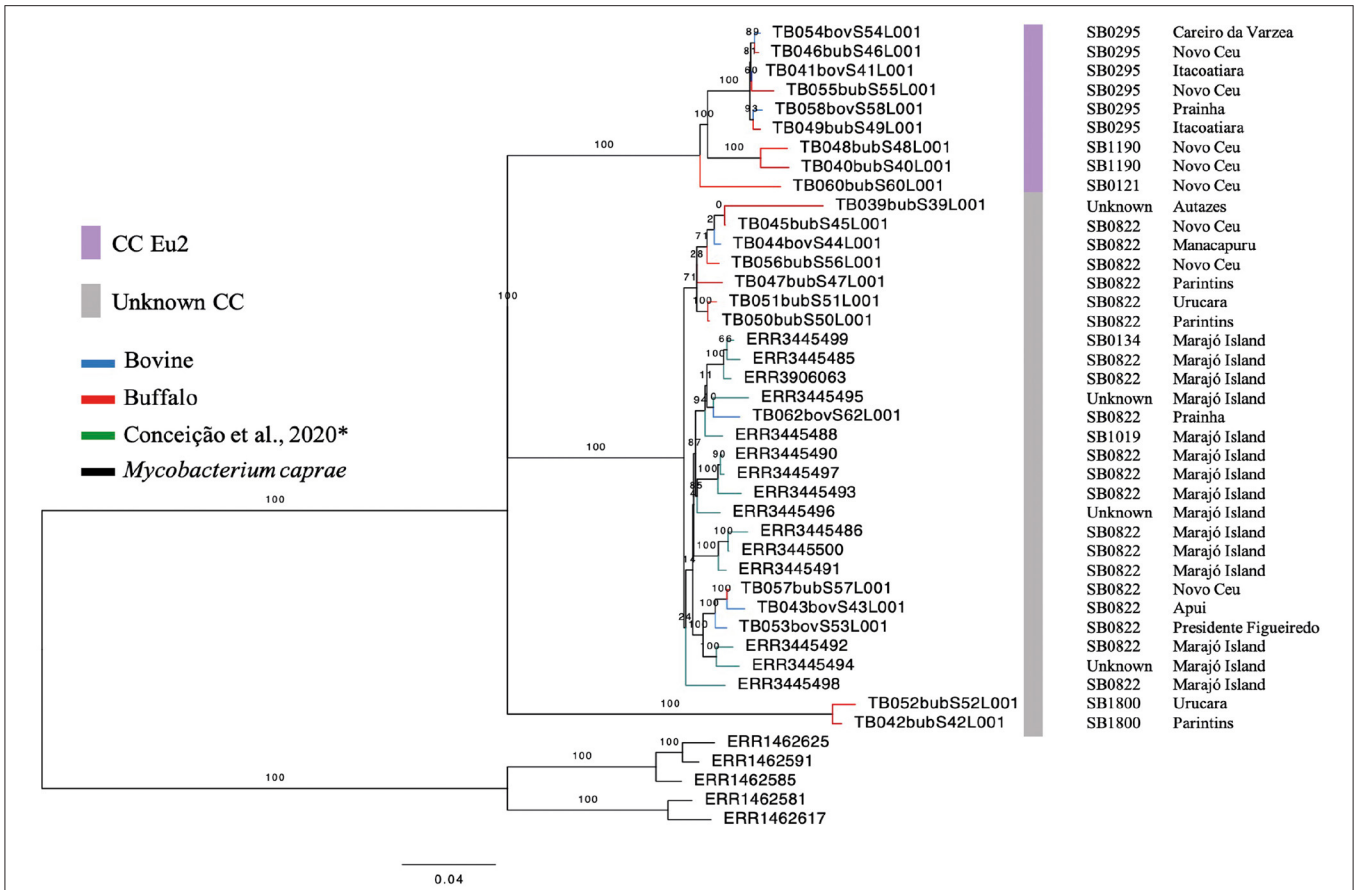

FIGURE 2 | Phylogenetic analysis of *Mycobacterium bovis* genomes from North of Brazil. Maximum likelihood (ML) phylogenetic tree from concatenated SNPs (single nucleotide polymorphisms) of *Mycobacterium bovis* genomes sequenced in this study and by Conceição et al. (24) from Marajó Island. Blue tips: *M. bovis* genomes obtained from cattle and sequenced in this study; red tips: *M. bovis* genomes obtained from buffalo and sequenced in this study; green tips: *M. bovis* genomes from unknown hosts sequenced by Conceição et al. (24) and obtained from cattle or buffalo from Marajó Island, Pará; black tips: *Mycobacterium caprae* genomes (outgroup). Purple bar, *M. bovis* genomes carrying markers of European 2 clonal complex; gray bar, *M. bovis* genomes carrying no markers of known clonal complexes. SB, spoligotype patterns. The phylogenetic tree was generated using RAxML and annotated using FigTree v1.4.3 (43). Horizontal bar shows substitutions per nucleotide. *unknown spoligotype pattern: we have submitted the pattern to M.bovis.org database, but up until this publication, a new SB number has not been provided.

(**Supplementary Data Sheet 1**). Genomes of *M. caprae* and *M. tuberculosis* H37Rv (outgroup) (**Supplementary Data Sheet 1**) were included to construct the phylogenetic tree using the ML approach as described above.

Pairwise SNP-Comparisons

From the SNP-distance matrix, pairwise distances distributions between *M. bovis* genomes obtained from cattle vs. buffalo (Amazon dataset) and between *M. bovis* genomes originating from the Lower Amazon River Basin and Marajó Island were compared using the non-parametric Mann Whitney-test in R software. A result was considered statistically significant when p-value ≤ 0.05.

RESULTS

Out of the 24 *M. bovis* isolates sequenced, two were excluded because of low coverage against the reference genome of *M. tuberculosis* H37Rv or >15% heterogeneous SNPs. These occurred because one isolate resulted in only 38 Kb of sequencing (i.e., failed to be properly sequenced) and the other showed the presence of mixed-strain infection, respectively. The 22 remaining quality-approved *M. bovis* genomes originated from 10 municipalities: Apui ($n = 1$), Autazes ($n = 1$), Careiro da Varzea ($n = 1$), Itacoatiara ($n = 2$), Manacapuru ($n = 1$), Novo Ceu ($n = 8$), Parintins ($n = 3$), Prainha ($n = 2$), Presidente Figueiredo ($n = 1$), and Urucara ($n = 2$) (**Figure 1**).

The genotypic characterization by spoligotyping revealed 5 distinct profiles (**Table 1**). The predominant spoligotype was SB0822, detected in 10 isolates, followed by SB0295, representing 6 isolates; the patterns SB1190 and SB1800 were identified in 2 isolates each; the pattern SB0121 had one representative; and one isolate without described spoligotype pattern. Surprisingly, out of 19 *M. bovis* isolates previously typed using an experimental technique (20),

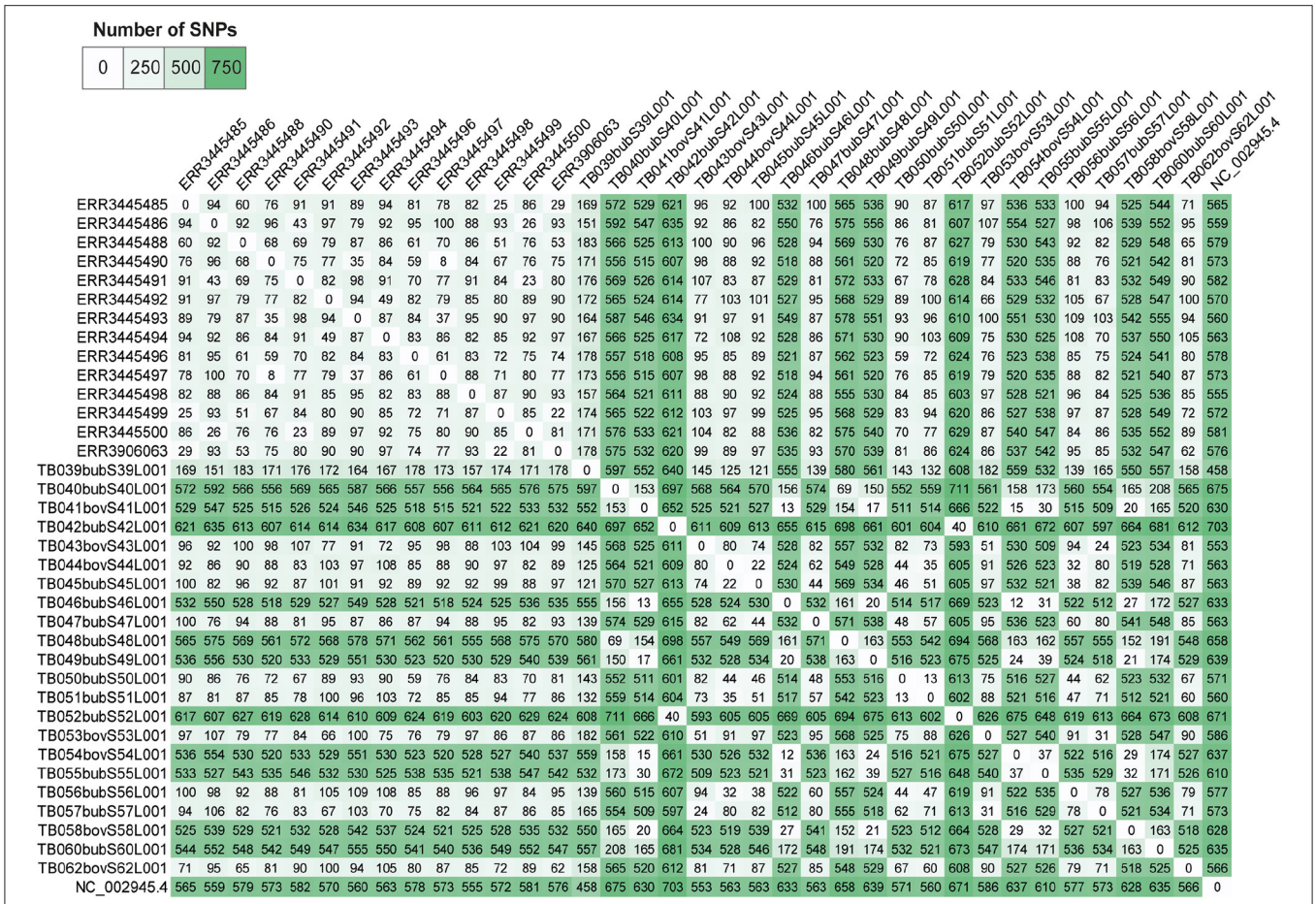

FIGURE 3 | Pairwise single nucleotide polymorphism (SNP)-distance between *Mycobacterium bovis* genomes of Northern Brazil. Genomes of *M. bovis* from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island [sequenced by Conceição et al. (24)] are included. Genomes starting with ERR are from Marajó Island, while genomes starting with TB are from the Lower Amazon River Basin. NC_002945.4: reference genome *Mycobacterium bovis* AF2122/97. Pairwise comparisons were generated from concatenated SNP matrix using PHYLOViZ (38).

12 were discordant. Disagreement between *in silico* and experimental spoligotyping has been previously described (41, 42). Finally, comparing hosts, our results show a higher number of spoligotypes patterns in buffalo when compared to cattle (**Table 1**). Given the low sample size, further studies should be conducted to confirm if buffalo have consistently higher diversity of spoligotype patterns when compared to cattle in this region.

Among the CCs, only the CC Eu2 was found among 41% (9/22) of the isolates. The remaining 13 samples (59%) were not identified as belonging to any known CC (i.e., Eu1, Eu2, Af1, or Af2). Within buffalo, 40% (6/15) of the samples were Eu2, 60% (9/15) had no CC marker. Within cattle, 42.8% (3/7) of the samples demonstrated the marker of CC Eu2, and the remaining of the isolates (4/7) were not classified within any described CC. In the generated phylogenetic tree, with *M. bovis* strains from the Lower Amazon River Basin and Marajó Island, the host classes (buffalo and cattle) are found dispersed among different clades (**Figure 2**), while *M. bovis* genomes from Marajó Island clustered together, appearing more closely related, and genomes from different Amazonas municipalities did not follow a clear clustering pattern according to geographic region.

Pairwise-SNP comparisons (**Figures 3**, **4**) and minimum spanning tree (**Figure 5**) of the *M. bovis* genomes obtained herein and from the Marajó Island (24) showed a highly diverse genomic dataset, with pairwise SNP-distances varying from 8 to 711 (from 8 to 100 among *M. bovis* genomes from Marajó Island and from 12 to 711 among *M. bovis* genomes of the Amazon) (**Figure 3**). Although the overall distribution was not significantly different (**Figure 4A**), *M. bovis* genomes from the Lower Amazon River region tend to show higher pairwise SNP-distances than *M. bovis* genomes from the Marajó Island (**Figure 3**). There was also no significant difference of *M. bovis* genetic diversity distribution using the dataset from the Lower Amazon River region as a function of host (**Figure 4B**; host information from Marajó Island was not available in ENA).

Reflecting this high diversity, based on current *M. tuberculosis*-based SNP threshold to infer recent transmission

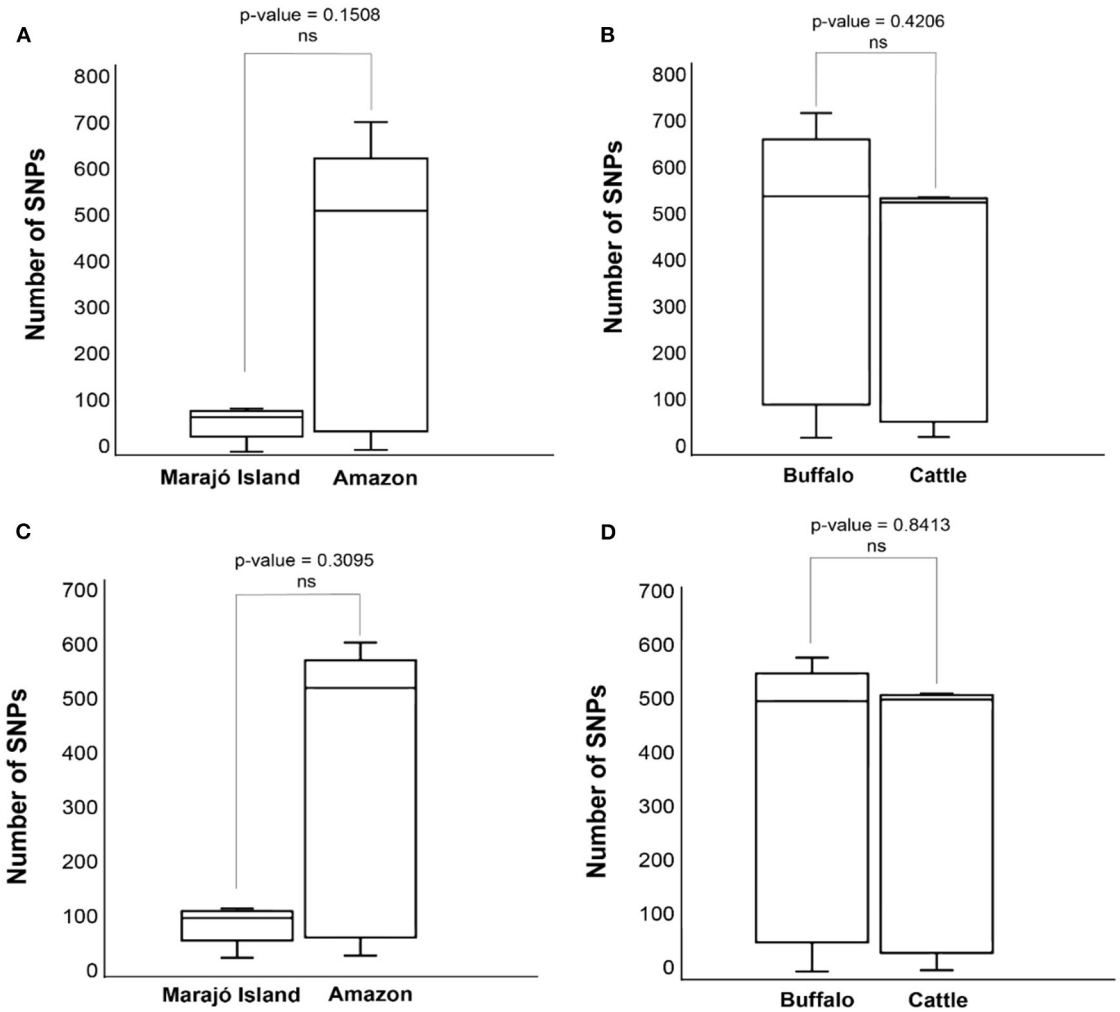

FIGURE 4 | Distributions of pairwise single nucleotide polymorphism (SNP)-distance distance of *Mycobacterium bovis* genomes Northern Brazil. **(A)** Comparison of *M. bovis* pairwise SNP-distance between genomes originating from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island [sequenced by Conceição et al. (24)]. **(B)** Comparison of *M. bovis* pairwise SNP-distance between genomes obtained from buffalo and cattle in the Lower Amazon River Basin. **(C)** Comparison of *M. bovis* lineage 3 (Lb3) pairwise SNP-distance between genomes originating from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island [sequenced by Conceição et al. (24)]. **(D)** Comparison of *M. bovis* Lb3 pairwise SNP-distance between genomes obtained from buffalo and cattle in the Lower Amazon River Basin.

links (∼12 SNPs) (44–46), only three possible active transmission clusters can be observed, one in Marajó Island (host unknown), one connecting the municipalities of Urucará and Parintins (between two buffalo, TB051 and TB050), and one connecting the municipalities of Careiro da Várzea, Novo Céu and Itacoatiara (involving cattle and buffalo, TB54, TB46, and TB41), demonstrating recent transmission between different herds and hosts (**Figure 5**). Isolates comprising each transmission clusters also had the same spoligotype pattern (SB0822, SB0822, and SB0295, respectively).

Based on the proposed four major global lineages of *M. bovis* (Lb1, Lb2, Lb3, and Lb4) (7) our setting would be composed by two lineages, Lb1 with two buffalo isolates (TB052 and TB042) and Lb3 with six buffalo and three cattle isolates with the CC marker Eu2 and seven buffalo and four cattle isolates without CC markers (**Figure 6** and **Table 2**). All isolates from Marajó Island were also identified as being from Lb3 without a CC marker. The presence of Lb1, infecting two buffalo, is the first description of this *M. bovis* lineage in Brazil and requires further investigation into the actual origin of these isolates.

In order to evaluate if the high genetic diversity observed in *M. bovis* from the Lower Amazon River region was only due to the presence of the two highly divergent Lb1 genomes, we compared the distributions of pairwise-SNP distances between the Lower Amazon River region and Marajó Island for the Lb3 only (**Figure 4C**). No significant difference in *M. bovis* genetic diversity was observed (**Figure 4C**). However, the maximum SNP-distance between two genomes observed in the Marajó Island was 100, while in the *M. bovis* genomes of the Amazon (Lb3 only) continued to be high. There was also no

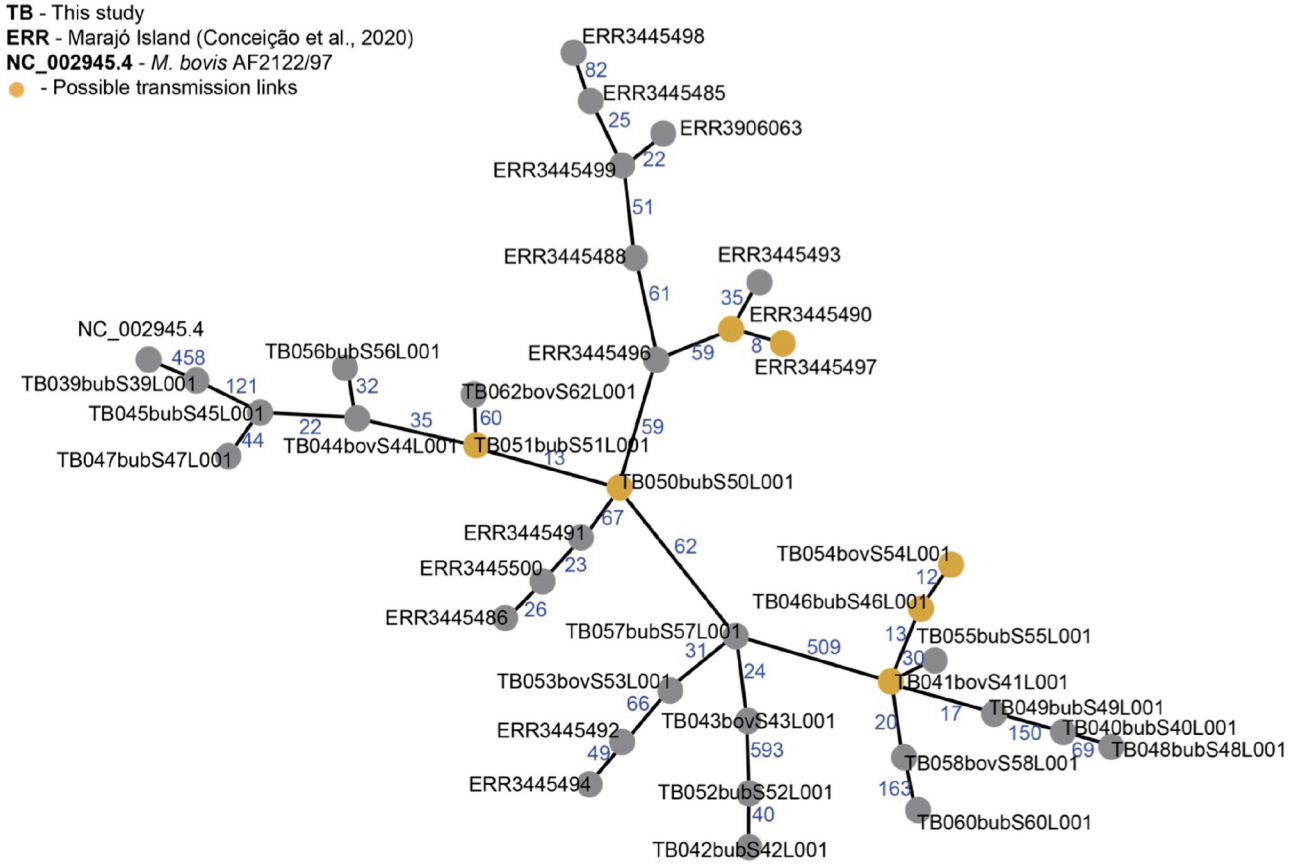

FIGURE 5 | Minimum spanning tree (MST) of *Mycobacterium bovis* genomes from Northern Brazil. Genomes of *M. bovis* from Amazonas state (sequenced in this study, starting with TB) and from Marajó Island [sequenced by Conceição et al. (24), starting with ERR] are included. NC_002945.4: reference genome *Mycobacterium bovis* AF2122/97. Nodes represent *M. bovis* genomes and edges the number of SNPs separating two genomes. Orange nodes represent possible transmission links using an approximate cut-off of 12 single nucleotide polymorphisms (SNP) distance between two *M. bovis* genomes. MST was generated from a concatenated SNP matrix using PHILOViZ (38). Possible transmission links: TB054, TB046 and TB041 are from the municipalities of C. da Varzea, Novo Ceu and Itacoatiara; TB051 and TB050 are from Urucará and Parintins; ERR3445490 and ERR3445497 are from Marajó Island.

significant difference in the genetic diversity of *M. bovis* Lb3 when comparing genomes obtained from cattle and buffalo (**Figure 4D**).

DISCUSSION

To investigate the clonality and population structure of *M. bovis* in the study area at first, we relied on the use of spoligotyping in the characterization of 22 *M. bovis* isolates from 15 buffalo and 7 cattle from June 2016 to October 2017. The spoligotype SB0822 in Brazil was first described in our previous study (20) and we agree with the recent study in Marajó Island in Pará state (24) which seems to demonstrate that SB0822 is the predominant spoligotype in the Amazon region, opposite to a national study with 143 samples from 10 states that found only one occurrence of SB0822 (21). Moreover, SB0822 has been previously described in France (47), cattle in Spain (48) and Portugal (49), and buffalo in Colombia (50), and overall agree with the history of the livestock in the region where the first animals introduced came from Cape Verde (a former Portugal's colony) initially to Marajó Island and from there expanded to the floodplains of the Lower Amazon, on the banks of the Amazon River (51).

Our results also show a higher number of spoligotype patterns of *M. bovis* in the Lower Amazon River Basin compared to the Marajó Island, which can be explained due to the frequent movement of animals in our area of study and the isolation of the herds in the Marajó Island. It is important to highlight that the diversity of *M. bovis* found within buffalo in our sampling is unlikely a result of recent introduction of animals from other Brazilian states since there has been no buffalo imported from other states to the Amazon region and this is probably maintained by constant reinfection from reservoir animals. Accordingly, SB0121 (the most prevalent spoligotyping in Brazil) was found in only one sample, which may reflect the low transit of animals from other states to the area of this study.

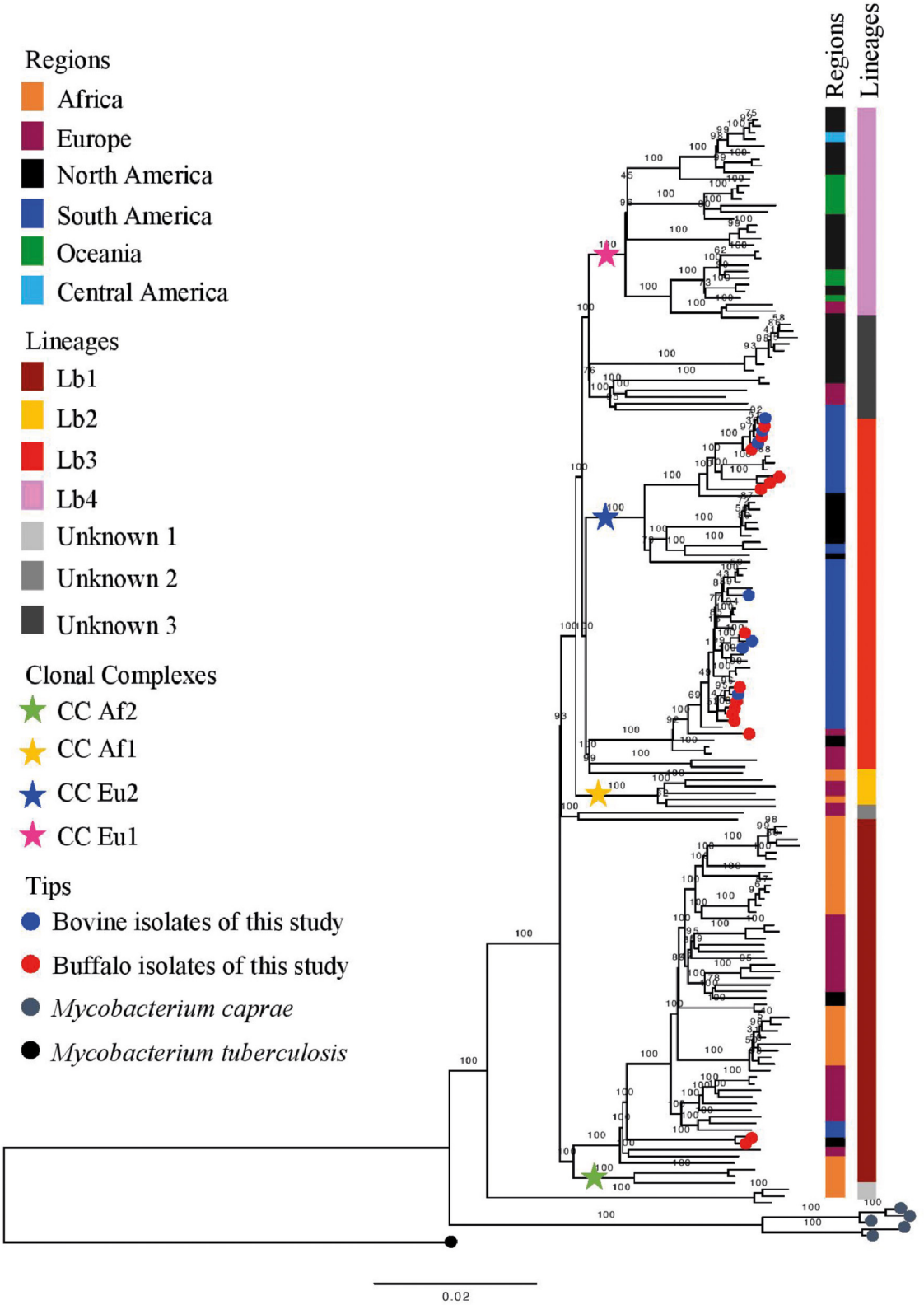

FIGURE 6 | Phylogenetic analysis of *Mycobacterium bovis* lineages. Maximum likelihood (ML) phylogenetic tree from concatenated SNPs (single nucleotide polymorphisms) of *Mycobacterium bovis* genomes from North of Brazil [Amazon, this study, and Marajó Island from Conceição et al. (24)] and worldwide isolates. *Mycobacterium caprae* genomes were included in the analysis, and *Mycobacterium tuberculosis* H37Rv was used as outgroup. The phylogenetic tree was generated using RAxML and annotated using FigTree v1.4.3 (43). Horizontal bar shows substitutions per nucleotide. Lineages are classified according to Zimpel et al. (9).

TABLE 2 | Distribution of *Mycobacterium. bovis* in Amazon by municipality, year, host, clonal complexes, and lineages.

Municipality	Year	Host species	Lb1	Lb3	
				Eu2	No CC marker
Apui	2017	Cattle	-	-	1
Autazes	2017	Buffalo	-	-	1
Careiro da Várzea	2017	Cattle	-	1	-
Itacoatiara	2017	Buffalo	-	1	-
	2017	Cattle	-	1	-
Manacapuru	2017	Cattle	-	-	1
Novo Ceu	2016	Buffalo	-	5	3
Parintins	2017	Buffalo	1		2
Prainha	2017	Cattle	-	1	1
Presidente Figueiredo	2017	Cattle	-	-	1
Urucara	2017	Buffalo	1	-	1
TOTAL			2	9	11

Eu2, Clonal Complex European 2; CC, Clonal Complex; Lb1 and Lb3, lineages of Mycobacterium bovis 1 and 3.

Our results show overall higher genetic diversity of *M. bovis* genomes obtained from different municipalities of Lower Amazon River region when compared to the Marajó Island. This is most likely due to the geographic isolation of the island with lower chances of animal importation over time. We also show possible transmission links between buffalo and cattle from different herds but from close geographic proximity. As wildlife reservoirs have not been identified in Brazil thus far, this transmission may have occurred due to infected animal transit and/or introduction into different herds, showing the presence of a two-host system allowing inter-species transmission in the region.

The detection of an inter-species transmission link, the intertwined phylogenetic dispersal of *M. bovis* obtained from cattle and buffalo, and the absence of significant difference in *M. bovis* genetic diversity in cattle *vs.* buffalo suggest that contact rate between different hosts and consequent geographic proximity likely played a more important role in determining the host range of *M. bovis* in this region than host species, agreeing with recent studies (10, 24). Currently the most accepted hypothesis is that *M. bovis* is not a specialized pathogen, i.e., can affect several host species irrespective of its genetic makeup. However, we cannot neglect the possibility of differential host susceptibility to different strains or lineages of *M. bovis*, which may allow one particular strain or lineage to thrive in a specific host. Herein and in the study in Marajó Island (24), for instance, there is only 40% power to determine a possible significant difference in distribution of *M. bovis* strains between buffalo and cattle. Therefore, studies with a higher sample size comparing the dynamics of *M. bovis* infection within buffalo and cattle are needed to definitively clarify this research question.

The results from our study support the fact that current CCs cannot represent the whole diversity of *M. bovis* strains. Interestingly, this is the first time that is described in Brazil isolates of *M. bovis* originating from the *M. bovis* lineage Lb1 (9). In a recent global phylogenomic study of *M. bovis*, strains of Lb1 were shown to emerge from older nodes than Lb3 in the phylogenetic tree and were detected in Eritrea, Ethiopia, Tanzania, Uganda, Tunisia, France, Spain, Italy, Switzerland, and the United States (9). However, some strains identified in this lineage carry the CC Af2 marker (9) which is found at high frequency in bTB cases from East Africa (12), demonstrating the important ties of this lineage to the African continent. One hypothesis to be looked at is that due to the proximity of these countries with Portugal and its colonies; these strains might have been first introduced in the Amazon region during Brazil colonization.

Results from this study, along with others (9, 16, 39, 40), can be used to refine the understanding of *M. bovis* lineage Lb1. With current genomic data, this lineage is composed of two main clusters: one made of *M. bovis* genomes carrying the CC Af2 marker, and the other without known CC markers (9, 16). Herein, the Lb1 cluster without CC Af2 marker includes 19 out of the 21 French *M. bovis* genomes of SB0120 sequenced by Hauer et al. (39) and included in this study. Recently, Branger et al. (52) suggested that this phylogenetic cluster be called CC European 3, also based on their previous work (39). In this study and in others (9, 16), this cluster is composed of *M. bovis* genomes of many spoligotype patterns (e.g., SB0120, SB0134, SB0828, SB0948, SB1517; **Supplementary Data Sheet 1**) and of BCG vaccine strains (16, 39). Although 19 specific SNPs have been provisionally suggested to be specific of Lb1 (9) and 5 SNPs of its non-Af2 cluster [i.e., CC Eu3; cluster I in Hauer et al. (39)], further studies using comprehensive global datasets should be conducted to confirm or identify definitive genetic markers. The remaining two French *M. bovis* genomes, SRR7851366 and SRR7851376, grouped with genomes of Lb3 and "unknown 3" group, respectively; their disparate position on the phylogenetic tree corroborates the finding of Hauer et al. (39).

The finding of isolates from Marajó Island belonging to Lb3 without CC marker reinforces the previously described (24) existence of a unique *M. bovis* clade in the island that was likely introduced in a single event. In contrast, our results suggest that *M. bovis* was introduced into the Lower Amazon River region as three different events, for which the temporal order remains to be evaluated. One introduction is related to the neighbor cities of Parintins and Urucara, with strains Lb1. Another introduction occurred with Lb3 strains without the CC Eu2 marker, probably with the same origin from the Marajó Island. And finally, an additional introduction is observed with Lb3 strains carrying the CC Eu2 marker, likely from cattle imported from other states and spreading to buffalo. In 2019, a study with 90 samples of cattle lesions suggestive of bTB from the states of Goias, Mato Grosso, Mato Grosso do Sul, Minas Gerais, São Paulo, Tocantins, and Pará found that 14.4% (13/90) belonged to the CC Eu1 and 81.1% (73/90) to the CC Eu2, while 4.65% (4/90) were not identified as any of the four known complexes (53). As the isolates without CC markers were not classified into lineages, the data collected seems insufficient to

reveal the true epidemiological picture of the bTB in Brazil. Knowing the genetic profile and understanding the transmission routes of *M. bovis* in the Amazon and elsewhere is essential in order to focus on public health and veterinary resources to contain bTB.

A limitation of this study is the sample size. The small number of *M. bovis* isolates may not be representative of the whole bacterial and animal populations of the Lower Amazon River Basin. Results must be interpreted in light of this fact, and efforts should continue to isolate and study additional *M. bovis* strains from the region.

From our study, we can make the following conclusions: (1) The *M. bovis* CCs classification cannot cover the whole diversity of *M. bovis* strains present in the Amazon region; (2) The presence of *M. bovis* strains Lb1 infecting buffalo requires further investigation into the actual origin of these isolates, showing that the true global diversity of *M. bovis* strains remains to be discovered, likely influenced by cattle trade over history; and (3) The *M. bovis* classification in lineages by SNP-based phylogenetic analyses seems to better cover the diversity of *M. bovis* strains present in the Amazon region compared to CC classification.

DATA AVAILABILITY STATEMENT

Sequencing reads were deposited in Sequence Read Archive (Bio Project Number PRJNA675550), NCBI and Accession Numbers are described in **Supplementary Data Sheet 1**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no animal was used in this study.

AUTHOR CONTRIBUTIONS

PC and JBK designed experiments, coordinated the research team, interpreted the data, wrote and revised the manuscript. HT and CS coordinated field sampling. CS coordination of field sampling. RA reviewed the manuscript. CZ designed experiments, assessed reads quality, performed phylogenomic analysis, interpreted the data, wrote and revised the manuscript. TTP performed pairwise SNP comparisons, constructed the minimum spanning tree, and interpreted the data. AG designed experiments, supervised CZ and TTP, interpreted the data, wrote and revised the manuscript. FA and TNP cultured *M. bovis*, and performed PCR and DNA extraction. AD performed whole genome sequencing. All authors contributed to the article and 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.2021.630989/full#supplementary-material

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REVIEW
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A Meta-Analysis of the Effect of Bacillus Calmette-Guérin Vaccination Against Bovine Tuberculosis: Is Perfect the Enemy of Good?

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More than 50 million cattle are likely exposed to bovine tuberculosis (bTB) worldwide, highlighting an urgent need for bTB control strategies in low- and middle-income countries (LMICs) and other regions where the disease remains endemic and test-and-slaughter approaches are unfeasible. While Bacillus Calmette-Guérin (BCG) was first developed as a vaccine for use in cattle even before its widespread use in humans, its efficacy against bTB remains poorly understood. To address this important knowledge gap, we conducted a systematic review and meta-analysis to determine the direct efficacy of BCG against bTB challenge in cattle, and performed scenario analyses with transmission dynamic models incorporating direct and indirect vaccinal effects ("herd-immunity") to assess potential impact on herd level disease control. The analysis shows a relative risk of infection of 0.75 (95% CI: 0.68, 0.82) in 1,902 vaccinates as compared with 1,667 controls, corresponding to a direct vaccine efficacy of 25% (95% CI: 18, 32). Importantly, scenario analyses considering both direct and indirect effects suggest that disease prevalence could be driven down close to Officially TB-Free (OTF) status (<0.1%), if BCG were introduced in the next 10-year time period in low to moderate (<15%) prevalence settings, and that 50–95% of cumulative cases may be averted over the next 50 years even in high (20–40%) disease burden settings with immediate implementation of BCG vaccination. Taken together, the analyses suggest that BCG vaccination may help accelerate control of bTB in endemic settings, particularly with early implementation in the face of dairy intensification in regions that currently lack effective bTB control programs.

Keywords: BCG vaccine, bovine tuberculosis, efficacy, cattle, control program

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle that is predominantly caused by *Mycobacterium bovis*, a zoonotic agent (1). The disease remains endemic in most low- and middle-income countries (LMICs) where it negatively impacts livestock productivity and represents a significant threat to public health. A live attenuated strain of *M. bovis*, the "Bacille de Calmette et Guérin" (BCG), has been used for experimental vaccination of cattle against bTB since 1913, well before its first trials in humans (2). Following Calmette and Guérin's promising early reports demonstrating safety of BCG and BCG-induced protection of cattle against experimental challenge with *M. bovis*, several trials were carried out in different countries in the early 20th century to better define its efficacy (3). Despite the repeated demonstration of BCG vaccine-induced protection in cattle, field use was not pursued because of the incomplete protection reported and, more importantly, because as a live attenuated vaccine, BCG sensitizes animals to the current World Organisation for Animal Health (OIE)-recommended purified protein derivatives (PPD)-based skin tests. This compromises the specificity of the standard tuberculin skin test and results in an inability to differentiate infected from vaccinated animals (DIVA) (4). Therefore, bTB control programs that use the OIE-prescribed tuberculin skin test also prohibit the use of BCG vaccination (5).

In the last decade, research in the field has focused on identifying antigens that are present in *M. bovis* and absent or not immunogenic in BCG. In particular, the antigens ESAT-6, CFP10 and Rv3615c have shown promise for differential diagnosis of bTB (4, 6, 7). The DIVA capability of these antigens has been demonstrated in both experimental and naturally infected animals, hence enabling the use of BCG vaccination as part of future bTB control programs (8, 9).

In order to assess the potential utility of BCG vaccination as a component of future bTB control programs, accurate estimates of its efficacy are first required. We sought to address this major knowledge gap through a systematic review and meta-analysis of the existing literature on efficacy of BCG vaccination against bTB in cattle. Vaccines can protect populations through two main modes of action—reducing the susceptibility of vaccinates to infection or by reducing the potential for transmission by vaccinates after infection. The latter effect is particularly relevant for BCG vaccination where reduction in pathology has been reported more frequently (10, 11) than sterilizing immunity. However, also estimating reduction in infectiousness requires either large scale field trials or carefully designed natural transmission studies (11). The vast majority of published efficacy studies of BCG in cattle have used experimental challenge models with a relatively high infectious dose that can only measure a reduction in susceptibility rather than assess the impact on transmission.

For our quantitative meta-analysis, we therefore focus on the effect of the vaccine to reduce susceptibility to infection (vaccine efficacy, ε_s) defined by the presence or absence of visible lesions and/or confirmed by culture. We also review the evidence that supports what the possible range of efficacy BCG may offer in terms of a reduction in infectiousness (ε_I) and explore the implications for disease control using a conceptual dynamic transmission model.

RESULTS

Characteristics of Included Studies

A total of 1,392 articles were screened, and 24 articles were included in the analyses (**Figure 1**) (12–35). In the instance that an article evaluated different doses and strains of BCG, different routes of vaccine administration, breeds of cattle, etc., each was considered as separate strata level data and included as a unique study. For instance, the study by Wedlock et al. (20) was extracted into three strata level data, representing the three different strains or variants of BCG tested (BCG Danish 1331, BCG Danish 1331 freeze-dried and BCG Pasteur 1173P2) (20). In total, 49 strata level data were extracted from the 24 publications included in the systematic review. The included publications spanned the time period from 1972 to 2018, and represented a total of 1,902 vaccinates compared against 1,667 control animals. All included studies are summarized in **Table 1**.

Meta-Analysis

A funnel plot of the log risk ratio against standard error was constructed to assess potential publication bias (**Figure 2**). This revealed a large degree of asymmetry suggesting the presence of publication bias. There was no obvious difference in symmetry between random-effects (RE) and fixed-effects (FE) funnel plots. Similarly, visual inspection of the predicted vs. empirical observations (Normal Q-Q plot) also did not show any major differences in data fit for RE and FE models (**Supplementary Figure 1**).

Given the suggestion of publication bias in the data, we focus on the RE model, which adjusts for variability between individual studies, as the more appropriate model to assess the relative risk

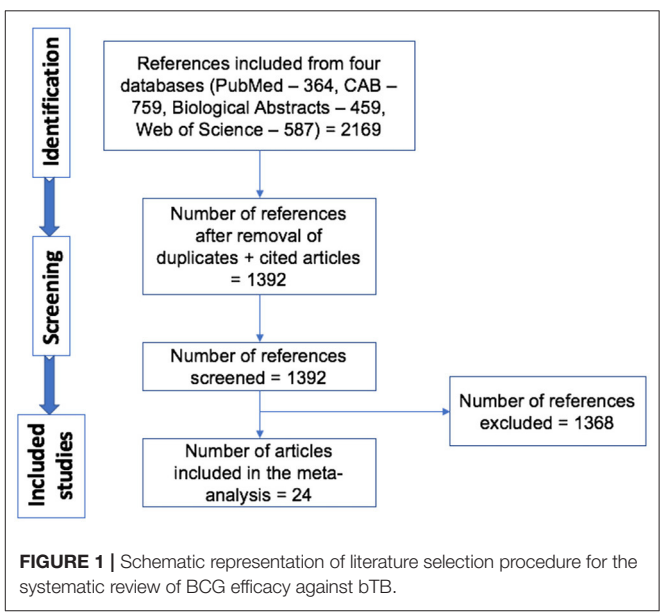

FIGURE 1 | Schematic representation of literature selection procedure for the systematic review of BCG efficacy against bTB.

TABLE 1 | List of strata-level data ($n = 49$) extracted from a total of 24 publications for inclusion in our meta-analysis.

Database #	Authors	Source	Location	Tpos	Tneg	Cpos	Cneg	BCG dose	Route	Method	Infection measurement method	Sample allocation method
101	Wedlock et al. (20)_1	BCG Pasteur 1173P2	New Zealand	9	1	10	0	1–4 × 10^6 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
101	Wedlock et al. (20)_2	BCG Danish 1331	New Zealand	8	2	10	0	1–4 × 10^6 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
101	Wedlock et al. (20)_3	BCG Danish freeze-dried	New Zealand	9	1	10	0	1–4 × 10^6 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
120	Buddle et al. (17)	BCG Pasteur 1173P2	New Zealand	16	2	7	2	1 × 10^5 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
579	Ameni et al. (35)	BCG Danish 1331	Ethiopia	14	9	22	4	1–4 × 10^6 CFU	Subcutaneous	Natural	Culture	Random Sample
802	Buddle et al. (16)	BCG Pasteur 1173P2	New Zealand	5	4	5	4	5 × 10^5 CFU	Subcutaneous	Experimental	Culture	Random Sample
826	Buddle et al. (14)_1	BCG Pasteur 1173P2	New Zealand	5	11	10	5	6 × 10^4 CFU	Subcutaneous	Experimental	Culture	Not specified
826	Buddle et al. (14)_2	BCG Pasteur 1173P2	New Zealand	4	12	10	5	6 × 10^6 CFU	Subcutaneous	Experimental	Culture	Not specified
828	Buddle et al. (15)_1	BCG Pasteur 1173P2	New Zealand	4	5	6	3	2 × 10^5 CFU	Subcutaneous	Experimental	Culture	Random Sample
828	Buddle et al. (15)_2	BCG Pasteur 1173P2	New Zealand	4	5	6	3	2 × 10^3 CFU	Subcutaneous	Experimental	Culture	Random Sample
828	Buddle et al. (15)_3	BCG Pasteur 1173P2	New Zealand	3	6	6	3	2 × 10^5 CFU	Intratracheal	Experimental	Culture	Random Sample
956	Berggren (13)	BCG Glaxo	Malawi	75	129	82	128	8–26 × 10^6 CFU	Subcutaneous	Natural	Culture	Alternate calves vaccinated
1065	Wedlock et al. (22)	BCG Pasteur 1173P2	New Zealand	5	5	7	3	1 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1080	Buddle et al. (21)_1	BCG Pasteur 1173P2	New Zealand	3	7	7	3	1 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1080	Buddle et al. (21)_2	BCG Pasteur 1173P2	New Zealand	2	8	7	3	1 × 10^9 CFU	Oral	Experimental	PM	Stratified Random Sample
1080	Buddle et al. (21)_3	BCG Pasteur 1173P2	New Zealand	3	7	7	3	*	Subcutaneous and oral	Experimental	PM	Stratified Random Sample
1213	Buddle et al. (18)_1	BCG Pasteur 1173P2	New Zealand	6	4	10	0	1 × 10^6 CFU	Subcutaneous	Experimental	Culture	Random Sample
1213	Buddle et al. (18)_2	BCG Pasteur 1173P2	New Zealand	9	1	10	0	1 × 10^6 CFU	Subcutaneous	Experimental	Culture	Random Sample
1213	Buddle et al. (18)_3	BCG Pasteur 1173P2	New Zealand	8	2	10	0	1 × 10^6 CFU	Subcutaneous	Experimental	Culture	Random Sample
1263	De Klerk et al. (24)	BCG Pasteur 1173P2	South Africa	8	6	9	4	3.2 × 10^7 CFU	Intramuscular	Combined	Culture	Random Sample
1266	Ameni et al. (23)	BCG Danish 1331	Ethiopia	4	9	11	3	1 × 10^6 CFU	Subcutaneous	Natural	Culture	Random Sample
1296	Thom et al. (28)_1	BCG Danish 1331	UK	6	3	8	1	1–4 × 10^6 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
1296	Thom et al. (28)_2	BCG Danish 1331	UK	9	0	9	0	1–4 × 10^6 CFU	Subcutaneous	Experimental	Culture	Stratified Random Sample
1303	Parlane et al. (31)_1	BCG Danish 1331	New Zealand	14	2	16	1	2–8 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1303	Parlane et al. (31)_2	BCG Danish 1331	New Zealand	11	4	16	1	2–8 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1304	Buddle et al. (29)_1	BCG Danish 1331	New Zealand	2	7	10	0	1–4 × 10^5 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1304	Buddle et al. (29)_2	BCG Danish 1331	New Zealand	3	6	10	0	1–4 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1337	Buddle et al. (33)	BCG Danish 1331	New Zealand	6	6	7	5	1.5 × 10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1358	Nugent et al. (34)_1	BCG Danish 1331	New Zealand	0	30	8	122	1 × 10^8 CFU	Oral	Natural	PM	Random Sample
1358	Nugent et al. (34)_2	BCG Danish 1331	New Zealand	1	33	8	122	3 × 10^5 CFU	Subcutaneous	Natural	PM	Random Sample
1358	Nugent et al. (34)_3	BCG Danish 1331	New Zealand	3	169	9	108	1 × 10^8 CFU	Oral	Natural	PM	Random Sample
1358	Nugent et al. (34)_4	BCG Danish 1331	New Zealand	11	166	12	74	1 × 10^8 CFU	Oral	Natural	PM	Random Sample
1358	Nugent et al. (34)_5	BCG Danish 1331	New Zealand	12	156	26	83	1 × 10^8 CFU	Oral	Natural	PM	Random Sample

(Continued)

TABLE 1 | Continued

Database #	Authors	Source	Location	Tpos	Tneg	Cpos	Cneg	BCG dose	Route	Method	Infection measurement method	Sample allocation method
1358	Nugent et al. (34)_6	BCG Danish 1331	New Zealand	4	93	8	81	1×10^8 CFU	Oral	Natural	PM	Random Sample
1366	Buddle et al. (19)_1	BCG Pasteur 1173P2	New Zealand	6	4	9	1	1×10^8 CFU	Oral	Experimental	PM	Stratified Random Sample
1366	Buddle et al. (19)_2	BCG Pasteur 1173P2	New Zealand	7	3	9	1	1×10^9 CFU	Oral	Experimental	PM	Stratified Random Sample
1366	Buddle et al. (19)_3	BCG Pasteur 1173P2	New Zealand	5	5	9	1	1×10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1371	Hope et al. (27)_1	BCG Danish 1331	UK	7	0	7	0	2×10^6 CFU	Subcutaneous	Experimental	PM and Culture	Not specified
1371	Hope et al. (27)_2	BCG Pasteur 1173P2	UK	3	4	7	0	2×10^6 CFU	Subcutaneous	Experimental	PM and Culture	Not specified
1373	Lopez-Valencia et al. (25)	BCG Tokyo	Mexico	6	59	15	51	1×10^6 CFU	Subcutaneous	Natural	Skin test and IFNg assay	Alternate calves vaccinated
1379	Buddle et al. (26)_1	BCG Danish 1331	New Zealand	2	7	6	4	1×10^6 CFU	Subcutaneous	Experimental	PM	Stratified Random Sample
1379	Buddle et al. (26)_2	BCG Danish 1331	New Zealand	5	4	6	4	1×10^8 CFU	Oral	Experimental	PM	Stratified Random Sample
1379	Buddle et al. (26)_3	BCG Danish 1331	New Zealand	5	4	6	4	1×10^7 CFU	Oral	Experimental	PM	Stratified Random Sample
1379	Buddle et al. (26)_4	BCG Danish 1331	New Zealand	6	3	6	4	1×10^6 CFU	Oral	Experimental	PM	Stratified Random Sample
1383	Dean et al. (30)	BCG Danish 1331	UK	4	6	9	1	1×10^6 CFU	Subcutaneous	Experimental	Culture	Not specified
1385	Dean et al. (32)_1	BCG Danish 1331	UK	8	2	8	1	1×10^6 CFU	Subcutaneous	Experimental	Culture	Random Sample
1385	Dean et al. (32)_2	BCG Danish 1331	UK	5	5	8	1	5×10^5 CFU	Subcutaneous and endobronchial	Experimental	Culture	Random Sample
1385	Dean et al. (32)_3	BCG Danish 1331	UK	5	5	8	1	1×10^6 CFU	Endobronchial	Experimental	Culture	Random Sample
1410	Nugent et al. (36)	BCG Danish 1331	New Zealand	2	518	8	289	3×10^5 CFU	Subcutaneous	Natural	PM and Culture	Block Randomization

**Buddle et al. (21)_3 used a combination of Oral and Subcutaneous BCG; Study no. 1263 (24) was performed in buffaloes. Tpos and Cpos are animals classified as positive for bTB in vaccinates and controls, respectively. Tneg and Cneg are animals that remained negative for bTB in vaccinates and controls, respectively.*

Multiple strata level data extracted from a single study are represented with numbers at the end in the Author column. For example, Database #101, Wedlock et al had 3 strata level data represented as Wedlock et al._1, Wedlock et al._2 and Wedlock et al._3. Buddle_a is Reference (14) and Buddle_b is Reference (15). a1, a2, and b1, b2 are strata level data of those respective studies.

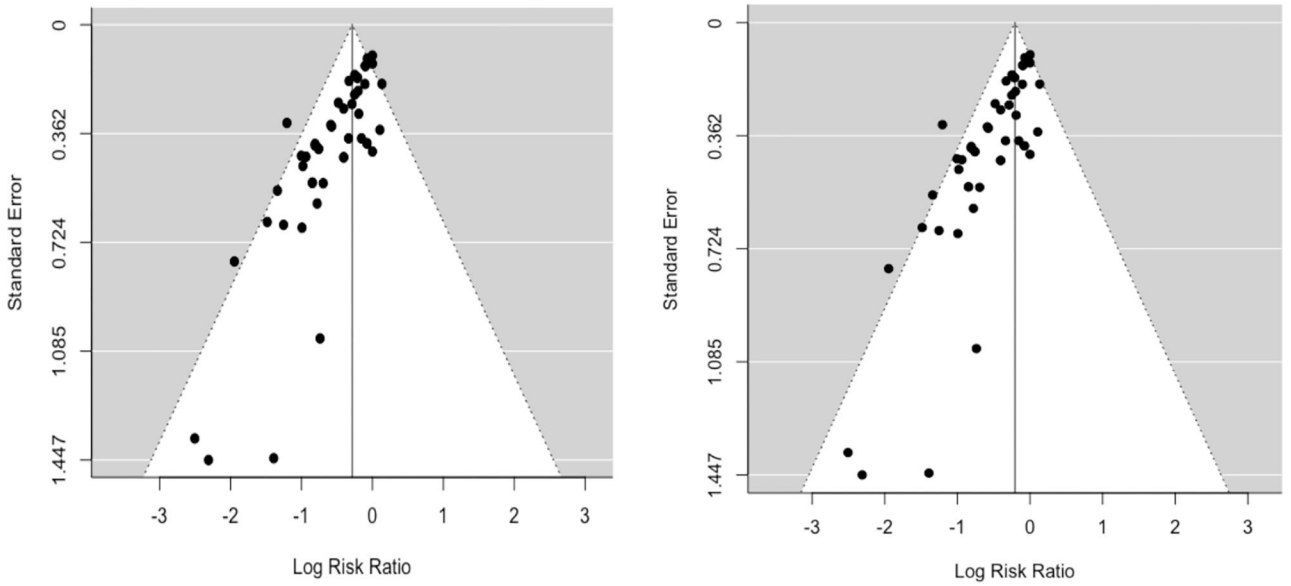

FIGURE 2 | Funnel plots (left: RE; right: FE) of the log risk ratio against standard error demonstrate potential publication bias.

ratio (RR). The relative risk ratio is defined as the probability of an outcome in an exposed group to that in an unexposed group (37). The RE model estimated RR to be 0.75 (95% CI: 0.68, 0.82), suggesting a 25% reduction in risk of infection (vaccine efficacy, ε_S) as measured by PM and/or culture in BCG vaccinates compared to control animals. Cochran's (Q) value ($Q = 76.1$, $df = 50$, and $p = 0.01$) and Higgins statistic ($I^2 = 32.1\%$) were computed to test for heterogeneity ($I^2 < 50\%$ represents low heterogeneity). While both the Q value and I^2 statistic are classical measures of heterogeneity, neither is comprehensive since Cochran's (Q) suffers low power and I^2 is imprecise in the case of meta-analyses of relatively small numbers of included studies. We used a forest plot to graphically summarize the variation in RR between studies (**Figure 3**).

To explore the potential impact of publication bias on the estimated RR, we carried out a sensitivity analysis using the trim and fill method (38). This is an algorithmic method to adjust for publication bias in a meta-analysis by imputing the values of missing studies (39). Here, the estimated number of missing studies on the right side of the funnel plot was found to be 21 (**Figure 4**). The Paul-Mantel method was used due to convergence issues with the maximum likelihood based methods (Maximum-Likelihood (ML), Restricted Maximum-Likelihood (40) and Empirical Bayes (EB) estimators). The adjusted RR estimate per this sensitivity analysis was found to be 0.84 (95% CI: 0.73, 0.98).

Meta-Regression

Several important biological factors varied between published studies, which *a priori* could have an impact on the estimated vaccine efficacy. Such confounding factors could also potentially lead to systematic patterns of bias as shown by the funnel plot analysis. We therefore constructed a multivariable meta-regression model using these factors to explore whether they could improve model fit and to assess the relative effect of these biological variables on estimates of vaccine efficacy. The factors included BCG source, dose, route, whether or not revaccination was performed, and challenge method (**Table 2**).

An omnibus test of all the moderator variables (QM = 23.7, df = 13, $p = 0.03$) indicates that the explained variance by the model is greater than the unexplained variance. However, this amounts to only 49.1% of the total heterogeneity and only one moderator (BCG Glaxo source) has a RR significantly different than 1 (at the 95% level). Given the relatively small sample sizes, reflective of the logistical constraints of experimental studies, this lack of statistical significance is unsurprising and does not rule out the potential biological importance of these variables and highlights the critical need for additional well-powered investigations to better assess the impact of these confounders on overall vaccine efficacy.

Implications for bTB Control

To explore the implications of the estimated efficacy of vaccination of $\varepsilon_S \sim 25\%$ for disease control, we carried out scenario analyses using a conceptual herd level transmission dynamic model. Given the potential density dependence of transmission rates of bTB within herds, intensification of production in emerging dairy industries is a particular concern for LMICs (41). Thus, we consider a scenario with an initial herd size of 30 but growing in size at a rate of 15% per year to 134 animals after 50 years. Motivated by estimates from India we consider an initial bTB prevalence (5, 10, and 15%) together with a set of higher prevalence scenarios (20, 30, and 40%) (42). There are currently no published quantitative estimates of transmission rates from LMICs, hence for this model population we use a

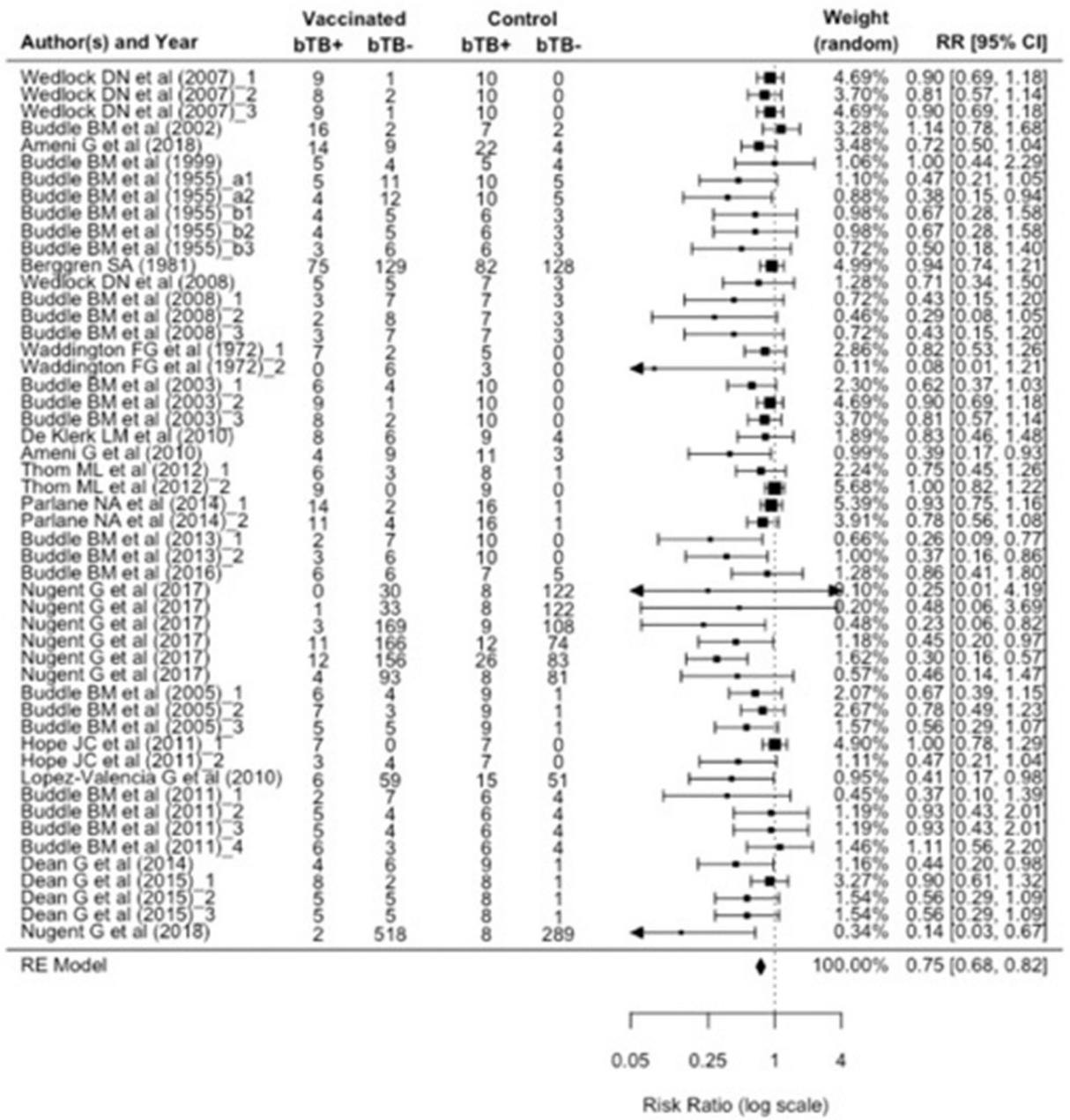

FIGURE 3 | Forest plot visualizing the relative risk ratio calculated for each included publication in the meta-analysis. The weight given to each included publication per the RE model is shown. "bTB+" refers to the number of animals reported to have infection and/or pathology (per culture and/or postmortem examination). And "bTB-" refers to the number of animals not reported to have infection and/or pathology.

density dependent transmission function estimated from herds in Great Britain (11).

In the absence of estimates of indirect BCG effects based on empirical trials, we used data from long-term natural challenge models to estimate the relative contribution of indirect effects when force of infection is low (representative of field setting). Overall efficacy in those recent field trials included in this meta-analysis [excluding the older Berggren (13) study] was estimated to be of 61% (95% CI: 40, 74) for natural transmission compared to 18% (95% CI: 11, 24) for experimental challenge studies (**Table 3**). The key distinction between experimental challenge and natural transmission studies—beyond being more representative of a field setting—is that the latter measures the total effect of vaccination (**Figure 5**). The total effect

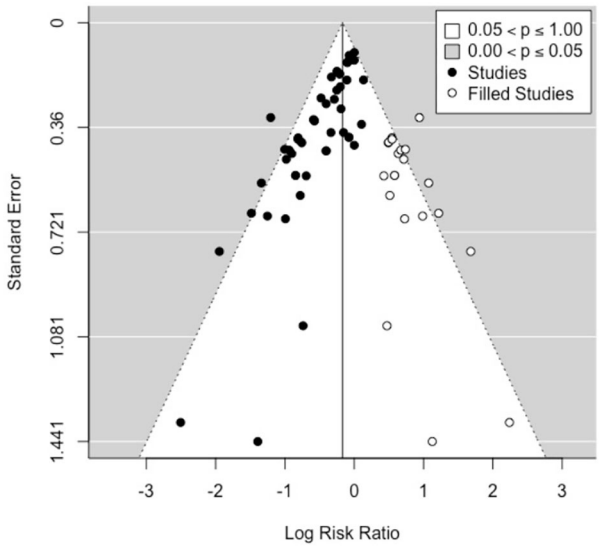

FIGURE 4 | Funnel plot showing the 21 studies (open circles, on the right side) imputed by the trim-and-fill method to carry out a sensitivity analysis for publication bias.

TABLE 2 | Multivariable meta-regression of the selected predictors on BCG efficacy ($R^2 = 49.10\%$, $n = 49$).

Moderators	Categories	RR (95% CI)	*p*-value (RE)
Revaccination	No	Reference	
	Yes	1.04 (0.79, 1.38)	0.77
BCG route	Endobronchial	Reference	
	Intramuscular	2.16 (0.55, 8.50)	0.27
	Intratracheal	1.01 (0.28, 3.67)	0.99
	Oral	2.04 (0.75, 5.53)	0.16
	Subcutaneous	1.46 (0.71, 3.00)	0.3
	Subcutaneous and Endobronchial	1 (0.38, 2.66)	1.00
Challenge method	Natural	Reference	
	Experimental	0.83 (0.40, 1.70)	0.61
BCG source	Danish 1331	Reference	
	Danish freeze-dried	1.14 (0.77, 1.68)	0.51
	Glaxo	12.14 (1.52, 97.09)	0.02
	Pasteur 1173P2	0.98 (0.77, 1.23)	0.84
BCG Dose		0.68 (0.31, 1.51)	0.35
Time from vaccination to challenge		1.00 (0.99, 1.001)	0.5
Length of exposure to challenge		0.998 (0.997, 1)	0.06

of vaccination will be greater than the direct protection of individuals (ε_s) due to the reduction in transmission from herd immunity effects, along with any additional protection from reduction in infectiousness (ε_I) of vaccinated individuals.

In the absence of control, the null model predicts a gradual increase in prevalence with herd size saturating around ∼70% for all initial conditions (**Figure 6**). We considered a vaccine efficacy to reduce infectiousness (ε_I) at two hypothetical thresholds: (i) ∼36%, approximating the difference between average overall efficacy in the natural transmission studies (0.61) and the overall direct efficacy as revealed by the meta-analyses of 0.25; and (ii) ∼49%—representing the difference between the upper bound of efficacy in the natural transmission studies (0.74) and overall direct efficacy as revealed by the meta-analyses of 0.25.

Our model scenarios illustrate the benefits of early intervention. Even with only a modest efficacy (ε_s) of 25%, a window of up to 25 years of slightly lower than the current disease prevalence levels may be achieved (**Figure 6A**), should BCG be implemented immediately in the low to moderate prevalence scenario (5, 10, 15%). With an additional reduction in infectiousness (ε_I) of vaccinates (49%), disease prevalence could be driven down close to Officially TB Free (OTF) status (<0.1% per the European Union) if BCG were introduced in the next 10-year time period in low to moderate prevalence settings. Immediate implementation, but with a lower ε_I of 36%, prevalence may start to rise again in 30–40-years, driven by increased herd size, suggesting that other strategies will also be required.

These scenarios predict considerable benefits in terms of reducing cumulative cases if BCG is implemented now. With an impact of vaccination on infectiousness, between ∼80 and 100% of cases can be saved in 50 years, driven by the strength of indirect effects on transmission (**Figure 6B**). Encouragingly, this model also predicts that from ∼50 to 95% of cumulative cases can be averted in higher disease burden settings (>15%) if acted upon now (**Figure 6**).

DISCUSSION

Following promising initial studies conducted by Calmette, Guérin and others that showed both safety of BCG and BCG-induced protection of cattle against both natural and experimental challenge with *M. bovis*, several trials were carried out through the early and mid 20th century in many different countries to better assess and define the efficacy of ancestral variants of BCG in cattle and showed varying levels of protection (3, 44) (**Supplementary Table 1**). While BCG went on to become the most widely used of all human vaccines (and the only available vaccine against human TB), it has not been considered for routine use in domestic livestock despite early promise. This is primarily because of the fact that BCG sensitizes animals to the widely used and OIE-recommended PPD based skin tests, which precludes its use where skin test-based control is being actively pursued (5), and it is with this context that an Expert Committee of the WHO/FAO Expert Committee on Zoonoses (45) stated that: "generally speaking, vaccination has no place in the eradication of tuberculosis in cattle."

The early proof-of-concept experiments conducted by Calmette and Guérin in the early 1900s to demonstrate safety of BCG and protection against progressive TB in cattle were replicated and refined by several other investigators in both Europe and the Americas (44, 46–53). Their findings led to considerable international interest in exploring the possibility

TABLE 3 | Natural transmission studies included in the meta-analysis.

Database #	Authors	Location	BCG source	BCG route	BCG dose	Controls_n	Vaccinates_n	Reported efficacy
579	Ameni et al. (35)	Ethiopia	BCG Danish 1331	Subcutaneous	1–4 × 10^6 CFU	26	23	30%
1266	Ameni et al. (23)	Ethiopia	BCG Danish 1331	Subcutaneous	1 × 10^6 CFU	14	13	60%
1358	Nugent et al. (34)	New Zealand	BCG Danish 1331	Oral and Subcutaneous	1 × 10^8 CFU	531	644	67%
1373	Lopez-Valencia et al. (25)	Mexico	BCG Tokyo	Subcutaneous	1 × 10^6 CFU	66	65	60%
1410	Nugent et al. (36)	New Zealand	BCG Danish 1331	Subcutaneous	3 × 10^5 CFU	297	520	85%

The studies conducted in Ethiopia by Ameni et al. (23, 35) used a reactor to sentinel ratio of >1. Nugent et al. (34) included 1,286 free-ranging cattle (676 vaccinates and 531 naïve controls) stocked at low densities, and challenged with a low force of infection, mimicking a more representative field setting. This was followed up with a subsequent field study (36) conducted in ∼800 free-ranging cattle that were followed up for over 3 years and similarly showed a relatively high overall protective efficacy of ∼85%. Lopez-Valencia et al. (25) did not conduct necropsy.

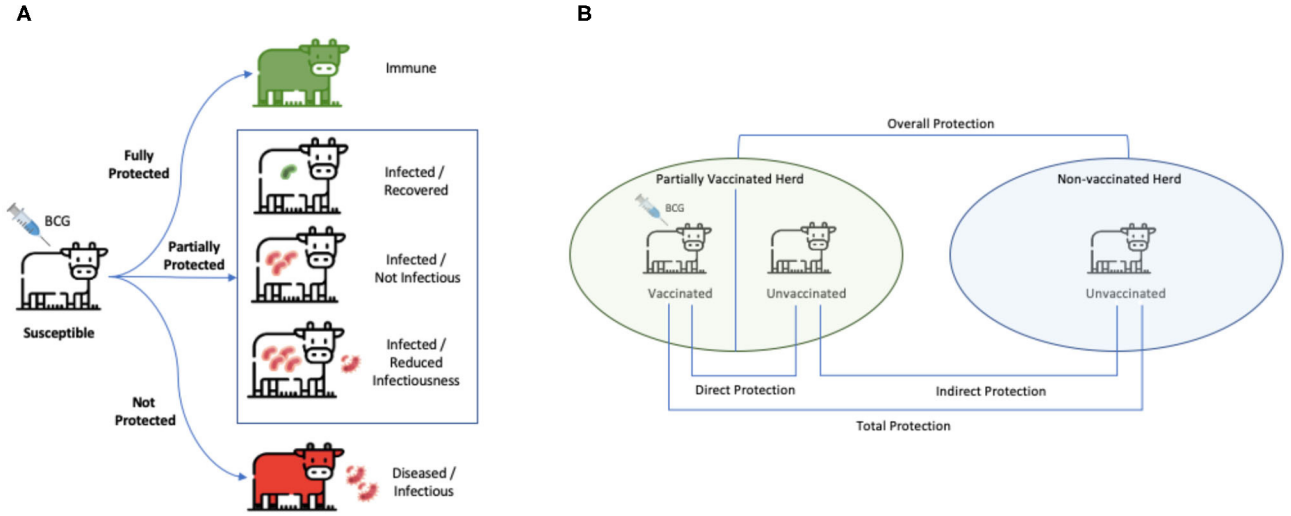

FIGURE 5 | Measures of vaccine-induced protection. **(A)** BCG vaccine confers a spectrum of protection ranging from complete (for a limited time period in certain population) to none. While completely protected individuals are immune, and unprotected ones succumb to infection and become heavily diseased, BCG can also be partially protective by reducing risk of disease transmission to unvaccinated individuals. **(B)** The green herd is partially vaccinated and the blue herd is unvaccinated. "Direct protection" is the reduction in susceptibility of vaccinated animals (as measured by ε_s). "Indirect protection" is a reduction in transmission due to any reduction in infectiousness of vaccinated individuals that become infected (as measured by ε_I) in addition to the reduction in transmission from herd immunity effects. "Total" protection is the risk of infection in vaccinated animals compared to that of unvaccinated animals. "Overall protection" is the reduction in rate of transmission in a population with vaccination program compared to that in a population with no vaccination program. This stems from both vaccinated and unvaccinated individuals and is used to evaluate the impact of a vaccination program at the population level. **(B)** Is adapted from Halloran et al. (43).

of eradicating bTB by vaccinating cattle with BCG as has been reviewed elsewhere (3, 10, 54–57). As part of the current systematic review, we extracted data from eight studies that were published between 1930 and 1972 (**Supplementary Table 1**). However, given that these studies used ancestral strains of BCG and doses that are not possible to accurately estimate, these studies were excluded from the formal analyses. However, it is noteworthy that these studies provided key foundational evidence that highlighted both the variable protection afforded by BCG in experimental and natural challenge, as well as showed that BCG vaccination sensitizes animals to tuberculins and hence would confound ongoing test and slaughter tuberculosis eradication programs. However, recent attempts to develop BCG-compatible diagnostic tests have shown great promise and paved the way for implementation of BCG-based control programs in LMICs and other endemic regions (7–9). Thus, there is renewed interest in exploring the use of BCG vaccination as a component of national control programs, particularly where test and slaughter is unfeasible (58, 59).

We systematically reviewed the literature to obtain estimates of the efficacy of BCG against bTB and assessed its potential utility in future control programs. The analyses suggest an overall vaccine efficacy of 25% (95% CI: 18, 32) as measured by the presence of visible lesions and/or culture. The potential contribution of vaccine strain as a confounder or predictor of BCG efficacy is intriguing. This is relevant since a majority of the studies that were included in the current review used either BCG Pasteur 1173P2

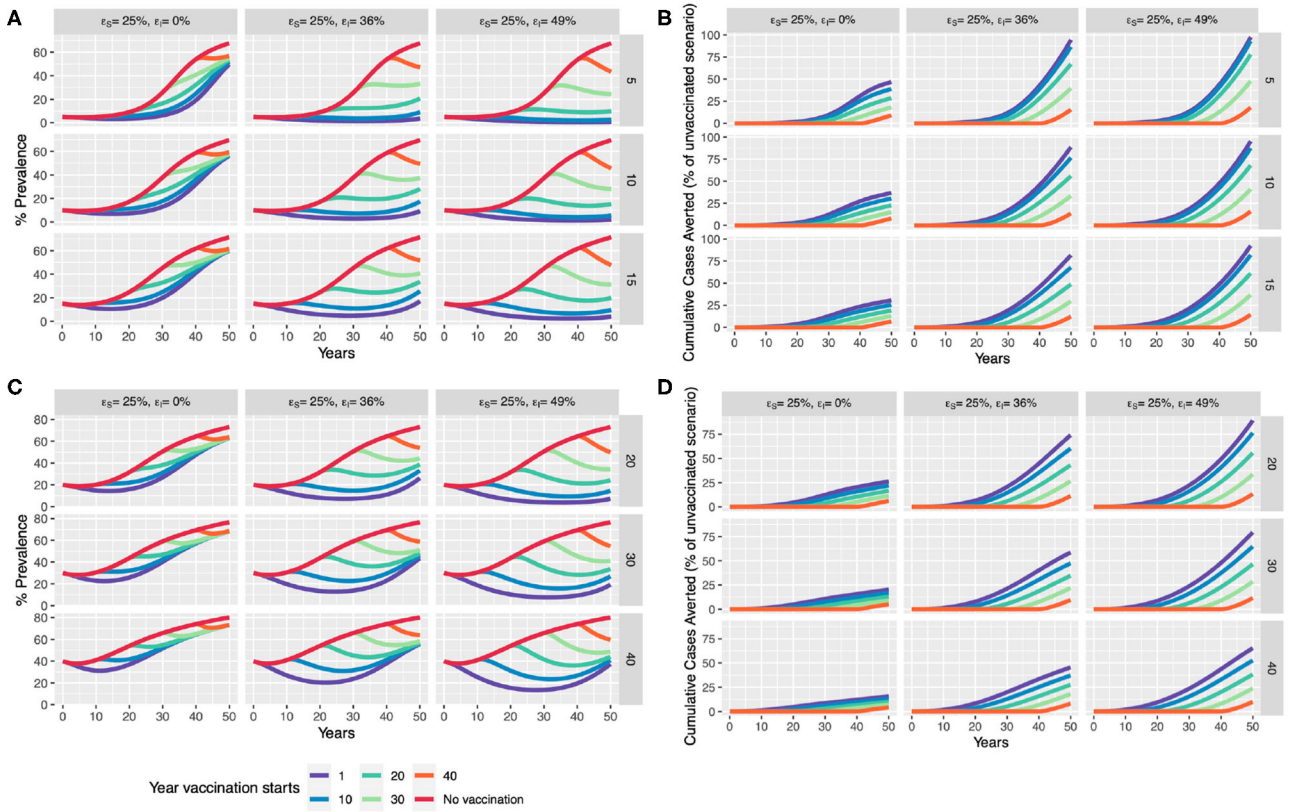

FIGURE 6 | Scenario analysis for impact of BCG vaccination in an expanding dairy herd. We compare a baseline "control" scenario (with no vaccination) to the introduction of whole-herd vaccination at 1, 10, 20, 30, and 40 years which only provides direct protection (ε_S of 25%), and one with an additional reduction in infectiousness of vaccinates (ε_I of 36 and 49%). We compare the effect of vaccination in terms of % prevalence and cumulative cases averted with assumed initial **(A,B)** moderate prevalence of 5, 10, and 15% (rows), and **(C,D)** high prevalence of 20, 30, and 40%.

(9 out of 24 studies) or BCG Danish 1331 (10 out of 24 studies). The meta-regression model suggests that neither of these strains had any significant effect on the observed heterogeneity. However, given the small sample size, further investigations may be warranted to assess the robustness of these inferences.

Given the large variability in the age at which animals were vaccinated in the included studies, the ideal timing for vaccination could not be subjected to rigorous analysis. While there is evidence in the published literature showing that BCG vaccination within a few days of birth (<1 month of age) can induce high levels of protective immunity (18, 60), there is a need for more studies in relevant settings, such as in bTB-endemic regions where high burden of environmental mycobacteria has been reported (61). In these settings, some studies suggest prior sensitization with environmental mycobacteria prevent induction of protection in calves, while others report that exposure to *M. avium* helps induce protection against *M. bovis* (17, 62). It is plausible that these seemingly contradictory results could be because exposure to different environmental mycobacteria could either enhance or reduce BCG's efficacy, and hence, future studies are needed to help clarify the role of these confounders to vaccine efficacy. Curiously, despite the early recognition by Calmette and Guerin and other investigators of the need for revaccination (at annual or 18 month intervals) in order to maintain protection (3), only a total of five studies performed revaccination, highlighting the scarcity of reliable data available on duration of immunity and revaccination intervals to formulate evidence-based approaches to control bTB (13, 16, 18, 24, 31). Thus, it is important to assess the true duration of immunity and role, if any, of revaccination to obtain better estimates of the efficacy of BCG and inform the parameterization of robust transmission dynamics and econometric models that may be used to guide development and implementation of future cost-effective vaccine-based intervention strategies (18, 31).

The roles of dose and type of challenge (experimental challenge or natural infection) were also investigated. A total of 17 studies that are part of this meta-analysis performed experimental challenge of their animals to infection. These studies call for high dose infection protocols in order to generate high levels of infection in control animals, which do not reflect the reality in natural conditions where animals most likely are not exposed to such high numbers of virulent bacteria as a single bolus. Such experimental challenge models with high infection doses do not allow BCG protection to be revealed. One reason for this may be that these models are often used

for the development of novel vaccines aimed at improving BCG efficacy and not *per se* to study BCG efficacy. This highlights the importance of conducting robust natural transmission studies in field conditions and in endemic settings where the real need for vaccination lies. It is noteworthy that most experimental trials have used *M. bovis* as the challenge strain. However, *M. bovis* is not the only causative agent of bTB since *M. tuberculosis* and other members of the *M. tuberculosis* complex are frequently isolated from cattle samples, and certain species of non-tuberculous mycobacteria (NTM) that contain virulence factors encoded in the Region of Difference-1 are also known to cause TB-like lesions (63, 64). Hence, future studies are needed to better estimate efficacy of BCG vaccine-induced protection with locally relevant bTB causing mycobacteria other than *M. bovis*.

It is important to note that there are several limitations to systematic reviews and meta-analyses. They can provide a summary of published evidence about a specific question, but our ability to answer it is limited by variation in experimental design and the consistency and completeness of reporting. This systematic review was limited to studies in English indexed in the four databases screened, and hence may have missed relevant investigations. Studies that did not report essential details were excluded. It is not surprising that systematic biases in the fit of the meta-analysis models suggest existence of publication bias and the potential to inflate or underestimate estimates of direct vaccine efficacy. One of the primary limitations of the included studies is that the reported measures of protection, evidence of lesions at necropsy and culture, together with immune reactions, are the current "gold standards," but have variable sensitivity and specificity (65). In order to address any potential bias in measurement of the outcome, we focused on studies which reported culture or necropsy data for calculation of RR estimation and further analyses. An exception was made however for the Lopez-Valencia et al. (25) study since it was one of only five estimates from a recent natural transmission study. This study considered an animal to be infected if there were positive reactions to the tuberculin skin test and the IFNγ release assay upon stimulation with PPD-B and recombinant ESAT6-CFP10 (25). While many studies followed a simple or stratified sampling strategy for randomization, two studies (13, 25) used a more systematic approach of vaccinating every alternate calf in the herd which may have introduced potential biases from the randomization process (**Table 1**). However, a rigorous assessment of other risks of bias due to deviations from intended interventions, missing outcome data and selection of reported result could not be performed due to lack of access to the complete data or detailed experimental protocols from these studies. Another limitation is that only the direct effect of vaccination (reduction in susceptibility, ε_S) could be estimated from the experimental challenge experiments (66). This is important, in particular, given our scenario analyses which demonstrate the transformative effect an additional reduction in infectiousness of vaccinates (ε_I) could have (67, 68). Thus, studies to assess the impact of BCG on infectiousness of vaccinates through natural transmission experiments or field trials are urgently needed (67).

Herd-size is perhaps the most important risk factor associated with transmission of bTB (69), leading to the common assumption that transmission is density dependent and increases with herd size (70, 71). This poses a particular challenge for bTB control in emerging dairy markets in LMICs where intensification of the dairy sector may make achieving control increasingly difficult over time. We developed herd level transmission dynamic models and performed scenario analyses using deterministic models to illustrate this challenge. The results of these analyses are striking—and suggest that despite the relatively modest direct protection afforded by BCG, a strong case may be made for implementing vaccination for bTB control sooner rather than later, with overall benefits reducing progressively over time to intervention (72) (**Figure 6**). Overall, a leaky vaccine like BCG could still play a pivotal role in disease control, particularly if implemented sooner than later in the face of dairy intensification efforts in bTB endemic countries.

Finally, even while scenario analyses are for illustrative purposes, there are several limitations to these analyses that we recognize and should be addressed in future investigations. To begin with, in the absence of surveillance programs in LMICs, transmission estimates are lacking and should be determined to accurately assess risk. Given that recent studies show increasing prevalence in intensifying cattle herds in endemic settings (69), these observations have major implications for informing and implementing disease control policy and suggest a potential role for BCG vaccination that has hitherto remained unexplored. Next, in order to realize herd-level benefits of vaccination, estimating the reduction in the risk of transmission from vaccinated individuals (ε_I) is crucial. Animal trials in relevant endemic settings are urgently needed to evaluate the potential of BCG to reduce onward transmission in the field (11).

It is important to note that in addition to cattle, tuberculosis affects a wide variety of livestock and both free-living and captive wildlife species including goats, sheep, pigs, cervids, wild boars, badgers, brushtail possums, and ferrets, many of which are recognized as potential reservoir hosts. Indeed, experimental and field trials to study the response to BCG vaccination of some these species suggests that, while vaccination confers incomplete protection, it's use in domestic and captive or free-living wildlife species should be seriously considered to reduce risk of cross-species spillover (73). Alternative approaches to control bTB, such as test-and-segregate and treatment with anti-mycobacterial agents including isoniazid have also been explored (74, 75). Treatment with isoniazid is uneconomical and not advised given the long duration of treatment needed and the need to withdraw milk together with reports that bacterial shedding may resume as soon as isoniazid is withdrawn (76). Moreover, use of a first line antimycobacterial agent in food animals is of major concern due to potential for contribution to the spread of drug resistant tuberculosis (77). Over the past decade, research in the field has also focused on improving TB vaccination using alternative approaches including the heterologous prime-boost strategy, introducing genetic modifications in BCG strains to increase immunogenicity, and also completely replacing BCG with attenuated *M. bovis* strains (78–82). Heat-inactivated *M. bovis* vaccines have also recently shown promise in wild boars,

pigs, red deer, badgers, and goats (73, 83–88). However, despite significant efforts and promising results in preclinical studies, there is only limited evidence from clinical and field trials for significant gains in efficacy of these newer generation or modified BCG vaccines. Interestingly, both heat-inactivated and attenuated vaccines for another major mycobacterial disease, paratuberculosis (Johne's disease), have been licensed and tested extensively for use in cattle and small ruminants, but their use is limited in countries because of potential for interference with tuberculin testing and diagnostic tools currently used in bTB eradication and control programs (89, 90).

In conclusion, in the short-term, in endemic regions where test and slaughter approaches to bTB control have not been shown to be effective or, BCG vaccination alongside a DIVA diagnostic test appears to be the most promising option in the near future. However, it is important to continue to develop and assess next generation vaccines as well as complementary strategies of disease control alongside that of BCG. For instance, the assessment of routine testing and segregation of reactor animals, mandatory pasteurization of milk fed to calves and sold for human consumption, enhanced husbandry practices, such as segregation of reactor or likely infected animals, herd certification policies for recruitment of disease-free animals, slaughterhouse surveillance, regulating movement and trade of reactor animals, etc., in conjunction with or as alternatives to BCG need to be rigorously evaluated in different production settings and epidemiological contexts. Together, our studies highlight an urgent need as well to perform sensitivity analyses and build econometric frameworks to assess the cost-benefit impacts of implementing vaccine-based control strategies to establish the business case for (or against) implementation of BCG vaccination as a component of a national bovine TB control program.

CONCLUSION

This systematic review and meta-analysis of the efficacy of BCG vaccination in cattle together with transmission dynamic model-based scenario analyses provides strong evidence for the consideration of implementation of BCG vaccine-based bTB control strategies, particularly in LMICs and other high burden settings. Despite a relatively small but positive protective effect, conservative transmission models suggest an important role for BCG in limiting spread of the disease and buying time for improvement of vaccine efficacy or the development of alternative approaches to disease control. Taken together with the predicted increase in prevalence associated with intensification of dairy production, our investigations suggest that BCG vaccination may indeed be simply good (enough) to accelerate control of bTB in endemic settings.

METHODS

Literature Search Strategy

A systematic search was performed for published articles reporting the effect of BCG against bTB in cattle as of February 24, 2020. Various combinations of Boolean operators and MeSH terms common to known articles of interest were evaluated before the following search terms were finalized: (BCG AND (("mycobacterium bovis" OR tuberculosis) AND (cows OR cattle OR bovine) AND (protect* OR effica* OR lesion* OR immune* OR vaccin*))). In order to minimize publication bias, search terms were kept uniform across the four databases (PubMed, CAB, Biological Abstracts, and Web of Science), which were selected for their inclusion of major and minor international journals. No limits were placed on publication date, and only published studies were considered. EndNote X8, a citation software program, was used to organize the articles generated by the four databases as well as remove duplicate publications. The study conforms to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (91).

TABLE 4 | Study inclusion/exclusion criteria.

Inclusion	Exclusion
Bovine TB	Wrong disease
Cattle or buffalo	Wrong species
Used BCG to vaccinate (and specified the strain used)	Wrong vaccine
Evaluated efficacy of BCG vaccination	Wrong type of study
English	Language limitation: not in English
Full text of publication obtanied	Full-text unavailable
	Other

Study Inclusion Criteria

Inclusion and exclusion criteria are detailed in **Table 4**. All included studies compared the potentially protective effects induced by a BCG variant against natural or experimental challenge with bTB in a vaccinated group of cattle and a control group of unvaccinated cattle. Studies with no control group, such as surveys evaluating the effectiveness of vaccination campaigns, were excluded. Additionally, articles that failed to report the BCG vaccine strain tested were excluded (summarized in **Supplementary Table 1**). While efforts were made to identify articles manually, all final included articles were represented in the formal database search. Primary studies were included when available. However, review articles were included when the primary article was inaccessible and adequate information on BCG strain, bTB challenge, and protection was detailed for vaccinate and control groups.

Data Extraction

A uniform data extraction form was developed based on information of interest from pre-identified articles and used by each of the extractors. Study identifiers included author, publication date, title, journal, study location, and study time period. Headings for study design included control and vaccinate group sizes; BCG strain (Danish 1331, Danish freeze-dried, Glaxo, Pasteur 1173P2, Tokyo); BCG dose; and BCG administration route (subcutaneous, endobronchial, intratracheal, intramuscular, oral); cattle age at time of vaccination; revaccination timing, frequency, and dose (if applicable); animal breed; and time from vaccination to

TABLE 5 | A summary of all studies included in the meta-analysis.

		Infection	
		Yes	**No**
Vaccination	Yes	362	1,540
	No	530	1,137

Out of a total of 1,902 BCG vaccinated animals, 356 were found to be positive (either by culture and/or presence of visible lesions) for bTB while 1,502 remained negative. Similarly, out of 1,667 control animals, 520 were infected while 1,104 animals neither had lesions nor any growth in culture.

challenge. Headings detailing bTB challenge methods included challenge type (natural or experimental) and duration (from challenge to slaughter or end of study period); as well as challenge strain, route (intratracheal, subcutaneous, oral, endobronchial) and dose if challenge type was experimental. Headings for protection included the infection measurement method (post-mortem, culture), number of vaccinates and controls affected, and the protection percentage reported. Vaccine efficacy was measured based on culture growth, and in the absence of culture data, presence or absence of visible lung or lymph node lesions in the control and treatment groups was used.

Prior to the formal review of all articles in the pool, data extractors (SS, LE, and CH) conducted a pilot run of 20 random articles in order to test inclusion/exclusion criteria and finalize the data extraction form. SS, LE, and CH independently performed the formal review of all articles, which comprised an initial screening of article titles and abstracts for inclusion or exclusion. Full-text review for data extraction from included studies was performed by SS and LE. When discrepancies arose in study inclusion/exclusion or extracted data between reviewers, studies were collectively revisited and discussed in order to reach a final decision.

Included studies used common protection measurement methods, primarily post-mortem examination (PM) and/or culture. In the case that a study used both PM and culture to measure protection, preference was given to culture (65); in the case that lung and lymph node lesions were reported, we considered positivity per lung lesions for effect measure. We only included studies that specified the challenge strain. A majority of the included publications performed experimental (17/24) challenge and did not revaccinate animals (18/24). A complete list of all included and excluded studies is publicly available at https://doi.org/10.26208/pykx-8s25.

Statistical Analysis

All statistical analyses were performed in R (version 3.5.0) using RStudio (version 1.1.442). Full source codes used for the statistical analyses are publicly available with this publication at https://doi.org/10.26208/pykx-8s25. Random effects (RE) (4) and fixed effects (FE) meta-analysis models were estimated using the "rma" function of the "metaphor" package and analyzed using additional functions from the "meta" package (92, 93). Funnel plots were constructed to assess for systematic bias in the fitted models. Cochran's Q statistic was computed to test for unexplained heterogeneity, and Higgin's statistic helped describe the inconsistencies in studies' results (94, 95). Key parameters used to define protocols for BCG vaccination trials that were judged on biological grounds to be important were assessed as moderators in a multivariate meta-regression model, estimated using the rma function of the metafor package.

Due to the variability between reporting of pathology between different studies, vaccine efficacy was measured as a binary dichotomous variable based on the presence or absence of culture growth, or visible lesions in the control and treatment groups. Vaccine efficacy is most commonly measured using the relative risk or risk ratio (RR) (4), defined as the ratio of attack rate in the vaccinated and control groups. As well as being more easily interpretable than other measures (such as odds ratios and risk differences), RR can be used to define vaccine efficacy (1-RR) and was estimated here per the RE model. The 2 × 2 contingency table below provides a summary of included sample size (**Table 5**).

Scenario Analysis

Thus far, no study on BCG efficacy has attempted to estimate the efficacy of BCG to reduce the infectiousness of vaccinates who subsequently become infected. In the absence of empirical data, the reduction in lesions in vaccinated animals estimated from natural transmission studies may be considered as a proxy for reduction in infectiousness. While how the extent of lesions directly relates to a reduction in infectiousness and thus transmission risk was not quantified, this significant reduction in lesion severity following BCG vaccination likely contributes to reduction in risk of transmission from vaccinates to susceptible cattle. Here, we conducted scenario analyses to explore the potential implications for disease control and the importance of estimating this neglected—but critical—aspect of BCG efficacy. We consider this proportionate reduction in lesions as a plausible upper bound for the total vaccine-induced protection.

For a well-mixed population, the critical vaccination threshold (V_c) for elimination of a disease can be calculated from the basic reproduction number (R_0) using the formula $V_c = (1 - 1/R_0)/E$, where E is the relative (combined direct and indirect) efficacy of the vaccine (96). For domesticated livestock species in organized settings, routine vaccinations are often performed for all animals, hence we can reframe the critical threshold in terms of the critical efficacy $E_c = (1 - 1/R_0)$. Thus, with a direct vaccine efficacy of 25% (95% CI: 18, 32), BCG could be expected to successfully eliminate infection from fully vaccinated herds when the within-herd R_0 is 1.32 (95% CI, 1.20–1.45) or lower.

A simple deterministic model for the within-herd transmission of bTB was used to explore the potential impact of BCG vaccination in an expanding dairy sector. The model assumes a well-mixed herd with animals stratified into four epidemiological compartments. In an unvaccinated population, animals are either susceptible (S) or infected and potentially infectious (I). Vaccinated individuals (V) have a reduced risk of becoming infected and we add a final infected after vaccination compartment to model a reduced infectiousness for these animals (I_V). Density dependence in transmission is modeled using the non-linear relationship estimated from herds in Great Britain (GB) using the so-called SOR model (71). The SOR model

subdivides the infectious class into an occult (O) and reactive (R) groups that differ in their reaction to the tuberculin skin test, but also assumes infected animals are potentially infectious immediately and is thus structurally equivalent to the $SI(VI_V)$ model used here.

The full model equations are:

$$\frac{dS}{dt} = (1-p)\,\mu N - \frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,S - \nu S$$

$$\frac{dI}{dt} = \frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,S - \nu I$$

$$\frac{dV}{dt} = p\mu N - (1-\varepsilon_s)\,\frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,V - \nu V$$

$$\frac{dI_V}{dt} = (1-\varepsilon_s)\,\frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,V - \nu I_V$$

$$\frac{dC}{dt} = \frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,S + (1-\varepsilon_s)\frac{\beta}{(N/N_m)^q}(I + (1-\varepsilon_I)\ I_V)\,V$$

$$\frac{dN}{dt} = (\mu - \nu)N$$

where ε_s is the efficacy of vaccination to protect against infection (reduction in susceptibility), ε_I is the efficacy of vaccination with respect to reducing the infectiousness of vaccinates that subsequently become infected and N_m is a constant centering parameter (=165) used for the estimation of q in the original GB model. β is a transmission parameter which we fix using the assumed initial prevalence (I_0) and initial herd size (N_0) for each scenario and q measures the strength of density dependence of transmission with $q = 0$ corresponding to density dependence and $q = 1$ frequency dependence. For the default scenario we use the point estimate from Conlan et al. (71) of $q = 0.15$ and present an alternative weaker density dependent scenario ($q = 0.71$, upper bound of approximate posterior distribution) in **Supplementary Material**.

For illustration we consider a herd with initial size $N_0 = 30$, a 20% per annum replacement rate ($\nu = 0.2$ per year) and per capita birth rate $\mu = 1.15\ \nu$ to give a population growth rate of 15% per year ($N = N_0 e^{-(\mu-\upsilon)t}$).

The basic reproduction ratio for this model (for fixed herd size N_0) can be calculated (next generation method) as:

$$R_0 = \frac{\beta}{\upsilon} N_0\,(N_0/N_m)^{-q}$$

We set the (97) value of β for each scenario by assuming that the initial population is at the equilibrium point of the constant population model. Thus, at $t = 0$, we assume that:

$$R_0 = 1/(1 - I_0/N_0)$$

and thus set:

$$\beta = \frac{\nu R_0\,(N_0/N_m)^q}{N_0}$$

Full source codes used for the statistical analyses are publicly available with this publication at https://doi.org/10.26208/pykx-8s25.

AUTHOR CONTRIBUTIONS

SS, LE, and VK designed the study. SS, LE, and CH performed data extraction. SS and AC compiled the final data and analyzed the data. SS drafted the paper. SS, AC, PD, MVe, GA, GR, JW, NJ, DB, MVo, and VK contributed to the writing. All authors contributed to the article and 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.2021.637580/full#supplementary-material

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Bovine Tuberculosis: The Emergence of a New Wildlife Maintenance Host in Ireland

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Despite advances in herd management, tuberculosis (TB) continues to affect ∼0. 5% of Ireland's national cattle herd annually. It is clear that any "final" eradication of TB in cattle will need to address all TB maintenance hosts in the same environment. In Ireland and the UK, European Badgers (Meles meles) are a known TB maintenance host, while deer are recognised as spillover hosts. However, deer have been identified as maintenance hosts in other countries and Sika deer, specifically, have been identified with TB in Ireland. We examined the power of cattle, badger and Sika deer densities (at the county level) to predict cattle TB-breakdowns in Ireland, at both the herd and the individual level, using data collected between 2000 and 2018. Our hypothesis was that any positive correlations between deer density and cattle TB-breakdowns would implicate deer as TB maintenance hosts. Using linear multiple regressions, we found positive correlations between deer density and cattle TB-breakdowns at both the herd and individual levels. Since Sika deer in County Wicklow are known to have TB, we ran further regressions against subsets of data which excluded individual Irish counties. Analyses excluding Wicklow data showed much weaker correlations between Sika deer density and cattle TB-breakdowns at both the herd and individual levels, suggesting that these correlations are strongest in County Wicklow. A similar effect for badger density was seen in County Leitrim. While locally high densities of Sika deer persist in Irish counties, we believe they should be considered an integral part of any TB-control programme for those areas.

Keywords: Sika deer, Cervus nippon, tuberculosis, TB, cattle, European badger, maintenance host, Ireland

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INTRODUCTION

Tuberculosis (TB), an infectious disease caused by members of the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex (MTBC) (1), is one of the leading causes of infectious disease mortality worldwide. As a zoonotic disease TB affects humans and multiple animal species and has been recognised as a major health risk to humans and animals for more than a century (2). In 2019, 1.4 million people died of tuberculosis (1). Bovine tuberculosis (bTB) is a chronic disease of cattle caused by members of the MTBC, primarily by *M. bovis*, but also *M. caprae* and, to a lesser extent, *M. tuberculosis* (3). In addition to infecting cattle and humans, the same Mycobacteria also infect other domestic animals and wildlife populations (4), causing general illness, pneumonia, weight loss and deaths. In countries where bTB is still common, and particularly where pasteurization of milk is not practiced, an estimated 10–15% of human cases of TB are caused by *M. bovis*.

Host density is a key driver of tuberculosis transmission rates and the aggregation of hosts (e.g., in large social groups) can create, or increase, opportunities for intra- or interspecific disease transmission (5). In Ireland bTB is most frequently caused by *M. bovis* (6). In cattle, TB transmission is most likely when stocking density is high (7). Depending on the infection dynamics of populations, infected wild animals are described as either maintenance or spillover hosts (8). In maintenance hosts, infection persists by intra-species transmission. By contrast, in spillover hosts, infection will not persist indefinitely, unless there is re-infection from another species.

The social structure of European Badgers (*Meles meles*) and their longevity, when infected, make them an ideal maintenance host for *M. bovis* (9). Indeed, badgers are recognised as wildlife reservoirs of *M. bovis* in the UK and Ireland (10). Other species are recognised as TB maintenance hosts in other countries; wild boar (*Sus scrofa*) in Iberia (11), African buffalo (*Syncerus caffer*) and Marsh antelope (*Kobus leche*) in Africa (12), Brushtail Possum (*Trichosurus vulpecula*) in New Zealand (13) and White-tailed Deer (*Odocoileus virginianus*) in the USA (14). In all cases, consideration of *M. bovis* (or other MTBC members) within these maintenance host populations is essential for bTB control programmes (15–19).

In general terms, disease emergence, or increased disease risk, is a frequent consequence of high density, close association and/or ungulate overabundance (20, 21). Red Deer (*Cervus elaphus*) and Fallow Deer (*Dama dama*), social species that naturally aggregate into groups, are considered TB spillover hosts in Spain (22), but recent work has shown that Red Deer, in Austria, Germany, and Italy, can also act as maintenance hosts for both *M. bovis* and *M. caprae* (another MTBC member) when they reach high densities (23–25). While regional density estimates appeared relatively low (e.g., 5.6 animals/km^2), aggregation behaviour of Red Deer was shown to increase local densities by an order of magnitude (up to 46.2 animals/km^2) at sites where supplementary winter feeding was provided (23). In the USA, White-tailed Deer numbers fluctuate from year to year in Michigan. In 1995 the population was estimated to be 2.2 million (26). Since that time the Michigan Department of Natural Resources (MDNR) have restricted supplementary winter feeding (27) and have attempted to reduce the state population to one million deer (in the spring herd). The MDNR have not been issuing estimates of deer populations for many years, but as the number of hunters and the deer harvest have both been on general declines since 1998 (28), we believe the White-tailed Deer population in Michigan is currently increasing. State-wide estimates of White-tailed Deer range from 4.5 to 8.8 animals/km^2, using population estimates from 1937 to 1995, respectively (26). Yet the density of White-tailed Deer at DeSoto National Wildlife Refuge, Michigan, USA has been recorded between 36.5 and 50.6 animals/km^2 (29). It is clear that aggregation behaviour in White-tailed Deer produces dramatic increases in local population density, in comparison to regional density estimates.

Estimates of Sika Deer (*Cervus nippon*) density for County Wicklow have risen from 7.8 animals/km^2 in 2000 to 31.4 animals/km^2 in 2018 (based on Sika Deer harvest or "bag" numbers published by the National Parks and Wildlife Service – NPWS–**Supplementary Table 1**). Although density calculations based on "bag" numbers are likely to be underestimates, as not all deer deaths (e.g., road deaths and natural causes) are reported in any given year, the technique has proved to be a good predictor of deer populations (30). The most recent estimate (31.4 animals/km^2 in 2018) for Sika in County Wicklow provides a regional density far greater than that of both Red Deer in an Austrian bTB hotspot (23) and that of state-wide estimates for White-tailed Deer in Michigan (26). If Sika Deer exhibit aggregation behaviour similar to Red and White-tailed Deer, it is likely that they have reached a threshold density in County Wicklow and are now acting as maintenance hosts for TB, rather than spillover hosts.

TB has been recorded in both farmed and wild deer in Ireland (31), but records from the Irish Department of Agriculture, Food and the Marine (DAFM) show that Wicklow is the only county in Ireland where TB has been confirmed in multiple hunted deer over many years (6). In 2007/08, within Wicklow, 80 Sika deer were culled and their entire carcases examined for the presence of typical TB lesions. Incidence of TB was 5% and the strains of TB in the deer were also found to be present in local badger and cattle populations (6). In 2014/15 a more thorough TB detection protocol identified 17% prevalence (23 of 133 deer) (6). Unfortunately, it is unclear whether the difference in prevalence between these two studies represents a rise in TB infection in Wicklow's Sika Deer, as detection protocols varied between years. Despite that uncertainty, it is clear that TB has persisted in Wicklow's Sika deer for at least the last decade. A recent study (32) has identified a high level of diversity of TB strains in Sika deer in County Wicklow. The same study also found that the TB strains in the deer were shared between badgers and cattle (32). This indicates that deer are sharing TB with other TB hosts in their environment and may, therefore, be acting as a source of infection for local cattle populations. Thus, we investigated whether Sika deer met the characteristics of a wildlife reservoir host for *M. bovis* (15, 19).

MATERIALS AND METHODS

We conducted two multiple linear regression analyses. The first analysis (herd-level analysis) used the density of cattle herds in each Irish county experiencing bTB breakdowns as the dependent variable and densities of cattle herds, Sika deer and badgers in each Irish county, as well as the year of recording (2000–2018), as predictive variables. The second analysis (individual-level analysis) used the density of cattle which were removed under the bTB eradication programme (i.e., number of "reactors") in each Irish county as the dependent variable, and densities of cattle, Sika deer, and badgers in each Irish county, as well as the year of recording, as predictive variables. The way in which the values for the dependent and predictive variables were determined is explained below.

Estimations of Deer Populations From Hunting Bags

A study of Sika Deer in the Wicklow Mountains National Park (WMNP) (33) identified a nett annual productivity of about

25% (including estimations of female productivity, double births, calf survival, and adult survival). Another estimate (34) puts the nett annual productivity of Sika Deer in County Wicklow at 28%. This means that in order to maintain stable numbers, the maximum sustainable yield of local Sika Deer would be 28% of the population.

Using hunting bag data from the NPWS (www.npws.ie), we considered any increases in county bag numbers in consecutive years as an indication of population increase, i.e., more deer were being born than being removed. In those circumstances, bag totals were considered to be 25% of the population. When consecutive years showed slight decreases in hunting bags, those bag numbers were considered to indicate more deer were being removed than being born. We assumed those bags to represent 33% of the deer population. While bag numbers appear to offer a relatively crude method of assessing deer populations (35, 36), they can provide good estimates of county-level populations (30) (**Supplementary Table 2**). A variety of Sika density estimates were produced for each county by dividing population estimates of each deer species by habitat areas derived from CORINE 2012 (37) (**Supplementary Table 3**). This technique provided a way of allowing for deer aggregations in preferred habitats (e.g., pasture and woodland). The Sika density estimates which best explained variation in the herd-level (**Supplementary Table 4**) and individual-level (**Supplementary Table 5**) breakdown data for cattle were selected for those models, respectively, although all Sika density estimates gave similar results (**Supplementary Tables 4**, **5**).

Badger Population Estimates

A survey of badger main setts in Ireland (38) was used to provide a baseline for badger densities across Ireland. Despite attempts at modelling fluctuations in badger populations using occupancy rates (39), nett productivity estimates (40) and removals over the study period, we were unable to produce credible population estimates. In County Wicklow, a seven-year study of badgers in an unculled population, found that the local density remained stable between 2010 and 2016, despite numerous badgers dying on roads in the study area (41). Indeed, this population showed a nett migration into surrounding areas, where DAFM culling was in operation. If such a pattern were repeated across the country, it seems likely that culled areas would show only temporary reductions in local population density. Bearing this in mind, we assumed that badger populations, at the county level, remained constant over the study period (**Supplementary Table 6**). This assumption tallies with anecdotal data.

A variety of badger density estimates were produced for each county by dividing estimates of badger numbers by habitat areas derived from CORINE 2012 (37). The badger density estimates which best explained variation in the herd-level (**Supplementary Table 7**) and individual level (**Supplementary Table 8**) breakdown data for cattle were selected for those models, respectively. As the badger populations in all counties were assumed to be stable, badger density values for individual counties were constant across the study period (2000–2018) in both the herd-level and individual-level models.

Cattle Population Densities

We used data from DAFM to identify the number of herds registered in each county (**Supplementary Table 9**), the number of infected herds within each county (**Supplementary Table 10**), the number of cattle removed as reactors in each county (**Supplementary Table 11**) and the number of individual cattle in each county (**Supplementary Table 12**), for each year of the study period (42).

Density estimates of herds, or individuals, were produced by dividing cattle numbers for each county by pasture areas derived from CORINE 2012 (37) (**Supplementary Table 3**).

Statistical Analysis

Analyses were performed in R (43) we used the *lmer* function from the **lme4** package (44) to perform Generalised Linear Models (GLMs). Data were centred and scaled, to remove any numeric bias from individual predictive variables, using the *standardize* function from the **arm** package (45). County cattle density, county badger density, county deer density, year and all two-way interactions of animal densities with year, were included in full models for herd-level and individual-level analyses. The R code for these models is included for the reference of readers (**Supplementary Figure 1**).

RESULTS

Herd-level Analysis

This analysis used the density of cattle herds, Sika deer and badgers to explain the variation in herds with breakdowns at the county level between 2000 and 2018. Cattle herd densities were calculated per square kilometre of pasture. Following a comparison of density alternatives which best explained the variation in herd TB-breakdown density (**Supplementary Table 4**), Sika deer densities were calculated per combined square kilometre of pasture and forestry. A similar comparison for badgers (**Supplementary Table 7**), identified densities calculated per square kilometre of pasture as most appropriate.

The GLM provided several details about the trends in herd breakdown density (**Table 1**). Over the period of the study (2000–2018), the number of herd breakdowns fell (Year; estimate $= -0.547431$, $t = -19.93$, $P < 0.001$). The density of cattle herds was strongly and positively correlated with herd breakdown density (Cattle Herds; estimate $= 0.319594$, $t = 6.877$, $P < 0.001$), and this correlation showed a slight (but statistically insignificant) weakening over the course of the study (Cattle:Year interaction; estimate $= -0.114097$, $t = -1.259$, $P > 0.05$). The density of badgers was also strongly and positively correlated with the herd breakdown density (Badgers; estimate $= 0.234398$, $t = 5.201$, $P < 0.001$), and this correlation also weakened over the course of the study (Badger:Year interaction; estimate $= -0.222166$, $t = -2.487$, $P = 0.0132$). The density of Sika deer was weakly (lacking statistical significance) positively correlated with herd breakdown density (Deer; estimate $= 0.05551$, $t = 1.674$, $P > 0.05$), but the strength of this correlation increased over the course of the study (Deer:Year interaction; estimate $= 0.139537$, $t = 2.134$, $P = 0.0334$).

TABLE 1 | Output from the GLM of herd-level analysis.

	Estimate	Std. Error	*t*-value	Pr(>|t|)	Significance
(Intercept)	−0.005468	0.01371	−0.399	0.6902	
Cattle (herd) density	0.319594	0.046474	6.877	<0.001	***
Badger density	0.234398	0.045066	5.201	<0.001	***
Deer density	0.05551	0.033163	1.674	0.0948	
Year	−0.547431	0.027464	−19.93	<0.001	***
Cattle: Year	−0.114097	0.090601	−1.259	0.2085	
Badger: Year	−0.222166	0.089342	−2.487	0.0132	*
Deer: Year	0.139537	0.065393	2.134	0.0334	*

*The nature of the correlations between explanatory variables and the dependent variable (cattle herd breakdown density) are indicated by the values in the Estimate column; positive values indicate a positive correlation and negative values indicate a negative correlation. The P-values of correlations are given in the Pr(>|t|) column, and the starred rating of these correlations is given in the significance column; *p < 0.05, **p < 0.01, ***p < 0.001.*

Individual-level Analysis

This analysis used the density of cattle, Sika deer and badgers to explain the variation in the density of "reactor" cattle from herds with breakdowns, at the county level, between 2000 and 2018. Cattle densities were calculated per square kilometre of pasture. Following a comparison of density alternatives which best explained the variation in "reactor" density (**Supplementary Table 5**), Sika deer densities were calculated per square kilometre of agricultural land. A similar comparison for badgers (**Supplementary Table 8**), identified densities calculated per square kilometre of pasture as most appropriate.

The GLM provided several details about the trends in "reactor" cattle density (**Table 2**). Over the period of the study (2000–2018), the number of "reactors" fell (Year; estimate = −0.43572, $t = -12.582$, $P < 0.001$). The density of cattle was strongly and positively correlated with "reactor" density (Cattle; estimate = 0.2498, $t = 4.831$, $P < 0.001$), but the strength of this correlation showed a dramatic reduction over the course of the study (Cattle:Year interaction; estimate = −0.39112, $t = -4.095$, $P < 0.001$). The density of badgers was positively correlated with "reactor" breakdown density (Badgers; estimate = 0.15049, $t = 2.983$, $P = 0.03$), but this correlation did not change over the course of the study (Badger:Year interaction; estimate = 0.03452, $t = 2.983$, $P > 0.05$). The density of Sika deer was strongly and positively correlated with "reactor" density (Deer; estimate = 0.18767, $t = 4.461$, $P < 0.001$). While the strength of this correlation increased over the course of the study, that change did not reach statistical significance (Deer:Year interaction; estimate = 0.13961, $t = 1.712$, $P = 0.0876$).

General Findings

Although there were differences between the two analyses, some general effects can be seen. Principally, the incidence of TB in cattle reduced dramatically during the study period. Despite this reduction, the local density of cattle, badgers, and Sika deer were all positively correlated with local TB density. While the correlation between local cattle density and local TB incidence, along with the correlation between local badger density with local TB incidence have been decreasing over time, the correlation between local deer density and local TB incidence has been increasing.

Having established that local Sika deer density was a useful predictor of local TB incidence, we investigated whether there were any regional aspects to this relationship. We ran further iterations of the herd-level and individual level analyses with subsets of the national data, excluding individual counties.

When Wicklow data were removed from the herd-level dataset (**Supplementary Table 13**), the resultant model no longer identified Sika deer density as an important predictor of TB breakdowns (Deer:Year interaction; estimate = 0.030361, $t = 0.431$, $P = 0.6665$). The same trend was seen when Westmeath data were removed from the herd-level dataset (**Supplementary Table 13**) (Deer:Year interaction; estimate = 0.130046, $t = 1.955$, $P = 0.0512$).

When Wicklow data were removed from the individual-level dataset (**Supplementary Table 14**), the resultant model no longer identified Sika deer density as an important predictor of TB reactors (Deer; estimate = −0.05224, $t = -1.258$, $P = 0.20896$). County Wicklow was the only county which affected the individual-level analysis in this way.

Curiously, when Leitrim data were removed from the herd-level dataset (**Supplementary Table 13**), the resultant model no longer identified badger density as an important predictor of TB breakdowns (Badgers; estimate = 0.040442, $t = 1.203$, $P = 0.2295$). The same trend was seen when Leitrim data were removed from the individual-level dataset (**Supplementary Table 14**) (Badgers; estimate = 0.05352, $t = 1.316$, $P = 0.1887$).

DISCUSSION

Herd-level Analysis

Higher local cattle densities and higher local badger densities correlate with higher local cattle herd TB-breakdown densities (**Table 1**). Such correlations are expected, since cattle and badgers are known maintenance hosts of TB in Ireland (46). The herd-level model shows the correlation between local badger density and local cattle herd TB-breakdown density has been decreasing over the last 19 years. This suggests that the national bTB

TABLE 2 | Output from the GLM of individual-level analysis.

	Estimate	Std. Error	*t*-value	Pr(>|t|)	Significance
(Intercept)	−0.01777	0.01719	−1.034	0.3016	
Cattle ("reactor") density	0.24980	0.05170	4.831	1.82 e−06	***
Badger density	0.15049	0.05045	2.983	0.0030	**
Deer density	0.18767	0.04207	4.461	1.01 e−05	***
Year	−0.43572	0.03463	−12.582	2.00 e−16	***
Cattle: Year	−0.39112	0.09552	−4.095	4.95 e−05	***
Badger: Year	0.03452	0.09598	0.360	0.7193	
Deer: Year	0.13961	0.8157	1.712	0.0876	

*The nature of the correlations between explanatory variables and the dependent variable (cattle "reactor" density) are indicated by the values in the Estimate column; positive values indicate a positive correlation and negative values indicate a negative correlation. The P-values of correlations are given in the Pr(>|t|) column, and the starred rating of these correlations is given in the significance column; *p < 0.05, **p < 0.01, ***p < 0.001.*

eradication policy in Ireland (47, 48), and in particular the control of TB in the badger population, has been achieving success. Indeed, recent monitoring of TB in culled badgers has shown a reduction in infection rate from 26% in 2007 to 11% in 2011 (49). Such progress may encourage a progressive shift from culling towards badger vaccination (50). However, the correlation between local cattle herd density and local cattle herd TB-breakdown density has remained static. This indicates that whatever controls on herds are currently in place have been insufficient to make significant reductions in herd-level TB infection over the last 19 years. A recent study has suggested that high risk herds should be monitored even more closely (50).

While the management of cattle and badgers appears to have reduced local rates of TB breakdowns in cattle herds, Sika populations do not appear to have been under management. Increases in local Sika deer densities were correlated with an increase in local rates of TB breakdowns in cattle herds (**Table 1**). From further analyses it appears that the data from Counties Wicklow and Westmeath were driving this correlation (**Supplementary Table 13**). These counties show the highest correlations of Sika deer density with TB levels in cattle. It is here that we believe Sika deer, as maintenance hosts of TB, pose the greatest threat to cattle.

Individual-level Analysis

Higher local cattle densities and higher local badger densities correlate with higher numbers of "reactor" cattle at test (**Table 2**). This is to be expected, as cattle and badgers are known maintenance hosts of TB in Ireland (46). Our individual-level model identifies the correlation between local cattle density and local "reactor" density has decreased over the study period. This suggests that herd management and monitoring practices have improved over the last 19 years. Potentially, it is these improvements which have prevented the influence of TB wildlife vectors (i.e., badgers or deer) changing over the course of the study. However, the local density of Sika deer was strongly positively correlated with the density of "reactor" cattle. It appears that data from County Wicklow were driving this correlation (**Supplementary Table 14**).

Sika Deer as Maintenance Hosts of *M. bovis*

While a growing body of evidence (6, 32) has identified TB in Sika deer in Wicklow, it has been difficult to identify Sika deer as maintenance hosts of TB. Several unique strains of *M. bovis* have been found in Sika deer within Wicklow (32), which suggests they act as wildlife reservoirs of TB. The data presented here provides clear evidence that higher levels of TB in cattle are associated with higher local densities of Sika deer. While this does not demonstrate Sika deer act as maintenance hosts of TB in Ireland, it adds further weight to the argument.

Sika deer in Wicklow now have regional densities comparable to other deer populations where TB maintenance has been demonstrated (23, 51). While no other deer species are implicated in our findings, Roe deer (*Capreolus capreolus*) are known to act as spillover hosts (52), and both Red deer (10, 23) and Fallow deer (53, 54) have been found to act as TB maintenance hosts at higher densities. So, while we would encourage active management of Sika Deer in Wicklow, we would also encourage the monitoring of Sika, Red and Fallow deer numbers in other counties. Such monitoring should be of particular interest to those who harbour the ambition of making Ireland TB-free by 2030 (48), or England TB-free by 2038 (55), as well as stakeholders in countries where the management of bovine TB continues to cost tax-payers eye-watering sums (48, 55).

It is difficult to offer clear guidelines regarding a "safe" Sika deer population density within Wicklow. Sika are not distributed evenly across the county (Wesley Atkinson *pers. comm.*), so assessment of density may require calculation at a finer scale (e.g., electoral district). Normal aggregation behaviour by deer means that local population densities may be an order of magnitude higher than the overall regional population densities (23). While we are unaware of a tradition of supplementary feeding of wild deer in Ireland, White-tailed deer in the United States (56) and Red deer in the Tyrol (23) may be encouraged to aggregate when they receive supplementary winter feed. The practice of providing supplementary feeding at pasture to farmed livestock grazing, in the vicinity of Sika habitat, may offer unintended supplementary feed for deer. This could promote aggregation behaviour and bring deer and cattle into contact at high density. Allowing

cattle access to woodland, or higher rough-grazing areas, would also increase cattle-deer interaction opportunities, as well as potentially increasing MTBC contamination in the environment where persistence of these diseases and exposure to susceptible species is a concern (4). Interspecies transmission of MTBC has been reported at interface areas between species (57–59).

Our findings serve as a timely reminder that the final eradication of TB in any national cattle herd, is likely to prove problematic unless all MTBC diseases are addressed in all livestock (60) and wildlife reservoirs (4, 16–19, 61).

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 authors.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because while the species considered in this study were vertebrates, we were not required to conduct any experiments with them. We relied on data which had already been collected and was freely available in the public domain.

AUTHOR CONTRIBUTIONS

DK formulated the idea, which was then developed by DK, MG, and EM. MG and EM sourced the data. DK developed the models to analyse the data and drafted the manuscript. MG fleshed out the outline. All authors continually reviewed and edited the manuscript, in order to produce the submitted draft.

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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.2021.632525/full#supplementary-material

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Multispacer Sequence Typing for *Mycobacterium bovis* Genotyping

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The molecular typing of *Mycobacterium bovis*, which causes bovine tuberculosis, can be accomplished by combining different polymorphic markers, contributing to its epidemiological investigation. Multispacer sequence typing (MST) is a sequencing-based method that employs intergenic regions susceptible to higher mutation rates given the low selection pressure. It has been applied to *M. tuberculosis*, but not to *M. bovis*. The aim of this study was to evaluate a MST for *M. bovis*. A total of 58 strains isolated from tissues with lesions suggestive of bovine tuberculosis, coming from cattle herds in six Brazilian states and four standard samples of *M. bovis* were typified employing the MST technique. Fourteen intergenic regions were used, and four types of genetic events were reported: single nucleotide mutation (SNP), insertion, deletion, and tandem repeat (TR). Seven loci were chosen for typing. Twenty-eight type sequences (ST) were identified, indicating type sequences (ST) were identified, indicating a 92.9% HGDI (Hunter Gaston Discriminatory Index). The data were used to analyze the evolutionary patterns of these isolates and correlate them to phylogeographic lineages based on the formation of clonal complexes generated from eBURST software. Later, we associated the MST with spoligotyping technique, currently considered the gold standard for classification of *M. bovis*. The results support the MST as an alternative method for genotyping of *M. bovis*. The method has the advantage of sequencing and the availability of sequences analyzed in public databases, which can be used by professionals around the world as a tool for further analysis. This was the first study to identify the variability of isolates of *M. bovis* by the MST method.

Keywords: *Mycobactereium bovis*, bovine tuberculosis, genotype, epidemiology, MST

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INTRODUCTION

Mycobacterium bovis is the main pathogen of bovine tuberculosis (TB), an important disease of domestic cattle, which can also be associated with an underestimated number of human TB cases (zoonotic TB) and serious threats to wildlife conservation (1–4). Bovine TB is a disease of high health and economic relevance for livestock and is part of the group of notifiable diseases listed by the World Organization for Animal Health (OIE) (5). Infection routes in the cattle are influenced by factors such as age, environment, and hygiene practices, as the bacterium can be eliminated via various routes, such as respiratory droplets and aerosol, milk, feces, urine, vaginal secretions

and/or semen (6). The presence of wildlife reservoirs in certain regions also hampers the control of the disease in livestock and translate into serious consequences for the preservation of wildlife species in multi-host systems (7). The disease directly affects livestock productivity, influencing international trade of animal products and, due to its zoonotic potential, is also of public health concern (8, 9).

Mycobacterium bovis belongs to the *Mycobacterium tuberculosis* complex (MTBC), a clonal group of mycobacterium species, lineages and/or ecotypes that cause similar diseases in a variety of mammalian hosts (10–12). Genomes of MTBC members are highly similar, with >99.9% identity over homologous regions, including the 16S rRNA gene, and absent remodeling through horizontal gene transfer or DNA recombination (10, 13, 14). This bacterial complex evolves only through SNPs (single nucleotide polymorphisms), short indels, large deletions, transposition of insertion sequence (IS) elements, and duplication of few paralogous gene families (10, 15, 16). Despite this high genetic similarity, species and lineages of the MTBC present variable phenotypes of host tropism and virulence (10–14).

Over the years, molecular genotyping techniques have significantly contributed to epidemiological studies of bovine TB, facilitating the knowledge of the spatial and temporal distribution of *M. bovis* strains, from populational structure to outbreak investigations (17, 18). Given the clonal nature of the MTBC, these techniques are based on few highly polymorphic regions of their genomes, including tandem repeats, a CRISPR locus, PGRS genes, and IS elements (17–21). The IS*6110*-based RFLP is a technique commonly used in *M. tuberculosis* genotyping, but of little application for *M. bovis* strains, as they carry only few IS*6110* copies in their genomes (22). Among the remaining techniques, spacer oligonucleotide typing (spoligotyping; based on the CRISPR locus) and variable number tandem repeat- mycobacterial interspersed repetitive units (MIRU-VNTR) have been the preferred combination for typing isolates of *M. bovis* in several countries (17–21). However, both techniques have limitations. Spoligotyping does not provide good resolution at the region or farm level, which limits its use for pathogen's transmission detection (17, 18). Spoligotyping is also sensitive to homoplasy, i.e., unrelated lineages presenting identical spoligotypes (23). This happens because the loss of a spacer region at the spoligotyping locus is a common event that can occur in strains that are not phylogenetically related (23, 24). While MIRU-VNTR has been reported to better discriminate among isolates at the farm level, there are 24 loci to choose from, and laboratories must frequently test which loci would better fit their reality for better discriminatory power (25). MIRU-VNTR is also influenced by homoplasy (24) and depending on the laboratory infrastructure, a 24 loci evaluation of *M. bovis* isolates to avoid homoplasy bias can be very laborious and time consuming (17).

Recently, *M. bovis* whole-genome sequencing (WGS) using next-generation sequencing platforms has been suggested to replace these genotyping assays (17, 18). However, WGS still faces many challenges related to its financial burden and requirement of specialized personnel, particularly in low and middle-income countries in which bovine TB is highly endemic. Most reported *M. bovis* WGS studies have been conducted in developed countries or in developing countries by researchers from high-income nations (26–32). Thus, albeit likely ideal, WGS use is still unreachable to many programs of bovine TB control and eradication around the world. Thus, there is a need for the continuous development of genotyping techniques with high resolution power. Accordingly, a multispacer sequence typing (MST) scheme for *M. tuberculosis* based on the Sanger sequencing of eight PCR-amplified intergenic regions, including four regions previously reported as VNTR regions, has been proposed (33). The aim of this study was to investigate the usefulness of this MST scheme to genotype 58 *M. bovis* isolates obtained from different Brazilian states.

MATERIALS AND METHODS

Bacterial Isolates

A total of 58 cultured isolates of *M. bovis*, deposited in the sample bank of the National Agricultural Laboratory of Minas Gerais (Lanagro/MG), Brazil, were selected. These isolates were obtained from cattle herds located in six Brazilian states [Minas Gerais ($n = 48$), Goiás ($n = 3$), Mato Grosso-MT ($n = 1$), Rio Grande do Sul-RS ($n = 1$), Paraná-PR ($n = 1$) and São Paulo ($n = 4$)] between the years 2006 and 2010. First isolation of these *M. bovis* from clinical samples were performed in Lanagro/MG according to previous publications (34). Isolated strains were identified as *M. bovis* by using a PCR assay described by Sales et al. (35). Four additional laboratory strains [*M. bovis* CR01; *M. bovis* AN5 (36); *M. bovis* BCG CR13; *M. bovis* México CR36] were also selected. All procedures were performed in a Biosafety Level 3+ Laboratory (BSL-3+) located at the Farming National Laboratory (LANAGRO) Minas Gerais, Ministry of Agriculture, Livestock and Supply, Brazil. Tubes containing DNA were properly disinfected, removed from BSL3+ and stored at −20°C until further analysis.

DNA Extraction and PCR Assays

A loop of each bacterial strain cultured in Stonebrink medium was subjected to a phenol-chloroform DNA extraction as previously described (37). Extracted DNA was subjected to conventional PCR assays of 14 intergenic spacer regions using previously described primer sequences (33). Each PCR assay was performed in a PX2 Thermal Cycler thermocycler (Milford, MA, USA). Briefly, 20 μL were prepared with the following concentrations of reagents: 10 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM dNTPs (Invitrogen, USA), 1 U Jump Start®Taq DNA Polymerase (Sigma, USA) and 1 X GoTaq Green® buffer (Promega, USA). DNA sample concentration added in each reaction was 100 ng/μL. As positive controls, we used DNA extracted from *M. bovis* strain AN5 (38), while negative controls consisted of ultrapure water. The conditions of the PCR assays determined for each pair of primers are shown in **Supplementary Table 1**. Amplification products were detected by gel visualization following electrophoresis.

Sequencing and Data Analysis

The PCR products were purified using the PureLink® PCR Purification Kit (Invitrogen, USA) and sequenced using Big Dye® Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's recommendations. Sequencing was performed in both directions in the 3130 Genetic Analyzer (Applied Biosystems, USA) equipment.

Sequence assemblies were performed with CAP3 program (39) using nucleotide data with quality higher than PHRED 20. Sequences were edited in Bioedit v.7.2.5 program (40). Subsequently, all sequences obtained for each primer pair were subjected to a multiple sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to verify the degree of identity between two or more sequences and the allelic diversity present in each spacer region. Each allele detected was assigned an identification number. For each individual DNA sample, the set of loci and their respective allele defined an allelic profile or sequence type (ST), which identified a clone. Bacterial isolates analyzed in this study were identified by numbers 1–58. The laboratory strains *M. bovis* CR01, AN5, CR13, CR36, and AF2122/97 (Genbank: NC002945.3) included in the analyses were represented by the numbers 59–63, respectively.

Spoligotyping

The spoligotyping was performed as described by Kamerbeek (41) using spolygotyping kit by Ocimum Biosolutions. The identification of individual spoligotypes and the appointment of unpublished patterns were made using Mbovis.org website (www.mbovis.org) (42).

Analysis of Genetic Relationship Among the Isolates

An evolutionary descent analysis of *M. bovis* isolates was performed using eBURST algorithm (43), available in Phyloviz software (44). Clonal complexes (CC) as defined by eBURST were identified and organized in dendrograms based on the comparison of the obtained allelic profiles of the tested *M. bovis* isolates. Briefly, CCs were determined by a simple model of clonal expansion and diversification based on the differences between these allelic profiles. Each CC was assigned a founder genotype that displays a more likely hypothetical pattern of evolutionary descent among the STs that make up this complex. The STs were organized into complexes by comparing the variation that exists among the loci of each founder genotype loci compared to other isolates (43). Accordingly, genotypic variations found in only one locus are called single locus variants (SLV), in two loci are called double locus variants (DLV), and in three loci are called triple locus variants (TLV) (43, 44). Confidence values for each SLV, DLV and TLV detected in each ST founder in a CC were assessed by bootstrapping (43). The geographic location and year of isolation of the strains were also included in the analysis.

Phylogenetic Analysis

The phylogenetic reconstruction of *M. bovis* isolates was performed by PubMLST program (http://pubmlst.org/) using the Neighbor-Joining model. The program uses PHILIP package to create Neighbor-Joining and UPGMA trees based on allelic profiles determined in the study.

Calculation of Allelic Diversity

The discrimination index (D) previously described by Hunter-Gaston (45) was used to calculate the allelic diversity at each locus and to assess genotypic diversity for each method employed (MST and spoligotyping).

RESULTS

Multispacer Sequence Typing (MST)

Fourteen intergenic spacer regions were analyzed in the selected *M. bovis* isolates. Regions MST1, MST2, MST3, MST6, and MST8 showed no sequence variation in an initial evaluation of 15 randomly chosen *M. bovis* isolates of the collection and were then excluded from the study. In addition, after multiple attempts, the PCR assay of the MST10 region failed to amplify appropriate PCR products, and this region was also excluded from the study. For the MST14 region, Sanger sequencing was not able to cover the whole extent of the region, hampering the identification of possible polymorphic positions for some of the samples and was also excluded from further evaluation. Finally, the remaining regions MST4, MST5, MST7, MST9, MST11, MST12, and MST13 proved quite variable, with a total of 28 mutational events, including single nucleotide polymorphisms (SNP), insertions, deletions, and tandem repeats. **Figure 1** shows the alleles identified for each of the seven evaluated spacer regions.

Definition of Genetic Profiles

Multiple sequence alignment of the DNA sequences of all *M. bovis* isolates for each locus allowed the identification of up to 8 different alleles which received individual identifier numbers (**Figure 1**). The grouping of all loci for each individual defined an allelic profile that was represented by a sequence type (ST). For the 63 isolates of *M. bovis* studied, 28 STs were found, where eight were represented by more than one *M. bovis* isolate, and 20 were described as orphan STs, as shown in **Table 1**.

Evolutionary Descent Analysis

The STs detected in the study were used to determine the genetic relationship of *M. bovis* isolates. A total of five CCs (ST6, ST10, ST11, ST17, and ST59) were detected (**Figure 2**). ST17, consisting of nine *M. bovis* isolates, being eight from Minas Gerais state and one from São Paulo state, was appointed as the founder CC of the whole group, as it had the highest number of SLVs (ST4, ST6, ST10, ST14, ST19, ST39, ST45, and ST54, including 24 *M. bovis* isolates from Minas Gerais, two from São Paulo and one from Mato Grosso) with diverging genotypes in only one of seven loci. ST6 was appointed the largest CC and presented the highest frequency of isolates with the same genotypic profile from different geographical locations: 10 *M. bovis* isolates from Minas Gerais, two from São Paulo, and one from Mato Grosso. This complex presented six SLVs, which expressed close relationship to ST1, ST11, ST12, ST17, ST25, and ST55, with 22 *M. bovis* isolates from the states of Minas Gerais ($n = 19$), São Paulo (n

FIGURE 1 | Alleles identified in seven intergenic spacers in *Mycobacterium bovis*. MST4 (ETR-B): eight alleles containing 1, 2, 3, 4, 5, and 7 copies of the 57 bp tandem repeat sequence (TR) and the presence of a SNP G47A (alleles 5 and 7). MST5: deletion of 67 bp. MST7: Five alleles containing from 2, 3, 4 and 5 copies

(Continued)

FIGURE 1 | of the 57 bp tandem repeat, a deletion of the first 11 bp repeat for all samples and a SNP G80A in allele 3. MST9: two alleles containing one and three repeat tandem copies of 54 bp, and a sequence represented by some polymorphisms in the genome of *M. bovis*, characterized by a different allele of a repeated region also present in the genome of *M. avium.* MST11: three alleles containing 3, 5, and 6 tandem repeats of 57 bp, the first and last repetition characterized by a deletion of 11 bp in all isolates. MST12: five alleles containing 3–5 repeat tandem copies of 77 bp with the first repetition characterized by a deletion of 37 bp in all isolates, the presence of a G76 base (allele 5), A424G SNP (allele 2–5), T485A SNP (allele 3) and a deletion of 18 bp after the last repeat (allele 1). MST13: three alleles containing from 1 to 3 copies of the 57 bp and a 23 bp deletion in the first repetition in all isolates.

TABLE 1 | Twenty-eight (28) ST identified by the comparison of genotypic profiles of 63 isolates of *M. bovis*.

ST	Number of *M. bovis* isolates	Allelic profile							%
		MST4	MST5	MST7	MST9	MST11	MST12	MST13	
6	12	4	2	4	1	2	2	3	19.05
17	9	4	2	4	1	1	2	3	14.29
1	5	3	2	4	1	2	2	3	7.94
10	5	4	2	4	1	1	2	2	7.94
19	4	4	2	4	1	1	5	3	6.35
11	4	6	2	4	1	2	2	3	6.35
14	2	4	2	4	1	1	3	3	3.17
25	2	4	2	4	1	2	5	3	3.17
45	1	1	2	4	1	1	2	3	1.59
30	1	2	2	4	1	1	2	2	1.59
61	1	3	2	1	1	2	2	1	1.59
54	1	3	2	4	1	1	2	3	1.59
31	1	4	1	4	1	2	2	1	1.59
5	1	4	2	2	2	2	2	3	1.59
39	1	4	2	4	1	1	2	1	1.59
9	1	4	2	4	1	3	4	3	1.59
4	1	4	2	4	2	1	2	3	1.59
13	1	5	1	4	1	1	2	2	1.59
12	1	5	2	4	1	2	2	3	1.59
52	1	6	1	4	1	2	2	3	1.59
63	1	6	1	4	1	2	4	3	1.59
29	1	6	2	4	1	1	2	2	1.59
62	1	6	2	4	1	2	1	2	1.59
59	1	6	2	4	1	2	2	2	1.59
60	1	6	2	4	1	2	4	2	1.59
58	1	7	2	3	3	2	2	3	1.59
55	1	7	2	4	1	2	2	3	1.59
34	1	8	2	4	1	2	2	1	1.59

ST identifies the sequence type featuring a genotypic profile (or allelic).
**Displays standard strains of M. bovis without source information. % Percentage of M. bovis isolates with corresponding ST.*

= 1), Goiás ($n = 1$), and Paraná ($n = 1$). ST11, consisting of four strains, coming from Minas Gerais and Goiás, is one of the SLVs of ST6 that has diversified producing their own SLV, characterized by members with unique genotypic profiles. Interestingly, the single *M. bovis* isolate from Rio Grande do Sul, the only state not bordering any of the states evaluated herein, having unique genotype (ST13) without associated SLVs, DLVs or TLVs.

Spoligotyping and MST

Spoligotyping was performed for 55 of the 63 *M. bovis* isolates that composed the study. A total of 22 spoligotypes were detected, 19 of which were already mentioned in the literature. A total of eight clusters (G1–G8) were formed, representing 74.54% (41/55) of the *M. bovis* isolates. The remaining 14 (24.45%) *M. bovis* isolates had the spoligotype described as orphans (**Table 2**). SB2211, SB2212, and SB2213, found in samples from Minas Gerais, São Paulo, and Mato Grosso, respectively, have never been described prior to this study. SB1135 was the most frequent spoligotype (n = 12), followed by SB0121 ($n = 9$), SB0295 ($n = 6$), and SB0881 ($n = 5$). SB1135 had samples only from Minas Gerais, while SB0121 and SB0295 were represented by Minas Gerais and São Paulo; and SB0881 by Minas Gerais and Goiás.

A separate analysis contrasting MST and Spoligotyping was also performed using samples from Minas Gerais only, which

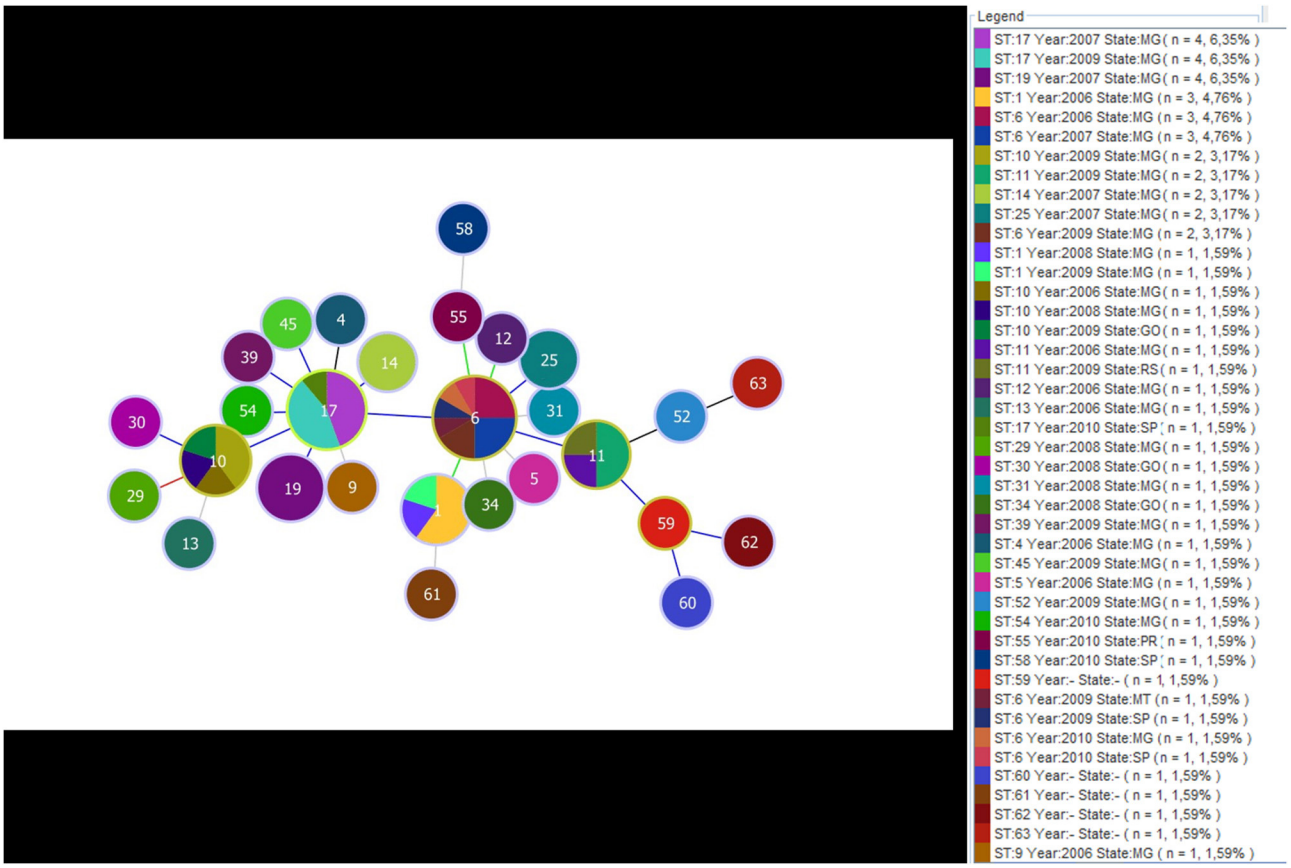

FIGURE 2 | Minimum spanning tree exhibiting the formation of clonal complexes according to genotypic profiles obtained by the multi sequence typing of *Mycobacterium bovis*. Distribution of 58 samples of *M. bovis* isolated from the years 2006 to 2010, four standard samples of *M. bovis* (CR01, CR02, CR13, and CR36) and the strain *M. bovis* (AF2122/97). The dendrogram reveals the formation of five clonal complexes ST6, ST10, ST11, ST17, and ST59 that formed from the comparison of 28 ST. In the subtitle "N" figure indicates the number of isolates with the same genotypic profile, as well as the size of the circles (the larger the circle, the larger the population represented). Circles with more than one color profile indicate the same genotype isolated from different regions and year of isolation of the pathogen.

corresponded to 43 of the 58 *M. bovis*. These samples came from 22 different towns and were isolated between 2006 and 2010. A total of 19 STs and 17 spoligotypes were identified. The eBURST displayed ST6 as the main clonal complex, showing for this CC three SLVs (ST17, ST11, and ST1). ST6 reported eight samples from seven different cities, and four spoligotypes (SB0295, SB0121, SB1135, and SB0333); ST17 reported seven samples from three different cities, and four spoligotypes (SB1135, SB0295, SB0333, and SB0881); ST1 composed five *M. bovis* isolates, from three cities, and three spoligotypes (SB1033, SB1050, and SB2212); and ST11, which depicted three samples, each one from a different city and showed orphans spoligotypes (SB0267, SB0849, and SB1802). The three most frequent spoligotypes were SB1135, represented by 12 samples isolated in 2007, all of them from a same town; SB0121 which was reported in eight samples, from seven cities and isolated in 2006, 2007, 2009, and 2010; and SB0295 reported in five samples, from four cities and isolated in 2008 and 2009 (**Figure 3**).

Phylogenetic Analysis

The phylogenetic tree was generated from 28 STs constituting a cladogram where each ST was represented by its number (**Figure 4**). Overall, *M. bovis* isolates were similarly clustered in the same groups formed by eBURST. It was observed that ST1, ST6, and ST12 were closely located in the center of the tree. When comparing this phylogenetic tree with the genotypic profiles of each ST, the genetic variation was limited by the difference in a single locus, admitting the close relationship between these isolates, which coincide with data observed previously by eBURST. The formation of five clades distributed in the tree edges was observed and the relationship between these clades was generated by variation of three or more loci identified by MST. Clade A was represented by the sequence type coming from Minas Gerais, São Paulo, and Paraná. Clade B was formed only by isolates from Minas Gerais. Clade C was represented by members of Minas Gerais and Goiás. And finally, clade D was represented by a single sample from Goiás and *M. bovis* BCG CR13, while

TABLE 2 | Spoligotypes of 55 *Mycobacterium bovis* isolates.

	Spoligotypes	# *M. bovis* isolates	Clustering	Orphan profiles	Patterns description	State
1	SB0120	2	G1	–	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□□	GO/*
2	SB0121	9	G2	–	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■■□□□□□	MG/SP
3	SB0295	6	G3	–	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■□■□□□□□	MG/SP
4	SB0333	2	G4	–	■■□■■□■■□■■■■□□□■■■■■□■■■■■■■■■■■■■■■■■□□□□□	MG
5	SB0881	5	G5	–	■■□■■■■■□■■■■■■□■■■■■□■■■■□□□□□□■■■■■■■□□□□□	MG/GO
6	SB1033	3	G6	–	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■□■■■■□□□□□	MG
7	SB1135	12	G7	–	■■□■■■■□□■■■■■■□■■■■■□■■■■■■■■■■■■■■■□■□□□□□	MG
8	SB1139	2	G8	–	■■□■■■■■□■■■■■■□■■■■■■■■□□□□□□□□□□■■■■□□□□□	MG/SP
9	SB0140	1		9	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□□	MG
10	SB0267	1		10	■■□■□■■■□■□■■■■□■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□	MG
11	SB0268	1		11	■■□■□■■■□■□□■■■□■■■■■■□■■■■■■■■■■■■■■■■■□□□□□	**
12	SB0486	1		12	■■□■■□■□□□□□□□■□■■■■■■■■■■■■■■■■■■■■■■■□□□□□	RS
13	SB0849	1		13	■■□■□■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□□	MG
14	SB1050	1		14	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■□□□□□■■■■□□□□□	MG
15	SB1093	1		15	■■□□■■■■□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■□□□□□	MG
16	SB1136	1		16	□□□□□□□□□□□■■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□□	MG
17	SB1145	1		17	■■□□□□□■□■■■■■■□■■■■■□■■■■□□□□□■■■■■■■■□□□□□	GO
18	SB1369	1		18	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■■□□□□■■■■□□□□□	PR
19	SB1802	1		19	■■□■□■■■□■□■■■■□■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□	MG
20	SB2211	1		20	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■□□■■■■■■■■□□□□□	SP
21	SB2212	1		21	■■□■■■■■□□■■■■■■□■■■■■□■■■■■■■□□□□□■■■■□□□□□	MG
22	SB2213	1		22	■■□□■■■■□□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■□□□□□	MT

**Laboratory strain M. bovis BCG CR13 **Laboratory strain M. bovis Mexico CR36. GO, Goiás; MG, Minas Gerais; RS, Rio Grande do Sul; SP, São Paulo; MT, Mato Grosso.*

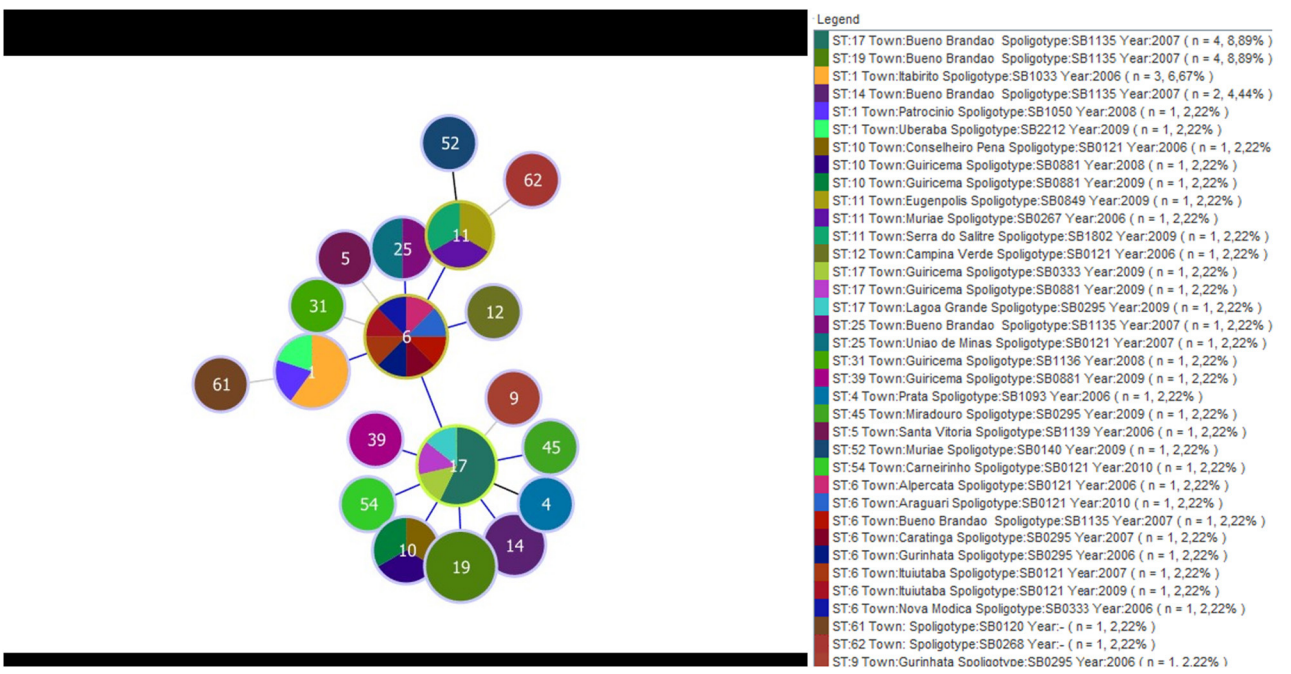

FIGURE 3 | Minimum spanning tree displaying the distribution of 19 type sequences and 17 spoligotypes determined by the MST and Spoligotyping, respectively. The data were obtained from 43 isolates of *M. bovis* from 22 municipalities in MG and isolates between the years 2006 and 2010. The colors represent the different spolitypes and '*n*' indicates the number of isolates with the same genotype profile.

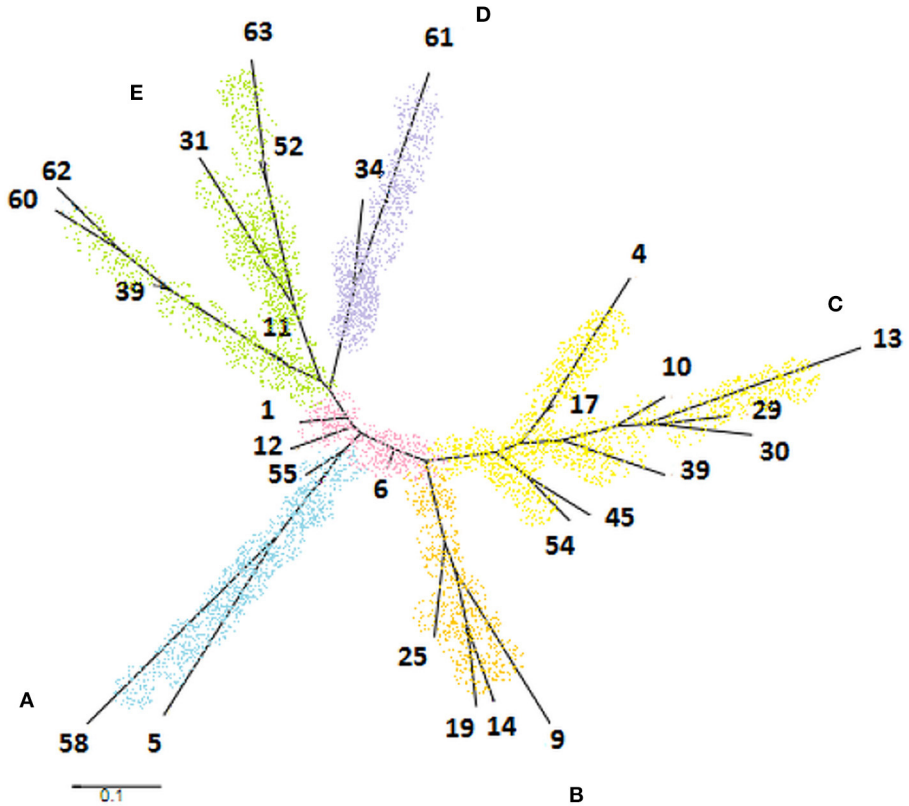

FIGURE 4 | Phylogenetic tree generated by PubMLST program based on 28 sequence types of *Mycobacterium bovis* obtained in the study. It was observed the formation of five clades identified by letters and colors as follows: clade **A** (blue), clade **B** (orange), clade **C** (yellow), clade **D** (purple), and clade **E** (green).

clade E was formed by laboratory strains *M. bovis* CR01, CR02, AN5, CR36, and AF2122/97.

The clade A, marked by the ST5 and ST58, showed the same pattern of spoligotype (SB1139), so as clade D, composed of ST34 and ST61 of spoligotype SB0120. The clade B, formed by ST9, ST14, ST19, and ST25, is composed of spoligotypes SB0295 and SB1135. These two spoligotypes differ only by the absence of the spacer eight in SB1135 compared to SB0295. Clade C includes eight different ST and four spoligotypes. The three ST located in the base of the tree were well-diversified in terms of spoligotypes patterns, corresponding to the high diversity of the ST6. Interestingly, although highly diverse in terms of ST types, these isolates differ only by SLVs. Clade E presented high diversity both of spoligotypes and of genotypic profiles described for ST (**Figure 4**).

Calculation Hunter and Gaston

The discriminatory power of MST and spoligotyping techniques were calculated according to the Hunter-Gaston discrimination index (45). The determination was based on the number of genotypic profiles defined during the test and the relative frequencies of each profile. For MST, the calculations were made for the 63 strains of *M. bovis* and 28 ST resulting in a 92.9% HGDI. Yet for spoligotype, reducing to 55 the number of isolates and to 22 the number of patterns, the index lowered to 91%.

DISCUSSION

The 14 spacer regions analyzed in this study for isolates of *M. bovis* were first described by Djelouadji et al. (33) to characterize *M. tuberculosis* isolates. The decision of analyzing them was taken based on the genetic similarity corresponding to 99.95% between these two species. The markers used are from intergenic regions susceptible to higher mutation rates given the low selection pressure. As the bovine bacillus has a broad host range compared to human germ and evolution occur under various conditions for both, we studied the 14 regions to find the most variable in *M. bovis*.

Genotyping by the MST included the evaluation of 14 intergenic spacer regions, 58 representative samples of *M. bovis*, four standard samples and AF2122/97 strain obtained from GenBank. A total of seven spacers showed genetic variability for the analyzed isolates. Four of them were previously described as Exact Tandem Repeat (ETR) defining ETR-B (MST4), ETR-C (MST11), ETR-D also called MIRU4 (MST12) (46, 47) and Mtub21 (MST13) (48). The other three spacers used were MST5, MST7, and MST9. The results showed the occurrence

of 28 genetic events, distributed among deletions, insertions, variation in the number of tandem repeats and point mutations. Sequencing shows the exact number of tandem repeats present in each sequence, and also determines stable markers within them such as SNPs (49) considered very important for studies of genetic events such as phylogeny.

Drancourt et al. (50) used ETR D-region (MST12) to differentiate eight *M. tuberculosis* complex members by sequencing. The region MST12 (ERT-D) in this study revealed the presence of five alleles characterized by a 40 bp sequence in all isolates, followed by 2–4 copies of the tandem repeat of 77 bp. It was also revealed G76 insertion of a base, an SNP A424G, an SNP T485A and a deletion of 18 bp after the last repetition of the first allele. These events were detected by comparison with the genome of the strain AF2122/97 analyzed *in silico* (**Figure 1**). These data, when compared to sequences characterized by (50) available in Genbank (EU180228- EU180234), showed that profiles found for *M. bovis* in this study were different from the profiles described by the author for the number of repetitions and for SNP.

MST performed for isolates of *M. tuberculosis* defined eight of 14 loci analyzed as variables (MST1, MST2, MST3, MST4, MST8, MST11, MST12, and MST13). In our study, four of these (MST1, MST2, MST3, and MST8) were excluded from our study due to low variability in the isolates of *M. bovis*. Djelouadji et al. (33) identified thirteen alleles in these regions that constituted genetic events of deletions and nucleotide substitutions. The other four spacers (MST4, MST11, MST12, and MST13) were characterized mainly by tandem repeats and showed a different profile found for *M. bovis*. A frequently observed event was the presence of a point mutation at the first base of many of the repetitions, which did not occur in any of the isolates of *M. bovis* studied in this work. MST12 and MST13 had a larger number of alleles for *M. tuberculosis*, while MST4 and MST12 were more variable for *M. bovis*.

The low genetic variability observed in the excluded MST may be related to the sampling bias in samples from Minas Gerais in relation to other states. The existing allelic diversity among *M. bovis* and *M. tuberculosis* may be due to the wide range of hosts of the bacillus of bovine, evolving in different environments and under different conditions (51). The population size and genetic diversity found in *M. tuberculosis* strains in relation to *M. bovis* can also be representative of these differences.

The most common spoligotypes in our study were SB1135 (21.8%), SB0121 (16.36%), SB0295 (10.9%) and SB0881 (9%) (**Table 2**). Of these, SB1135 has been described so far only in Brazil (Mbovis.org). Except for SB1135, the other three SB were also reported by Parreiras et al. (52), as well as SB0120, SB1802, SB1145, SB1033, and SB0267 described as an orphan or less frequent. Corroborating our findings, Rodriguez et al. (53) described SB0295 and SB0121 as the majority SB in a study conducted on a national level, as well as (38) which also recorded the occurrence of SB0295 spoligotype in the country. There have been reports of both in many Brazilian isolates and other Latin American countries (54). SB0881 was described previously in Brazil (55), France (56) and Spain (57). Haddad et al. (56) described the SB0295 and SB0121 in Holland in Belgium. SB0120 and SB0295 were also reported among animal wildlife and domestic species, in southern Spain (58), while SB0121 was identified in cattle and wild fauna in the Iberian Peninsula (59) and had also occurred in Mexico and Venezuela (54).

On the less frequent spoligotypes or orphans, SB0140 was described by Smith et al. (60, 61) as one of the most frequent patterns found throughout the world, particularly in Europe and the British Isles; however, the pattern was observed only in a single isolate in our study. SB0140 was also the most frequent pattern according to a study by (54) using isolates from Latin America. SB1145 and SB1802 were only isolated in Brazil, previously reported in Minas Gerais, also with low frequency (52).

CCs were formed and organized from the association of seven genetic regions. The use of clonal complexes as a tool for population analysis is important because it allows us to recognize specific subpopulations or clones that have unique genetic characteristics. Epidemiologically related isolates are derived from the clonal expansion of a single cell, resulting in common characteristics (62). By analyzing MST in association with spoligotyping we observed that some isolates were closely related with exclusive MST profiles and spoligotypes while others, coming from the same region and year, showed different profiles and patterns. The results identified 15 orphans' profiles for MST, 14 orphan spoligotypes and eight groups represented by more than one isolate with equal genotypes in both techniques (**Figure 2**). Similar results were obtained by Parreiras et al. (52) by associating spoligotyping and MIRU-VNTR. The procedure used for this kind of occurrence typed by VNTR in association with spoligotyping is to rearrange the isolates identified by the same VNTR profiles groups geographically located at spoligotyping (60), thereby obtaining more data on the phylogeny of the bovine bacillus.

The importance of finding an alternative method for genotyping *M. bovis* is a result of the occurrence of remarkably diverse populations in different countries, making it difficult to determine a set of useful markers that meets all regions (59). Regional differences in the discriminatory power of genetic markers make it necessary to define an optimal combination of markers that can be used in a particular region or country (63). The different patterns obtained indicate the variety of strains that infect cattle from different geographical areas.

In this study, seven polymorphic loci MST were characterized as variables for *M. bovis*. The observed molecular variation was explored to determine the evolutionary relationships of this pathogen. The results showed that the tested regions can be a promising tool in epidemiological studies. IS*6110* RFLP techniques, spoligotyping, and VNTR have been used as a reference for studying the diversity of species of the *M. tuberculosis* complex (55). However, these methods do not recognize the genetic diversity of strains analyzed as sequencing-based methods capable of identifying all the genetic events present in the molecular marker. The MST method proposed offers the advantage of sequencing, which allows for the detection of different genetic events observed in one sequence. The typing methods performed based on sequencing provide important data

on evolutionary forces that shape bacterial populations (64). Bing et al. (65) determined that a methodology based on sequencing of non-constotive genes (MLSA) can be used for molecular typing, in addition to being an excellent method for use in phylogenetic analyzes, for epidemiological and surveillance studies.

Our results demonstrate that the number of samples needs to be increased to provide additional data to monitor the formation and expansion of clonal complexes according to geographic location and year of isolation. This was the first study to identify the variability of isolates of *M. bovis* by the MST method. The results indicated that the method works to typify differentiate isolates of *M. bovis* using fewer than those used in VNTR or special reagents like the membranes used in spoligotyping.

CONCLUSIONS

Genotyping by MST can contribute to epidemiological studies of *M. bovis*. The method was efficient to detect the genetic variability present in the sequences analyzed and to infer evolutionary relationships in the short term. The method was also suitable to correlate geographical location and phylogenetic data of *M. bovis* isolates used in this study.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ES, CG, GA, HG, ND, and PS performed the all-laboratory tests. ES, AF, and AL performed the interpretation of DNA sequencing results. AF, MH, and AL wrote the manuscript and AG did the translation. MH, AG, PS, and JF accurately reviewed the manuscript. All authors have read and approved the final version of the 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.2021.666283/full#supplementary-material

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Real-Time PCR Validation for *Mycobacterium tuberculosis* Complex Detection Targeting IS*6110* Directly From Bovine Lymph Nodes

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Rapid and accurate diagnostic tools, such as Real-Time PCR (qPCR), need to be implemented as a confirmatory test in the framework of bovine tuberculosis (bTB) surveillance and control programs, shortening the turnaround time to confirm bTB infection. The present study aimed to evaluate a direct qPCR from fresh tissue samples targeting the insertion sequence IS*6110* using individually homogenized bovine lymph nodes compared with microbiological culture. Retropharyngeal, tracheobronchial, and mesenteric lymph nodes fresh tissue samples (n = 687) were collected from 230 different cattle carcasses at the slaughterhouse. Only 23 of the 230 examined animals showed tuberculosis-like lesions, with 62 of 230 considered as positive. Among these 62 animals, 61 resulted as culture-positive, whereas 48 were qPCR-positive. Thus, this qPCR targeting IS*6110* showed an apparent diagnostic sensitivity and specificity values of 77.1% [95% confidence interval (CI): 66.5–87.6%] and 99.4% (95% CI: 98.3–100.6%), respectively, and a positive predictive value of 97.9% (95% CI: 93.9–102.0%) and negative predictive value of 92.3% (95% CI: 88.4–96.2%). Positive and negative likelihood ratios were 130.2 and 0.2, respectively, and the agreement between microbiological culture and this qPCR was almost perfect (κ = 0.82). These results highlight this qPCR targeting IS*6110* as a suitable complementary method to confirm bTB in animals with either tuberculosis-like lesions or non-tuberculosis-like lesions, decreasing the number of samples subjected to microbiological culture and, hence, its overall associated costs and the turnaround time (under 48 h) to confirm bTB infection. Besides, sampling mesenteric lymph node, which is uncommonly sampled, together with tracheobronchial and retropharyngeal ones, is advisable during postmortem inspection in bTB surveillance programs at the slaughterhouse, especially in areas with a low bTB prevalence scenario.

Keywords: bovine tuberculosis, fresh lymph node, IS*6110*, direct qPCR, *Mycobacterium tuberculosis* complex

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INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease caused by *Mycobacterium bovis* and other members of the *Mycobacterium tuberculosis* complex (MTC) (1, 2) that affects various species of mammals, including humans (3, 4). bTB is still one of the largely neglected zoonotic diseases, particularly in developing countries, as the control and surveillance programs for this disease are inadequate or are not carried out, and domestic and wild animals, which act as reservoirs, often share pasture areas. Thereby, it has been estimated that a quarter of the world's population has latent tuberculosis, requiring a global effort to develop new tools for the diagnostic and treatment of this disease (5). In the European Union (EU), bTB primarily affects livestock, which is of economic importance due to its impact on trade. Indeed, bTB is subjected to national eradication programs based on skin testing of all registered cattle herds, slaughtered policy, and abattoir surveillance (Council Directive 64/432/EEC). According to the EU legislation, the official diagnosis of bTB is based on the detection of the cellular immune response (single intradermal tuberculin testing) in reactor animals (skin test-positive animals), which is followed by slaughtering, histopathological examination of atypical or enlarged lymph nodes or parenchymatous organs with tuberculosis-like lesions (TBLs), and/or culture of MTC in primary isolation medium (6). Although a substantial economic expenditure is addressed to ensure efficient surveillance systems and control programs, the detection and confirmation of bTB infection in cattle herds should be more reliable and swifter (7).

Microbiological culture is considered the reference technique for bTB diagnosis with recovery rates ranging from 30 to 95% (8–10) and sensitivity (SE) and specificity (SP) values of 78.1 and 99.1%, respectively (7). It is reported that culture is an imperfect, laborious, and time-consuming technique that requires high biosecurity facilities and relatively high expertise (7, 8), whose performance can, moreover, be affected by several factors (8–11). A major drawback is the delayed culturing process (up to 2–3 months), making the time required to reach a final diagnosis longer (7, 11, 12).

In the current landscape, rapid, cost-effective, and accurate diagnostic tools could pave the way for managing and controlling bTB in cattle herds (13). Although enzyme-linked immunosorbent assay testing is useful to detect anergic tuberculous cattle as a complement to single intradermal tuberculin testing, this assay is not routinely applied in bTB control programs because of its reduced SE (14–16). By contrast, real-time PCR assays [so-called quantitative PCR (qPCR)] have been shown to directly detect MTC in fresh bovine tissue samples with moderate to high estimates of SE and SP (7, 11, 16). Direct qPCR can detect small amounts of MTC DNA independently of its viability with a turnaround time of 24–48 h, shortening the required time to reach confirmatory results (7, 15, 16).

IS*6110* is a target sequence with multiple copies only present in pathogens belonging to MTC, commonly used for MTC detection by PCR (7, 12, 17, 18). Besides IS*6110*, other targets have also been used for the same purpose, including IS*1081* (16, 19), hupB (20, 21), 16S-23S rRNA internally transcribed spacer (22), p34 gene (23), TbD1 (24), or mpb70 (11) with varying results.

In the light of the earlier mentioned, rapid and accurate diagnostic tools, such as qPCR, may be implemented as a confirmatory test in the framework of bTB surveillance and control programs at the slaughterhouse to shorten turnaround time and inform decision-makers on time. Therefore, the present study firstly aimed to evaluate the diagnostic performance of a direct qPCR from fresh tissue samples targeting IS*6110* using individually homogenized lymph nodes and, secondly, to validate the IS*6110* qPCR for the detection of MTC positive samples and animals in the framework of the bTB eradication campaign.

MATERIALS AND METHODS

Samples Selection and Processing

Fresh retropharyngeal, tracheobronchial, and mesenteric lymph node tissue samples ($n = 687$) were collected from 230 cattle carcasses at the slaughterhouse from 2018 to 2019. All samples were collected during routine postmortem veterinary examination within an official context and agreeing with national and European regulations. No purpose killing of animals was performed for this study, so no ethical or farmer's consent approval was required.

Every lymph node was independently sliced, and the presence of visible-TBL or non-tuberculosis-like lesions (NTBLs) was recorded. Individual homogenization was carried out to obtain a uniform mixture of every lymph node independently using a tissue homogenizer (Fisherbrand, Fisher Scientific, Madrid, Spain). Briefly, 4–7 g of each lymph node tissue was placed into a 15-ml Falcon™ tube (Corning, Madrid, Spain) with the same volume (w/v: 1/1) of 0.85% sterile sodium chloride and ground until a homogeneous mixture was obtained. Tissue homogenate was used for DNA isolation and selective bacterial culture.

Mycobacterium tuberculosis Complex Microbiological Culture

Selective bacterial culture was performed in the BSL3 facilities of the Production and Animal Health Laboratory of Córdoba (LPSACo, Regional Government of Andalusia). Briefly, the homogenate was decontaminated with an equal volume of 0.75% (w/v: 1/1) hexadecyl pyridinium chloride solution in agitation for 30 min (25). Samples were centrifuged for 30 min at 1,500×*g*. The pellets were collected with swabs and cultured in liquid media (MGIT™ 960, Becton Dickinson, Madrid, Spain) using an automatized BD Bacter™ MGIT™ System (Becton Dickinson). The culture was considered positive when isolates were confirmed as MTC by qPCR (26).

DNA Extraction From Homogenized Lymph Nodes

DNA extraction from homogenized tissue samples was performed using DNA Extract VK (Vacunek, Bizkaia, Spain) according to the manufacturer's guidelines with several modifications. In brief, a mix of 300 mg of homogenate, 250 µl of sterile distilled water, and 250 µl of sample lysis buffer

VK-SB were added in a 2-ml tube containing 300 mg of 0.5-mm glass beads and submitted to mechanical disruption at 30 Hz during 20 min. Then, the lysed tissue was centrifuged for 5 min at 7,000×*g*, transferring 200 µl of supernatant to a new 1.5-ml tube. Enzymatic digestion was carried out with 25 µl of 20 mg/ml proteinase K at 56°C for 3 h in a thermo-shaker at 750 rpm. After that, 200 µl of the lysis buffer VK-LB3 were added, and the mixture was incubated for 10 min at 70°C. Finally, 210-µl ethanol (96–100%) was added to the sample that was applied in a spin column following the manufacturer's guidelines. DNA elution was run using 100 µl of Tris/hydrochloride buffer supplied with the kit pre-heated at 70°C. Positive and negative extraction controls were also included. All the DNA extraction products were stored at −20°C until use.

Quantitative Real-Time PCR From Fresh Tissue Samples

The transposon IS*6110*, which is present in all species of the MTC, was the target of this qPCR. Specific primers (IS*6110*-forward: 5′-GGTAGCAGACCTCACCTATGTGT-3′; IS*6110*-reverse: 5′-AGGCGTCGGTGACAAAGG-3′) and a probe (IS*6110*- probe: 5′-FAM-CACGTAGGCGAACCC-MGBNFQ-3′) targeting a conserved region of IS*6110* transposon were used (27). The diagnostic performance of the qPCR was conducted using the QuantiFast® Pathogen PCR + IC Kit (QIAGEN, Hilden, Germany). Amplifications were run in duplicate for each sample in the MyiQ™2 Two-Color qPCR Detection System (Bio-Rad, Hercules, CA, USA) under the following cycling conditions: 95°C for 5 min followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Following the manufacturer's guidelines, an exogenous inhibition heterologous control [internal amplification control (IAC)] supplied with the kit was included. An inter-run calibrator with a known Ct value of 32 was introduced in each assay to self-control intra-assay repeatably and accuracy. Complete inhibition of amplification was considered when IAC did not amplify and partial inhibition when it showed a cycle threshold (Ct) > 33. When any inhibition was detected, samples were diluted up to a final concentration of 450 ng/µl, and qPCR was run again. Serial 10-fold dilution series of *M. bovis* genomic DNA with known quantities, ranging from 10^6 to 10^0, were used as standards to estimate the limit of detection (LOD) or analytical SE. The reactions were carried out in triplicate per dilution in three different assays, and LOD was determined as the lowest concentration in which 95% of replicates were positive according to the Clinical and Laboratory Standards Institute guidelines.

In the case of culture-positive and qPCR-negative samples, DNA extraction and qPCR were repeated to verify the results. Then, proteinase K digestion was increased up to 12 h (overnight incubation) at 56°C in a thermo-shaker at 750 rpm. Positive (MTC confirmed sample) and negative (MTC negative sample) controls were included, as well as an inter-run calibrator. The IS*6110* PCR product of culture-negative and PCR-positive samples were EtOH precipitated, purified using ExoSAP-ITTM (Thermo Fisher Scientific, Barcelona, Spain), and further analyzed by Sanger sequencing (performed at STABvida, Lisbon, Portugal). The obtained sequences were studied using the Bioedit software version 7.1.3.0. Samples confirmed by sequencing were considered as true positives and used to recalculate the diagnostic parameters of the qPCR targeting IS*6110*.

Validation of Diagnostic Tests

The results of qPCR targeting IS*6110* were compared with microbiological culture ones (*gold standard*) to estimate the diagnostic SE and SP, positive and negative predictive values (PPV and NPV, respectively), and positive and negative likelihood ratios (PLR and NLR, respectively) (WinEpi software 2.0, Faculty of Veterinary Medicine, University of Zaragoza, Spain). Moreover, an agreement between culture and qPCR results was assessed using Cohen's kappa coefficient (κ) (values ≤ 0 indicated no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement) (WinEpi software 2.0).

RESULTS

Topographical Distribution of Tuberculosis-Like Lesion

A total of 687 retropharyngeal, tracheobronchial, and mesenteric lymph node samples belonging to 230 cattle carcasses were analyzed to serve as evidence of the presence of MTC using microbiological culture and qPCR directly from lymph nodes. Due to the logistic of the slaughterhouse and the timing of slaughtering, it was not always possible to collect the three lymph node samples from all 230 carcasses, lacking one retropharyngeal and two mesenteric lymph nodes. Before being analyzed, every single tissue sample was subjected to a visual inspection to disclose gross lesions, with 26 of 26/687 (3.8%) tissue samples belonging to 23 different cattle (23/230, 10.0%) showing TBL (**Table 1**). Most of the lesions were evidenced in only one lymph node (tracheobronchial = 11; retropharyngeal = 9), whereas in three animals, TBLs were observed in two lymph nodes (tracheobronchial–retropharyngeal = 1; tracheobronchial–mesenteric = 1; retropharyngeal–mesenteric = 1).

Mycobacterium tuberculosis Complex Microbiological Culture Results

Seventy-three of 687 tissue samples (10.6%) were positive to microbiological culture, whereas 614 were negative (89.4%). Bacteria were detected in tracheobronchial lymph nodes (42 of 73; 57.5%), followed by retropharyngeal (22 of 73; 30.1%), and mesenteric ones (9 of 73; 12.3%) (**Table 1**). An animal was considered culture-positive when MTC was detected by culture in at least one lymph node. Thus, 61 of 230 animals (26.5%) were positive to culture, whereas 169 were negative (73.5%). MTC was detected in most of the animals (50, 82.0%) in one lymph node (tracheobronchial = 31; retropharyngeal = 16; mesenteric = 3), whereas in 10 animals (16.4%), MTC was detected in two lymph nodes (retropharyngeal–tracheobronchial = 5; tracheobronchial–mesenteric = 5) and in one animal in the three lymph nodes.

TABLE 1 | Evaluation of the microbiological culture and direct qPCR targeting IS*6110* results obtained upon analyzing 687 lymph nodes, according to the presence or absence of tuberculosis-like lesions.

		TBL				NTBL			
		RF	**TB**	**MS**	**Total**	**RF**	**TB**	**MS**	**Total**
Culture	+	5	13	2	20	17	29	7	53
	–	6	0	0	6	201	188	219	608
qPCR	+	8	13	2	23	9 (10)	22 (24)	3	37
	–	3	0	0	3	209 (208)	195 (193)	223	624
Total		11	13	2	26	218	217	226	661

+, Positive; –, Negative; qPCR, real-time PCR; TBL, tuberculosis-like lesion; NTBL, non-tuberculosis-like lesion; RF, retropharyngeal lymph nodes (n = 229); TB, tracheobronchial lymph node (n = 230) s; MS, mesenteric lymph nodes (n = 228).
Culture-positive and qPCR-negative samples that were finally positive and/or negative to qPCR after DNA extraction was repeated (in brackets).

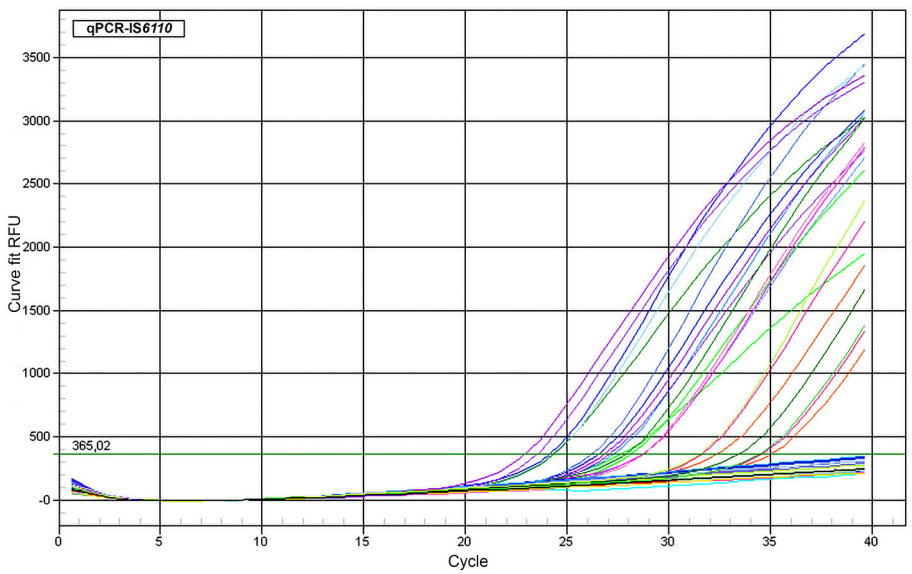

FIGURE 1 | Amplification plot of representative samples. qPCR targeting IS*6110* assay using representative fresh lymph node tissue samples belonging to different cattle. ΔRFU (Y axis) of the reaction was plotted against the Ct value (X axis). Samples with lowest DNA concentration could be detected ranging from 34 to 36 cycles roughly.

Quantitative Real-Time PCR Targeting IS*6110*

Fifty-seven of 687 tissue samples (8.3%) were detected as positive by means of qPCR targeting IS*6110*, with Ct values ranging from 24.2 to 37.5 (**Figure 1**). The IAC amplified in most of the samples without partial inhibition, showing complete inhibition in eight of the 687 samples due to the high yield of DNA (over 1,000 ng/μl). These samples were diluted up to a final concentration of 450 ng/μl and re-evaluated by qPCR, keeping a negative result for all of them for MTC but with IAC amplification. The LOD for this qPCR-IS*6110* was determined to be ranging from 10 to 100 genomic equivalents, and the cutoff was established to Ct < 38. Most of the qPCR-positive results were obtained from tracheobronchial lymph node (35 of 57; 61.4%), followed by retropharyngeal (17 of 57; 29.8%), and mesenteric (five of 57; 8.8%) lymph nodes, reflecting the same trend as observed in the microbiological culture (**Table 1**).

Following the same criterion used for the microbiological culture, an animal was considered PCR-positive when at least one of the examined lymph nodes yielded a positive result to the qPCR. Thus, 44 of 230 cattle (19.1%) were qPCR-positive, whereas 185 were negative (80.4%). Briefly, 36 of the 44 qPCR positive animals (81.8%) were detected in only one lymph node (tracheobronchial = 24; retropharyngeal = 11; mesenteric = 1), seven animals (15.9%) in two lymph nodes (retropharyngeal–tracheobronchial = 5; tracheobronchial—mesenteric = 2), and one animal (2.3%) in the three lymph nodes.

Diagnostic Performance of Quantitative Real-Time PCR Compared With Microbiological Culture

Fifty-three of the 73 lymph node samples positive to culture were also positive to qPCR targeting IS*6110*. Because extraction is a rate-limiting factor determining the success of downstream

TABLE 2 | Diagnostic performance of direct qPCR targeting IS*6110* compared with microbiological culture as gold standard analyzing 687 lymph nodes belonging to 230 cattle.

			True positives			Measures of diagnostic accuracy (95% CI)			
		Result	+	–	Total	Sensitivity	Specificity	Reliability	*k* value
Lymph nodes	qPCR	+	56	4	60	76.7% (67–86.4%)	99.3% (98.7–100%)	96.9% (95.7–98.2%)	0.83
		–	17	610	627				
		Total	73	614	687				
Animals	qPCR	+	47	1	48	77.1% (66.5-87.6%)	99.4% (98.3%-100.6%)	93.5% (90.3–96.7%)	0.82
		–	14	168	182				
		Total	61	169	230				

+, Positive; –, Negative; qPCR, real-time PCR; 95% CI, 95% confidence level.

TABLE 3 | Validation of direct qPCR targeting IS*6110* for the detection of MTC analyzing 687 lymph nodes belonging to 230 cattle.

MTC corrected positive results Culture/Sanger sequencing						Measures of diagnostic accuracy (95% CI)			
		Result	+	–	Total	Sensitivity	Specificity	Reliability	*k* value
Lymph nodes	qPCR	+	60	0	60	77.9% (68.7–87.2%)	100% (100%)	97.5% (96.4–98.7%)	0.86
		–	17	610	627				
		Total	77	610	687				
Animals	qPCR	+	48	0	48	77.4% (67.0–87.8%)	100% (100%)	94% (90.8–97.0%)	0.83
		–	14	168	182				
		Total	62	168	230				

+, Positive; –, Negative; qPCR, real-time PCR; 95% CI, 95% confidence level.
MTC-corrected positive results: (i) samples positive to culture and (ii) culture-negative samples and PCR-positive after Sanger sequencing.

bTB detection by PCR, this step was repeated in those culture-positive and qPCR-negative samples (20/73 samples, 27.4%) to verify the results. Thereby, previous extraction conditions were changed by a proteinase K digestion up to 12 h at 56°C in a thermo-shaker at 750 rpm (overnight incubation), obtaining this time three positive samples of the 20 and remaining the rest ($n = 17$) negative to qPCR. Hence, an apparent SE of 76.7% (95% CI: 67–86.4%) was found. On the other hand, only four of the 614 samples negative to culture were positive to qPCR, with the remaining samples also giving a negative result to qPCR, with an apparent SP value of 99.3% (95% CI: 98.7–100%). The PPV and NPV values were 93.3% (95% CI: 87.0–99.6%) and 97.3% (95% CI: 96.0–98.6%), respectively. In addition, the PLR and NLR were 117.8 and 0.23, respectively. Finally, the concordance or level of agreement between both diagnostic assays for tissue samples was substantial ($\kappa = 0.83$) (**Tables 1**, **2**).

Considering this re-run of the extraction step, 47 of 61 MTC culture-positive animals were also positive for qPCR targeting IS*6110*, resulting in an apparent SE of 77.0% (95% CI: 66.5–87.6%). Only one of the 169 MTC culture-negative animals was positive to qPCR, finding an apparent SP of 99.4% (95% CI: 98.3–100.6%). The measures of PPV and NPV were 97.9% (95% CI: 93.9–102.0%) and 92.3% (95% CI: 88.4–96.2%), respectively. The PLR and NLR were 130.2 and 0.23, respectively. The agreement between microbiological culture and qPCR at the animal level was almost perfect ($\kappa = 0.82$) (**Table 2**).

Validation of IS*6110* Quantitative Real-Time PCR for the Detection of *Mycobacterium tuberculosis* Complex

Because microbiological culture is considered an imperfect test for bTB diagnosis in which SE may be affected by several factors (7, 8, 16), the combination of culture and IS*6110* qPCR was validated to detect MTC positive samples or animals. In this sense, culture-negative and PCR-positive samples obtained in our study could be considered as MTC positives. This way, the four IS*6110* qPCR-positive and culture-negative lymph node samples were further subjected to Sanger sequencing, and the presence of MTC DNA was evidenced in all of them. Consequently, the diagnostic estimates of the direct qPCR for MTC detection were evaluated, considering as MTC-corrected positive samples, culture-positive samples, and those in which MTC was revealed by Sanger sequencing. For tissue samples, 60 of the 77 MTC-corrected positive samples were successfully amplified by means of qPCR targeting IS*6110* with a corrected SE of 77.9% (95% CI: 68.7–87.2%), SP of 100% (95% CI: 100–100%), and reliability of 97.5% (95% CI: 96.4–98.7%). The PPV and NPV values were increased to 100% (95% CI: 100–100%) and 97.3% (95% CI: 96–98.6%), respectively. The PLR and NLR were 160 and 0.22, respectively, with a level of agreement between assays almost perfect ($\kappa = 0.86$) (**Table 3**).

At the animal level, 48 of the 62 MTC-corrected positive animals were also positive for qPCR targeting IS*6110* with a corrected SE and SP of 77.4% (95% CI: 67–87.8%) and 100%

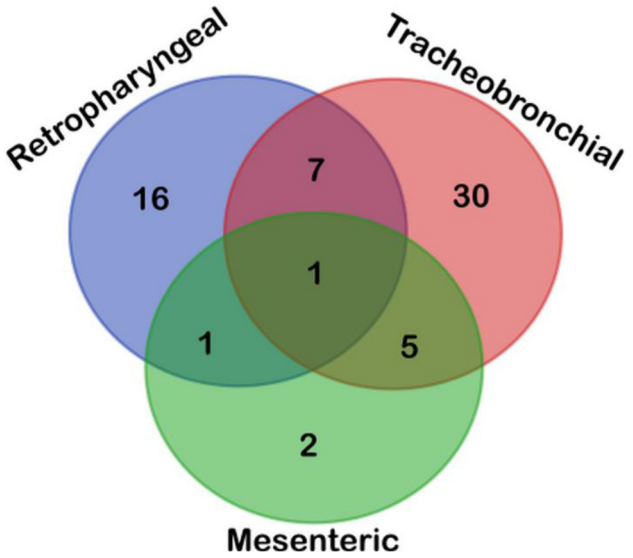

FIGURE 2 | Venn diagram depicted the topographical distribution of positive lymph nodes ($n = 77$) according to their results obtained upon analyzing samples by microbiological culture, qPCR-IS*6110*, and Sanger sequencing. Most positive cattle were disclosed in tracheobronchial lymph node, followed by retropharyngeal and mesenteric lymph nodes, with most positive animals presenting only one positive lymph node highlighting the choice of tissue is a cornerstone for performing an accurate direct diagnosis of MTC.

TABLE 4 | Assessment of the microbiological culture and direct qPCR targeting IS*6110* results obtained upon analyzing 687 lymph nodes belonging to 230 cattle, according to the presence or absence of tuberculosis-like lesions.

		Microbiological culture			
		+	−	Total	
NTBL	qPCR +	36	1	37	661
	qPCR −	17	607	624	
TBL	qPCR +	20	3	23	26
	qPCR −	0	3	3	

+, Positive; –, Negative; qPCR, real-time PCR; 95% CI, 95% confidence level; NTBL, non-tuberculosis-like lesion; TBL, tuberculosis-like lesion.

(95% CI: 100–100%), respectively, and reliability of 93.9% (90.8–97.0%). The measures of PPV and NPV were 100% (95% CI: 100–100%) and 92.3% (95% CI: 88.4–96.2%). The PLR and NLR were 100.2 and 0.2, respectively, and the κ value was 0.83 (almost perfect agreement of both assays) (**Table 3**).

Finally, according to the distribution of MTC-corrected positive results for the lymph nodes (77), the majority of MTC-corrected positive animals (48) were detected in only one lymph node (tracheobronchial = 30; retropharyngeal = 16; mesenteric = 2), 13 animals in two lymph nodes (retropharyngeal–tracheobronchial = 7; tracheobronchial–mesenteric = 5; retropharyngeal–mesenteric = 1), and one animal in the three lymph nodes, as showed in **Figure 2** (**Table 4**).

Bacteriology and Quantitative Real-Time PCR Results Distribution According to Tuberculosis-Like Lesion

Analyzing MTC and qPCR results together with the presence of TBL, 26 of 687 tissue samples (3.8%) showed TBL, and 20 of these 26 samples (76.9%) resulted in both culture and qPCR positive. The remaining six were negative to culture, being three of them also negative to qPCR. In contrast, the other three were positive to qPCR and subsequently confirmed by Sanger sequencing.

Thirty-six of 661 NTBL tissue samples (5.4%) were positive to either microbiological or qPCR assays, 17 were only culture-positive (2.6%), and 1 only qPCR positive (0.2%). Of note, this culture-negative and qPCR-positive sample was confirmed as positive after Sanger sequencing. Thus, 607 NTBL tissue samples (91.8%) were negative for both techniques (**Table 3**).

DISCUSSION

bTB is one of the oldest and most relevant zoonoses worldwide, where its eradication is the main objective of the EU. As a consequence, rapid, cost-effective, and sensitive tools for the diagnosis of different pathogens belonging to MTC play a pivotal role in controlling and preventing its transmission in countries where it is still especially present in dairy and meat cattle herds (13, 15, 28). Therefore, direct qPCR from tissue samples could work as an accurate and rapid diagnostic alternative in animal health (7, 11, 13, 23), which could be implemented by public health agencies not only to reduce the turnaround time on reaching a confirmatory diagnosis compared with microbiological culture but also to shorten the time of exposure to MTC, facilitating the decision-making process. In this context, the main objective of the present study was to evaluate a qPCR targeting IS*6110* to detect MTC directly from fresh tissue bovine lymph node samples.

In the present study, the direct qPCR targeting IS*6110* showed an apparent SE and SP for individual tissue samples of 76.7 and 99.3%, respectively, when compared with microbiological culture. In addition, the agreement between both assays was almost perfect (κ = 0.83). Several factors make it challenging to run a direct detection of MTC, such as the paucibacillary nature of this complex, the extremely hardy disruption of mycobacterial cells, or the extensive necrosis, fibrosis, and mineralization associated with TBL, interfering all of them with mycobacterial DNA isolation and leading to false-negative results, which limits the final diagnosis performance (19, 29, 30). In our case, samples were individually homogenized before the process to reach a uniform distribution of MTC in the whole matrix but also trying to restrict a dilution effect beyond the detection limit of the qPCR. Despite that, 20 samples were positive to microbiological culture but negative for qPCR. It is well-known that the yield and quality of DNA after extraction could depend on multiple factors (11, 15); consequently, DNA isolation was repeated in all qPCR-negative samples increasing proteinase K digestion up to 12 h at 56°C (overnight incubation), obtaining three additional qPCR-positive samples, and slightly improving SE from 71.2 to 76.7%. A similar approach was conducted to improve diagnostic

SE and SP of direct qPCR targeting mpb70 from 88.4 and 92.3 to 94.5 and 96.0%, respectively (11). These results suggest that DNA extraction protocol is certainly relevant, impacting directly on diagnostic SE of direct qPCR from fresh tissue samples.

Several alternative methods have been previously performed to improve diagnostic SE of direct qPCR from tissues. Thus, nested-PCR targeting TbD1 (24) or IS*6110* (17) have been suggested as a method to improve the detection of MTC in bovine tissue samples with a diagnostic SE and SP ranging from 76.0 to 98.2% and from 88.7 to 100%, respectively. Nevertheless, a nested-PCR requires two different amplification steps, increasing the concern about cross-contamination, which could negatively affect diagnostic SP values. On the other hand, Parra et al. (22) used a manual extraction method with capture probes targeting 16S-23S internally transcribed spacer region to isolate a higher yield of mycobacterial DNA from tissue homogenate samples obtaining a diagnostic SE ranging from 61.1% for samples with NTBL to 80.6% for TBL samples, with an average SE of 73.8%. In this sense, Taylor et al. (19) reported an increase of diagnostic SE from 70.1 to 91.2% targeting *IS*1081 and carrying out DNA isolation only from TBL, ruling out positive samples without readily macroscopic lesions. In our case, 23 of 26 lymph nodes with TBL (88.5%) were amplified targeting IS*6110*; nevertheless, when all samples were considered, both TBL and NTBL samples, an SE of 76.7% was obtained, highlighting the potential of using this target for qPCR screening not only in TBL but also in NTBL.

Previous studies targeting mpb70 (11) or IS*6110* (7) have reported higher SE and SP results than those herein reported; however, it is noteworthy to mention that in those studies, there was a high proportion of the evaluated samples with TBL (39.8 and 100%, respectively). This feature evidences that animals included in those studies were in more advanced stages of bTB infection (15, 31). Unlikely, in the present study, most of the tissue samples lacked TBL (661/687), with only 3.6% of them presenting TBL, which points to animals were sampled in earlier stages of the infection. In addition, qPCR targeting IS*6110* showed a moderate diagnostic SE and high SP. These results highlight the diagnostic potential of direct qPCR from fresh tissue to detect MTC at early stages of infection and, therefore, when the mycobacterial load is lower.

Regarding the topographical distribution of the lesions, most of the TBL samples were disclosed in the tracheobronchial lymph node, followed by retropharyngeal and mesenteric lymph nodes, with most positive animals presenting only one affected lymph node (77.4%). One of the strengths of the present study is that a detailed evaluation of the topographical distribution of the results was made, as the choice of tissue samples at the abattoir is a key player for carrying out an accurate direct diagnosis of MTC. In addition, the diagnosis from a pool of lymph nodes from reactor animals with TBL or NTBL is probably to have a dilution impact on the results. According to our results, most of the true positive animals reacted in one single lymph node, highlighting that not only tracheobronchial and retropharyngeal lymph nodes but also mesenteric lymph node, which is uncommonly sampled during postmortem inspection in bTB surveillance systems at the slaughterhouse, should be evaluated and collected for TB diagnosis. These results turn out to be relevant in areas with a low TB prevalence scenario to enhance the diagnostic accuracy of direct detection methods.

Although microbiological culture is considered the *gold standard* for bTB confirmation, this technique is time-consuming and imperfect, inducing false-negative results (8), and SE and SP will always be biased (7, 32); therefore, combination with other techniques is required to truly identify MTC positive samples. Thereby, three of six samples with TBL and culture-negative were detected as positive for direct qPCR and confirmed by Sanger sequencing, displaying a SE and SP of 77.9 and 100%, respectively, for MTC detection. It is worthy of note that the other three culture-negative and qPCR-negative TBL samples presented pyogranulomatous lesions and Ziehl–Neelsen negative results when examined under the light microscope (data not shown). These results suggest that other microorganisms may be involved in the production of these lesions, as has already been demonstrated in pigs (33), which could be taken into account for future studies.

Several factors impact the success of microbiological cultures, such as decontamination process (8) or encapsulation of the granulomas (34); however, DNA amplification of MTC can be successfully performed from fibrotic and encapsulated granulomas. Our results highlight that direct qPCR can detect more positive samples from fresh lymph nodes tissue with TBL than microbiological culture (23 vs. 20), resulting in a faster and effective confirmatory method for MTC during official postmortem inspection at the slaughterhouse. Nonetheless, microbiological culture remains a required method to mycobacterial isolation and molecular epidemiology studies so far (7).

Finally, direct qPCR targeting IS*6110* was also used to run the diagnostic performance of all animals included in this study, showing diagnostic SE and SP values of 77.0 and 99.4%, respectively, which are very close to those previously reported for microbiological culture (7). In addition, predictive values of 97.9% PPV and 92.3% NPV, together with PLR and NLR of 130.2 and 0.23, respectively, point to a qPCR-positive animal could be considered as true positive. Previous reports on animals have indicated either a barely higher SE for qPCR targeting IS*6110* (7) or lower SE for *IS*1081 (16) compared with the diagnostic SE herein reported. In addition, if diagnostic estimates for MTC detection were considered, SE and SP values could be increased up to 77.4 and 100%, respectively. The disparities among studies may be attributed to differences in the approach for data analysis, epidemiologic situation, or the sample size. On the other hand, gross postmortem examination is a critical stage for the detection of bTB-infected animals at slaughterhouses. Nevertheless, the number of reactors with TBL is currently reduced at the slaughterhouse due to the success of surveillance and control programs decreasing bTB prevalence in cattle herds. Therefore, in the present framework, qPCR assay targeting IS*6110* might work as a suitable complementary method to confirm bTB in reactor animals with either TBL or NTBL, decreasing the number of samples subjected to microbiological culture and, hence, the overall associated cost as well as the turnaround time, $<$48 h, for confirming bTB infection.

CONCLUSION

The present study revealed that qPCR targeting IS*6110* is an efficient confirmatory test that may be implemented in bTB surveillance and control programs, shortening turnaround time to keep decision-makers noticed promptly, as well as reducing economic costs.

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.

AUTHOR CONTRIBUTIONS

JS-C and ÁG-R performed all experiments in this study and Animal Health and Production Laboratory of Córdoba supported with microbiological culture and technical assistance. ÁG-R, JS-C, IR-T, EV, FJ-M, FL-M, FC-T, and LG-G were involved in obtaining and processing tissue samples. LC, IL, JG-L, and CT designed the study. JS-C and ÁG-R wrote the manuscript down with the invaluable insights of JG-L, IL, IR-T, IR-G, FJ-M, LG-G, FC-T, LC, CT, and AM. IL and JG-L directed and supervised the whole study. All authors contributed to the article and approved the submitted version.

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Heterogeneity of Pulmonary Granulomas in Cattle Experimentally Infected With *Mycobacterium bovis*

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Mycobacterium bovis is the cause of tuberculosis in most animals, most notably cattle. The stereotypical lesion of bovine tuberculosis is the granuloma; a distinct morphological lesion where host and pathogen interact and disease outcome (i.e., dissemination, confinement, or resolution) is determined. Accordingly, it is critical to understand host-pathogen interactions at the granuloma level. Host-pathogen interactions within individual granulomas at different stages of disease have not been examined in cattle. We examined bacterial burden and cytokine expression in individual pulmonary granulomas from steers at 30, 90, 180, and 270 days after experimental aerosol infection with *M. bovis*. Bacterial burdens within individual granulomas examined 30 days after infection were greater and more heterogenous (variable) than those examined 90 to 270 days after infection. Bacterial burdens did not correlate with expression of IFN-γ, TNF-α, TGF-β, granuloma stage, or lung lesion score, although there was a modest positive correlation with IL-10 expression. Granuloma stage did have modest positive and negative correlations with TNF-α and IL-10, respectively. Heterogeneity and mean expression of IFN-γ, IL-10 and TNF-α did not differ significantly over time, however, expression of TGF-β at 90 days was significantly greater than that seen at 30 days after infection.

Keywords: cytokine, granuloma, *in situ* hybridization, *Mycobacterium bovis*, pathology, tuberculosis

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INTRODUCTION

Bacteria of the genus *Mycobacterium* are Gram-positive, acid-fast bacilli. Among the many mycobacterial species, several are important human and animal pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium bovis* (1). Tuberculosis in humans is primarily caused by *M. tuberculosis*; however, the zoonotic pathogen, *M. bovis*, which most commonly affects cattle can also produce tuberculosis in humans (2). The *M. tuberculosis* (*M. tb.)* complex includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *M. canetii*, *M. pinnipedii*, *M. orygis*, *M. suricattae*, *M. mungi*, the dassie bacillus and the chimpanzee bacillus (3–9). Of all these species, *M. bovis* has the broadest host range, which includes most mammalian species, most notably cattle [bovine tuberculosis, (bTB)].

The hallmark lesion of tuberculosis, regardless of host or tissue, is the granuloma. A granuloma resulting from infection with a member of the *M. tb.* complex may also be referred to as a tuberculoid granuloma (10).

The tuberculoid granuloma is a morphologically distinctive microscopic lesion dominated by a modified epithelial-like (epithelioid) macrophage, with lesser numbers of lymphocytes and multinucleated giant cells. In humans and animals, granulomas represent the host-pathogen interface where disease outcome (i.e., dissemination, confinement, or resolution) is determined; therefore, it is critical to understand host-pathogen interactions at the granuloma level (11–13). The granuloma paradox, however, is that although it is crucial for controlling and containing infection, it also contributes to early proliferation of bacilli (14–17).

The pathogenesis of bovine tuberculosis recapitulates many aspects of human tuberculosis; indeed, cattle have been used a model of human tuberculosis (18, 19). In humans and cattle, tiny residues, $<5\,\mu m$, of evaporated droplets (droplet nuclei) can be generated by an infected host while coughing, or in the case of humans, talking or singing (20–22). Such nuclei remain airborne for hours, and once inhaled can be deposited in the terminal bronchioles and alveoli. Resident alveolar macrophages phagocytose the bacilli, which in turn induces the production of cytokines, chemokines and enzymes. Expression of both pro- (e.g., IFN-γ, TNF-α,) and anti-inflammatory (e.g., IL-10, TGF-β) cytokines activate innate immune cells such as neutrophils, monocytes, macrophages and dendritic cells (14, 23). A coordinated exchange of cells between the lung and draining lymph nodes ensues as dendritic cells containing bacilli migrate from the lung to the lymph nodes. There, naïve T cells are activated through antigen presentation. Activated T cells home to the site of infection and cytokine and chemokine signals attract and activate monocytes and macrophages. As cells accumulate, the granuloma forms. The typical tuberculoid granuloma has a central region of caseous necrosis, surrounded by a zone of epithelioid macrophages and multinucleated giant cells, with the outermost zone containing increasing numbers of lymphocytes and occasional plasma cells. As infection persists, a partial to complete fibrous capsule may be produced. In addition to typical granuloma morphology, granulomas may be non-necrotizing or highly mineralized.

In cattle the heterogenous nature of bovine tuberculoid granulomas has been described in terms of morphology, as well as cellular composition and cytokine expression (24–27). Comparisons have also been made between tuberculoid granulomas arising in different organs from the same animal (25, 27). What has been less studied are granuloma changes over the course of disease, from early to late infection, including the bacterial burden within individual granulomas. Research in non-human primate (NHP) models of human tuberculosis reveals that individual granulomas are microenvironments in which local immune responses drive the bacterial burden, disease progression, and overall disease status (12, 28) indicative of disease control at the granuloma level.

The objective of the current study was to describe individual granuloma changes, in terms of morphologic stages, bacterial burdens and cytokine composition at increasing intervals after experimental aerosol infection. We hypothesized that granuloma heterogeneity may be reflected not only in morphology, but also in bacterial burden, and the concomitant cytokine environment.

MATERIALS AND METHODS

Mycobacterium bovis Aerosol Challenge

Mycobacterium bovis strain 10-7428 was used in this experiment. This field strain was of low passage (<3) and had been shown previously to be virulent in the calf aerosol model (29). Inoculum was prepared using standard techniques (30) in Middlebrook's 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% oleic acid-albumin-dextrose complex (OADC; Difco, Detroit, MI, USA) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA). Mid log-phase growth bacilli were pelleted by centrifugation at 750 X g, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2) and stored at-80^0 C until used. Frozen stock was warmed to room temperature and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson). A single dose was determined to be $1.12 \text{ X } 10^4$ CFU per steer.

Aerosol infection of steers with virulent *M. bovis* has been described in detail previously (24, 29, 31). Briefly, 20 Holstein steers (9 months of age) were obtained from a source with no history of *M. bovis* infection. Steers were infected with a single dose of virulent *M. bovis* strain 10-7428 by nebulization of inoculum into a mask (Equine AeroMask®, Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. All experimental animal procedures were conducted in accordance with recommendations in the Care and Use of Laboratory Animals of the National Institutes of Health and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (32, 33). Animal-related procedures were also approved by the USDA-National Animal Disease Center Animal Care and Use Committee.

Sample Collection

Five steers each were euthanized at 30, 90, 180, and 270 days after infection. Steers were humanely euthanized by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microscopic analysis as described previously (29). Each lung lobe was examined separately and sectioned at 0.5 – 1.0 cm intervals. Lesions suspected to be granulomas were dissected out and processed as individual samples. In cases where numerous granulomas were present, a maximum of 5 granulomas each from five lung lobes were collected for a total of 25 granulomas per steer.

Individual granulomas were divided with one half being processed for mycobacterial isolation, as described below, and the other half processed for microscopic evaluation by formalin fixation. Individual granulomas and other tissue samples ($\leq$0.5 cm in width) were fixed by immersion in 10% neutral buffered formalin ($\geq$ 20 volumes fixative to 1 volume tissue) for approx. 24 h and transferred to 70% ethanol, followed by standard paraffin embedding techniques. Paraffin embedded

samples were cut in 4 μm thick sections, transferred to Superfrost Plus™ charged microscope slides (Thermo Fisher Scientific, Pittsburg, PA) and stained with hematoxylin and eosin (H&E). For all individual granulomas, adjacent sections were stained by the Ziehl-Neelsen technique for visualization of acid-fast bacilli (AFB) and numerous near adjacent unstained sections were used for *in situ* hybridization (ISH).

Lesion Scoring

Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semiquantitative gross pathology scoring system described previously (34). Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were assessed individually based on the following scoring system: 0, no visible lesions; 1, no external gross lesions, but lesions seen on slicing; 2, <5 gross lesions of <10 mm in diameter; 3, >5 gross lesions of <10 mm in diameter; 4, >1 distinct gross lesion of >10 mm in diameter; 5, gross coalescing lesions. Cumulative mean scores were then calculated for each entire lung.

Granuloma Bacterial Burden

Quantitative assessment of mycobacterial burden of individual granulomas was evaluated as described elsewhere (35). Only granulomas from the right and left caudal lobes, for a total of 10 granulomas per steer, were processed. Briefly, granulomas were homogenized in phenol red nutrient broth using a blender. Logarithmic dilutions (10^0-10^9) of homogenates in PBS were plated in 100 ml aliquots on Middlebrook 7H11 selective agar plates (Becton Dickinson) and incubated for 8 weeks at 37 °C. Isolates were confirmed to be *M. bovis* by IS6110 real time PCR as described elsewhere (36).

Microscopic Examination

All individual granulomas were staged according to criteria described previously (24, 25, 37, 38). Stage I (initial) granulomas were characterized by accumulations of epithelioid macrophages admixed with low numbers of lymphocytes and neutrophils. Multinucleated giant cells were sometimes present, but necrosis was absent. When present, AFB were seen within macrophages or multinucleated giant cells. Stage II (solid) granulomas were characterized by accumulations of epithelioid macrophages surrounded by a thin, incomplete fibrous capsule. Infiltrates of neutrophils, lymphocytes and multinucleated giant cells were sometimes present. Necrosis was minimal to mild and centrally located. When present, AFB were seen within macrophages or multinucleated giant cells. Stage III (necrotic) granulomas were characterized by necrotic cores, some with small foci of dystrophic mineralization, surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. As distance from the necrotic core increased, the relative number of lymphocytes also increased and the number of epithelioid macrophages and multinucleated giant cells decreased. The entire granuloma was surrounded by a thin to moderate fibrous capsule. When present, AFB were seen within the necrotic core and, to a lesser extent, within macrophages and multinucleated giant cells. Stage IV (necrotic and mineralized) granulomas were characterized by a variably thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores, often with foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surrounded necrotic areas; these cellular infiltrates were bordered by a zone of large numbers of lymphocytes. When present, AFB were seen most often within the necrotic core.

mRNA Chromogenic ISH

Only granulomas from the right and left caudal lobes, for a total of 10 granulomas per steer, were processed for ISH. ISH was performed on individual granulomas from 30, 90- and 270-days post-infection. RNAscope® ZZ probe technology (Advanced Cell Diagnostics, Newark, CA) was used to perform mRNA ISH in formalin-fixed paraffin-embedded (FFPE) tissue sections using the RNAscope® 2.5 HD Reagents – RED kit (Advanced Cell Diagnostics) on samples from 30, 90 and 270 days after infection. Proprietary ZZ probes complementary to mRNA sequences of interest were used for visualization of mRNA transcripts for the following cytokines: IFN-γ (Cat. No. 315581), TNF-α, (Cat. No. 316151), TGF-β (Cat. No. 427271) and IL-10 (Cat. No. 420941). A positive control probe targeted the *Bos taurus*-specific *cyclophilin B* (*PPIB*) housekeeping gene (Cat. No. 319451), while a probe targeting *dapB* of *Bacillus subtilis* (Cat. No. 310043) was used as a negative control. The RNAscope® labeling technique has been shown to be capable of single mRNA molecule detection (39).

Formalin-fixed paraffin embedded tissue pretreatment was performed with manual antigen retrieval according to the manufacturer's instructions. Slides were baked in a dry oven for 1 hr at 60°C to promote tissue-to-slide adherence, deparaffinized and rehydrated in fresh xylenes and 100% ethanol, and air dried. RNAscope® hydrogen peroxide (Advanced Cell Diagnostics) was next applied to each tissue section for 10 min at room temperature (RT, 20^0 C) to block endogenous peroxidase activity, followed by rinsing with fresh distilled water (dH_2O). Disruption of formalin cross-linking and unmasking of antigenic epitopes was achieved by submerging slides in a boiling 1X RNAscope® target retrieval solution (Advanced Cell Diagnostics) for 15 min, followed by rinsing with fresh dH_2O and 100% ethanol. Once slides had completely air dried, a hydrophobic barrier was drawn around each tissue using an ImmEdge® pen (Vector Laboratories, Burlingame, CA), and slides were stored at RT overnight with desiccants. The following day, RNAscope® Protease Plus was applied to each tissue section and incubated in a humidifying tray at 40°C in a HybEZ™ Hybridization System oven (Advanced Cell Diagnostics) for 30 min. Slides were then rinsed with fresh dH_2O before proceeding to probe hybridization.

Probe hybridization, amplification, and detection were performed according to manufacturer's instructions. All incubations were carried out in a humidifying tray either at RT or in a HybEZ™ oven at 40°C. Between each incubation step, slides were washed with fresh 1X Wash Buffer (Advanced Cell Diagnostics). To allow binding of the ZZ probe to target mRNA, customized probe prewarmed to 40°C was applied to each tissue section and incubated at 40°C for 2 hrs. Branched amplification and detection of the probe with Fast Red chromogen (Advanced

Cell Diagnostics) was achieved by incubating slides with kit reagents (Advanced Cell Diagnostics) as follows: AMP 1 (30 min), AMP 2 (15 min), AMP 3 (30 min), and AMP 4 (15 min) at 40°C; AMP 5 (30 min) and AMP 6 (15 min) at RT; and a 60:1 solution of RED-A: RED-B (Advanced Cell Diagnostics) at RT for 10 min.

Following RED detection, slides were rinsed with fresh dH_2O before being transferred to a 1:1 Gill's hematoxylin I:dH_2O (American MasterTech, Lodi, CA) counterstain. Slides were submerged in hematoxylin solution for 2 min, rinsed with fresh dH_2O thrice, submerged in 0.02% ammonia water for bluing, and dry baked at 40°C for 20 min or until completely dry. Tissue dehydration was not completed due to the alcohol-sensitive nature of the Fast Red chromogen. To mount the tissue samples, slides were dipped in fresh xylenes, 1–2 drops of aqueous EcoMount mounting medium (Biocare Medical, Pacheco, CA) was applied to each tissue section, and a #1 thickness cover slip was applied over top of the tissue section. Slides were dried at RT in the dark overnight before microscopic examination.

Morphometry

Slides were scanned at 40X magnification and digitized using the Aperio ScanScope XT workstation (Aperio Technology, Inc., Vista, CA, USA). Digitized images of individual granulomas were analyzed using image analysis software (HALO™, Indica Labs, Inc., Corrales, NM). Using the RNA ISH HALO™ module, the chromogenic reaction of Fast Red was identified and the signal (i.e., red dots) quantified. The RNA ISH module algorithm identifies both cells and signal allowing the quantification of signal number per area occupied by cells within the granuloma (i.e., signal/total cell area). The entire section of each granuloma was analyzed.

Statistical Analysis

Mean values for lesion scores, tissue weights and cytokine expression at each time point were evaluated using one-way analysis of variance, followed by Tukey's *post-hoc* multiple comparison test (GraphPad Prism 8.0, GraphPad Software, San Diego, CA, USA) to compare differences between means. Correlations between multiple variables were analyzed using Pearson correlation coefficients (GraphPad Prism 8.0). For all analyses a p-value < 0.05 was considered significant.

RESULTS

Gross Lesions, Lesion Scores and Granuloma Stages

Gross lesions were seen in lungs of all infected steers at all time points. The spread and variability (i.e., SD) of lung lesion scores within groups was similar and the means did not differ significantly (**Figure 1**). Granulomas examined at 30 days post-infection were predominantly stage I (**Figure 2**). At 90, 180- and 270-days post-infection all 4 granuloma stages were represented, albeit to varying degrees (**Figure 2**).

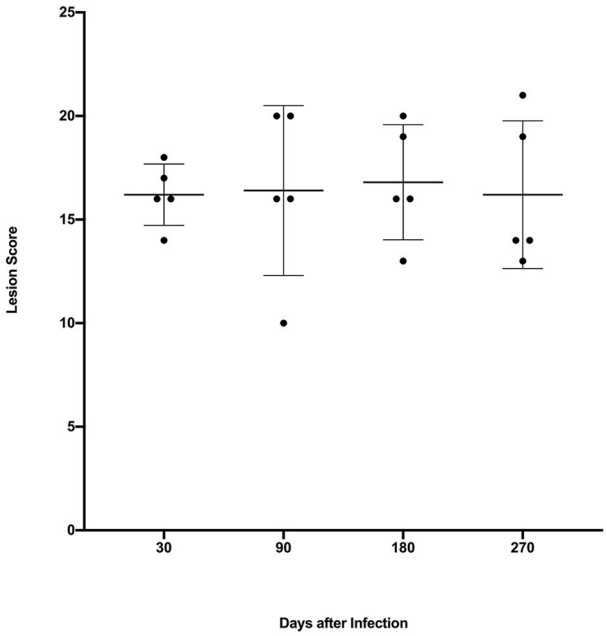

FIGURE 1 | Lung lesion scores from calves experimentally infected with *M. bovis* and examined at various time points after infection. Values are expressed as individual scores with mean ± SD.

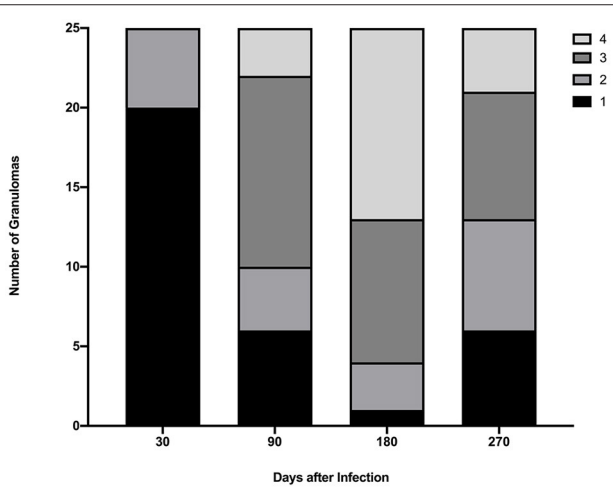

FIGURE 2 | Granuloma stages collected from lungs of steers euthanized 30, 90, 180, and 270 days after experimental infection with *M. bovis*. A maximum of 5 granulomas were collected from each of 5 lung lobes (right cranial, left cranial, right caudal and middle, left caudal, and accessory).

Bacterial Burden of Individual Granulomas

As a group, steers examined 30 days after infection had a significantly higher bacterial burden ($p < 0.0001$) than cattle examined at 90, 180 and 270 days after infection (**Figure 3**). Additionally, as groups, the bacterial burdens of cattle examined 90, 180 and 270 days after infection did not differ significantly from each other (**Figure 3**). Examination of individual granulomas revealed the bacterial burdens of

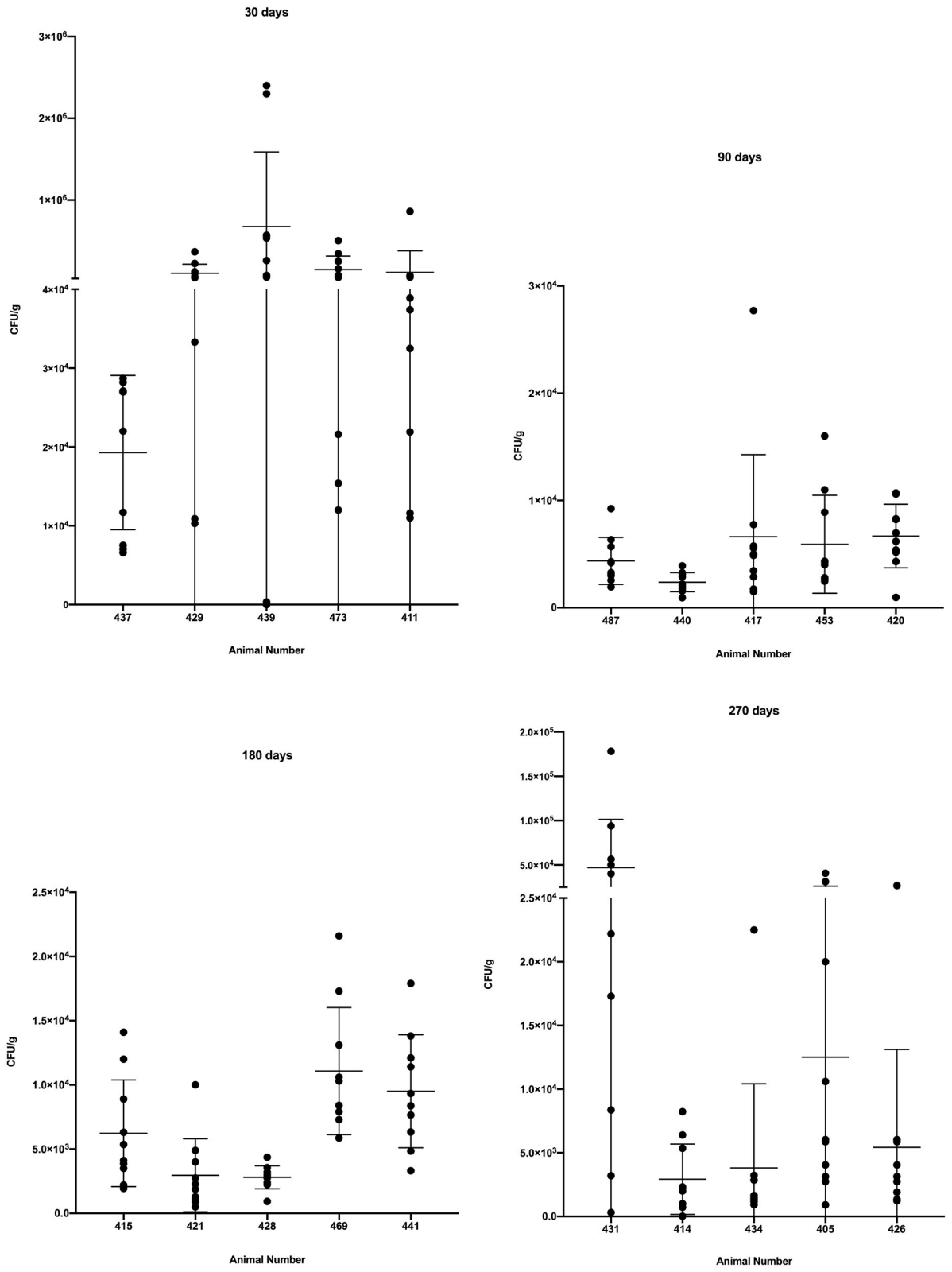

FIGURE 3 | Bacterial burden in colony-forming units/g (CFU/g) in individual granulomas from steers experimentally infected with *M. bovis* and examined at 30, 90, 180, and 270 days after infection. Values represent CFU/g within individual granulomas from right and left caudal lobes with individual animal means ± SD.

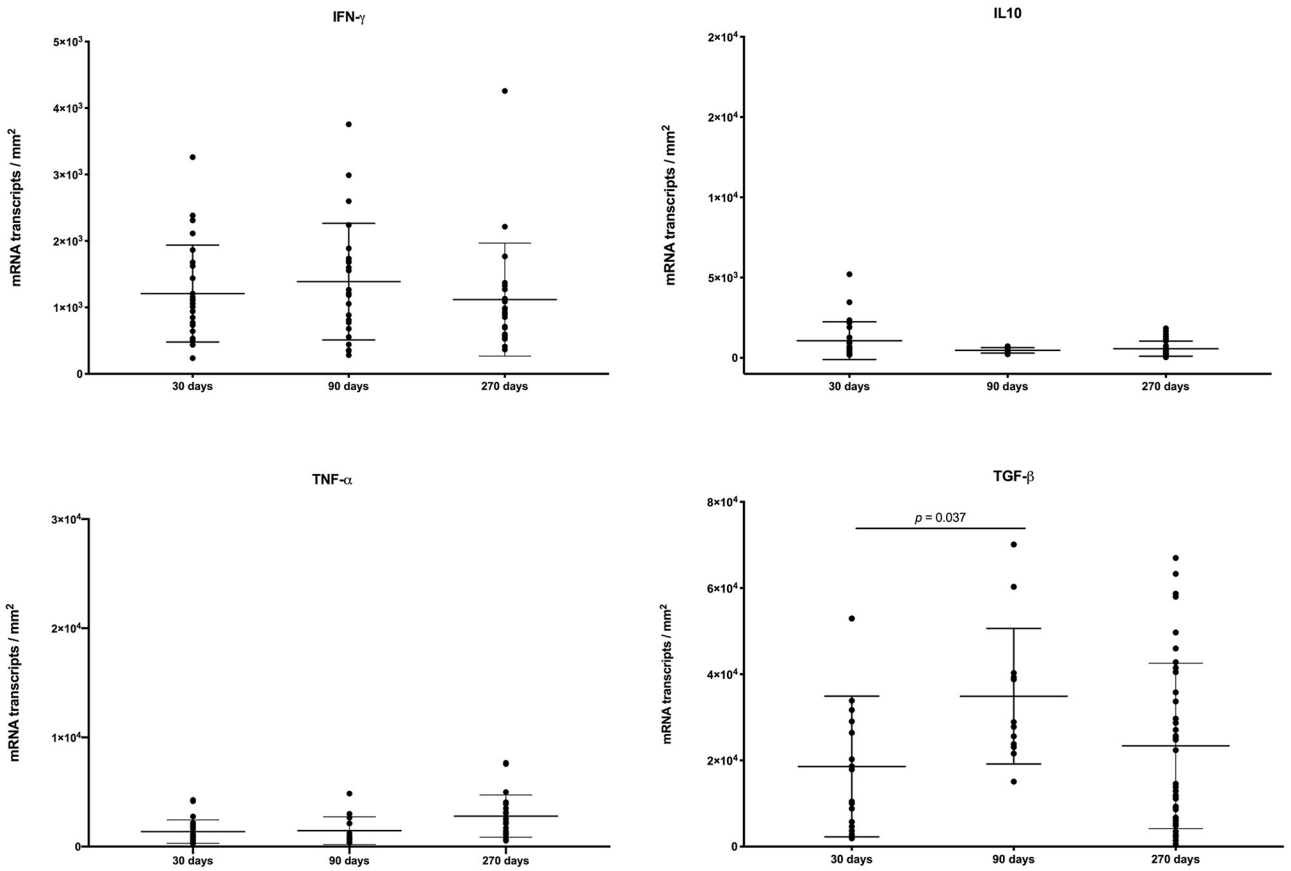

FIGURE 4 | Expression of IFN-γ, IL-10, TNF-α, and TGF-β, as measured by *in-situ* hybridization in individual granulomas from right and left caudal lung lobes of steers experimentally infected with *M. bovis* and examined at 30, 90, and 270 days after infection. Values represent mRNA transcripts/mm^2 with group means ± SD.

granulomas in all animals at all time points were heterogenous with varying degrees of dispersion. The burden range was high, as a number of granulomas from animals examined 30 days post-infection had bacterial burdens $>10^5$ CFU/g while a single granuloma from steer #439, 30 days post-infection contained no culturable *M. bovis* (i.e., sterile granuloma). Although no additional sterile granulomas were noted, a single granuloma with <100 CFU/g was seen in steer #414 at 270 days post-infection.

In situ Hybridization

The balance of pro- and anti-inflammatory cytokines likely contribute to the dynamic nature of granulomas over the course of infection (40). We assessed the expression of various cytokines within granulomas from different time points after infection. Individual granuloma cytokine expression was heterogenous and the variability was greater for IFN-γ and TGF-β compared to IL-10 and TNF-α (**Figure 4**). However, we observed no significant differences in mean expression of IFN-γ, IL-10 or TNF-α at any time points examined, with the exception of TGF-β where expression was significantly greater at 90 days compared to 30 days.

Correlations

When all timepoints were combined, there a was marginally positive correlation between bacterial burden and IL-10 expression; otherwise at any individual time point there was no correlation between bacterial burden and gene expression for any cytokine (**Table 1**). Granuloma stage was marginally positive correlated with TNF-α, and negatively correlated with IL-10 gene expression when all time-points were combined; however, no correlation was seen at individual time points (**Supplementary Tables 1–3**). No correlation was noted between lung lesion score and bacterial burden at any time point.

Cytokine gene expression using all time-points combined showed marginal positive correlations between IFN-γ and both IL-10 and TNF-α (**Table 1**). These marginal positive correlations were also seen at 30 days post-infection (**Supplementary Table 1**), however, only the positive correlation between IFN-γ and TNF-α carried over to the 90-day post-infection time point (**Supplementary Table 2**). No correlations between cytokine gene expression were noted at 270 days post-infection (**Supplementary Table 3**).

TABLE 1 | Pearson correlation coefficients (r) for cytokine expression, bacterial burden (CFU/g) and granuloma stage in pulmonary granulomas examined at all time-points combined (30, 90, 180, and 270 days after infection) from calves experimentally infected with aerosolized *M. bovis*.

	IFN-γ	IL-10	TNF-α	TGF-β	CFU/g
IL-10	0.35[a]	–	–	–	–
	0.007[b]				
TNF-α	0.26	0.03	–	–	–
	0.043	0.790			
TGF-β	0.079	-0.042	−0.127	–	–
	0.556	0.757	0.346		
CFU/g	−0.085	0.250	−0.148	−0.055	–
	0.484	**0.032**	0.248	0.676	
Granuloma Stage	−0.04	−0.35	0.36	0.203	−0.129
	0.714	**0.003**	**0.003**	0.121	0.138

[a] *Pearson coefficient (r).*
[b] *p-value (<0.05 are highlighted in bold text).*

DISCUSSION

Prior studies in cattle have described the morphological heterogeneity of tuberculoid granulomas in both experimental and natural infections (26, 37, 41, 42). The present study examined bacterial burdens of individual pulmonary tuberculoid granulomas at 30, 90, 180 and 270 days after experimental aerosol infection and revealed heterogeneity in bacterial burdens at all time points. Heterogeneity of both morphology and bacterial burdens of individual granulomas has not been examined in cattle. Such heterogeneity suggests that bovine pulmonary tuberculoid granulomas may have their own behavior and trajectory as has been demonstrated in NHP models of human tuberculosis (12, 28). In spite of this independence, as a group the bacterial burden at 30 days was significantly greater than later time points. A decrease in bacterial burden beyond 30 days after infection is consistent with previous studies in cattle demonstrating evidence of a delayed type hypersensitivity (DTH) response to *M. bovis* antigens 3–4 weeks after infection (43–46). The present data would suggest that after the time required for a DTH adaptive immune response to develop there is an overall 1-2 log decrease in bacterial burdens. Nevertheless, there was only a single granuloma from which no *M. bovis* was isolated. Such sterile granulomas are not uncommon in NHP models of human tuberculosis (11, 12, 40). The NHP model has also demonstrated a higher bacterial burden in lesions arising at earlier time points and a significant reduction in bacterial burden after the onset of adaptive immunity, consistent with the findings in this study (12).

Previous studies of tuberculoid granulomas of cattle have suggested that bacterial burdens are increased in late stage IV granulomas, while others have found no correlation between bacterial numbers and granuloma stage (26, 41). Those studies, however, estimated bacterial burden by enumerating AFB observed in microscopic tissue sections. In the current study, we saw no correlation between bacterial burden and granuloma stage and there was not an observable increase in bacterial burdens in granulomas collected at later time points. To the contrary, we found bacterial burdens higher at 30 days post-infection than at later time points. Bacterial burdens did not correlate with expression of IFN-γ, TNF-α, TGF-β, granuloma stage, or lung lesion score, although there was a modest positive correlation with IL-10 expression. Granuloma stage did have modest positive and negative correlations with TNF-α and IL-10, respectively.

Considered a key cytokine, IFN-γ is critical to macrophage activation and pivotal in a protective Th1 response to mycobacteria. Therefore, an interesting finding of the present study was the steady state of IFN-γ expression over time, which did not correlate with either bacterial burden or granuloma stage. Prior studies in cattle have produced inconclusive results in terms of IFN-γ expression in granulomas. Some of these differences may be in technique, inoculation dose, duration of disease, or tissue (i.e., lymph nodes vs. lung). Previous cattle studies using IHC to measure IFN-γ protein within granulomas showed no difference in expression based on granuloma stage; however, there was increased IFN-γ expression in calves receiving a higher dose (1,000 CFU vs. 1 CFU) (42). A separate study using IHC showed an increase in IFN-γ protein immunolabeling between stages I and IV (41). Yet, another study, also using IHC, showed IFN-γ in granulomas of all stages, with a qualitative, but not significant decrease in labeling in late stage IV granulomas compared to earlier stages (47). A study using mRNA extracted from formalin-fixed paraffin-embedded lymph node cross sections showed no significant differences in expression levels of IFN-γ, TNF-α or IL-10 at 5, 12, and 19 weeks after infection and no correlation between lesion severity and individual cytokine expression (48). Supporting the present findings, a study using ISH methods similar to those used here, demonstrated that IFN-γ expression did not differ between stage I and stage IV granulomas examined at a single time point (150 days) after experimental infection (24).

In contrast to IFN-γ expression, there was a mild, but not significant trend of increasing TNF-α gene expression from 30 to 270-days post-infection. Increasing TNF-α levels, may be associated with the increased presence of necrosis within granulomas as they progress from stage I, non-necrotizing

granulomas to stage IV granulomas with significant necrosis. Necrosis is a consistent feature of many tuberculoid granulomas, regardless of mycobacterial species, host species or target organ, intimating the importance of this process in the pathogenesis of tuberculosis. Tissue destruction facilitates spread of disease as bacilli gain entrance to vascular elements, which carry them to distant sites. Other studies using granulomas from cattle collected at a single time-point after experimental infection, or from naturally infected cattle of unknown disease duration have shown decreasing trends, increasing trends, or no difference, respectively, between TNF-α expression in granulomas of stage I to IV. (24, 41, 47, 49).

In tuberculosis, TNF-α has been shown to be critical, with low levels associated with fatal disease; however, excessive TNF-α induces a hyper-inflammatory environment (50, 51). TNF-α induces cytokine production by macrophages, activates macrophages for killing, and modulates macrophage apoptosis (52). Considered pro-inflammatory, cytokines such as TNF-α and IFN-γ if left uncontrolled can lead to excessive tissue damage, increased inflammation, and deteriorating disease status. To balance inflammation and tissue destruction, IL-10 deactivates macrophages, resulting in diminished Th1 cytokine production and decreased production of reactive nitrogen and oxygen species (53). The present study suggests that early in infection, when bacterial burdens are higher and inflammation is increasing, IL-10 production is stimulated. This stimulation appears less critical at later time points when bacterial burdens are lower. In mice, overexpression of IL-10 is associated with increased bacterial burdens, while in humans with tuberculosis, elevated levels of IL-10 have been associated with more active disease and more severe clinical signs (54, 55).

Examinations of TGF-β expression in granulomas from experimentally infected cattle have yielded conflicting results. In one study of lymph node granulomas from experimentally infected cattle examined 29 weeks after infection TGF-β expression was greater in more advanced granulomas compared to early stage granulomas (37). Two other studies found lower expression in late stage granulomas compared to early stage granulomas (49, 56). TGF-β is a key cytokine implicated in fibrinogenesis and new collagen synthesis. In macaques, TGF-β has been suggested as a critical driver of tissue repair in active tuberculosis (57). As more advanced granulomas are often characterized by a peripheral fibrous capsule, the presence of increased TGF-β expression at 90 days compared to 30 days, as seen in the present study, is consistent with a role in fibrosis.

In NHPs positron emission tomography-computed tomography (PET-CT) imaging has allowed serial imaging during the course of infection producing a dynamic map of individual granuloma activity that can be correlated with postmortem exam findings (11, 12). The present study in cattle attempted to follow the course of infection by examining individual animals at increasing intervals after infection. This method yields multiple snap shots of granuloma activity at defined time points, but does not allow observation of individual granuloma development, activity and disease progression within an animal. Thus, a shortcoming of the present study is that we are unaware of the state of individual granulomas before or after the defined time points. Nevertheless, examination of individual pulmonary granulomas of cattle did reveal significant heterogeneity in bacterial burdens, however it is less clear how bacterial burdens relate to granuloma development or cytokine production within granulomas.

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 USDA-National Animal Disease Center Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MP and TT: experiment design. MP and CK: sample collection and experiments. MP and PB data analysis. MP: manuscript preparation. MP, TT, CK, and PB: manuscript editing. All authors contributed to the article and approved the submitted version.

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RNA-Seq Transcriptome Analysis of Peripheral Blood From Cattle Infected With *Mycobacterium bovis* Across an Experimental Time Course

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Bovine tuberculosis, caused by infection with members of the *Mycobacterium tuberculosis* complex, particularly *Mycobacterium bovis*, is a major endemic disease affecting cattle populations worldwide, despite the implementation of stringent surveillance and control programs in many countries. The development of high-throughput functional genomics technologies, including RNA sequencing, has enabled detailed analysis of the host transcriptome to *M. bovis* infection, particularly at the macrophage and peripheral blood level. In the present study, we have analysed the transcriptome of bovine whole peripheral blood samples collected at −1 week pre-infection and +1, +2, +6, +10, and +12 weeks post-infection time points. Differentially expressed genes were catalogued and evaluated at each post-infection time point relative to the −1 week pre-infection time point and used for the identification of putative candidate host transcriptional biomarkers for *M. bovis* infection. Differentially expressed gene sets were also used for examination of cellular pathways associated with the host response to *M. bovis* infection, construction of *de novo* gene interaction networks enriched for host differentially expressed genes, and time-series analyses to identify functionally important groups of genes displaying similar patterns of expression across the infection time course. A notable outcome of these analyses was identification of a 19-gene transcriptional biosignature of infection consisting of genes increased in expression across the time course from +1 week to +12 weeks post-infection.

Keywords: biomarker, cattle, gene expression, host-pathogen interaction, immune response, time series, tuberculosis, *Mycobacterium bovis*

INTRODUCTION

Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis* and other intracellular bacterial pathogens of the *Mycobacterium tuberculosis* complex (MTBC), which display 99.9% DNA sequence identity at the genome level (1–3). Each member of the MTBC has a distinctive host spectrum, such that tuberculosis (TB) affects a wide range of mammals including humans (4). In addition, BTB has been classified as the fourth most important disease of livestock in terms of zoonotic and economic impact globally (5, 6). It has also been conservatively estimated that BTB costs $3 billion annually and imposes a large financial burden on farmers with infected herds (7, 8). Furthermore, as a zoonosis, *M. bovis* infection has important implications for human health; transmission from cattle to humans does occur and is responsible for a small but significant number of human TB cases, particularly in developing countries (9–11).

Tuberculous mycobacteria—primarily *M. bovis* and *M. tuberculosis*, the main cause of human TB—are generally inhaled from the environment within aerosol droplets and are phagocytosed by host alveolar macrophages (AMs); therefore, infection is normally initiated within, and restricted to, lung tissues (12–15). Tuberculous mycobacteria have evolved a wide range of mechanisms to modulate, suppress, and manipulate specific host immune mechanisms, including inhibition of phagosomal maturation, detoxification of reactive oxygen and nitrogen species (ROS and RNS), repair of ROS- and RNS-induced cellular damage, resistance to antimicrobial and cytokine defences, modulation of antigen presentation, and induction of cellular necrosis and inhibition of apoptosis (16–19). Tuberculous disease is characterised by lesions located at the site of infection, which are formed when AMs and other immune cells engage and eliminate most of the bacilli. The remaining intact mycobacterial cells are confined in granulomas that act to contain the infection, but may, under certain conditions, actually facilitate expansion and dissemination of mycobacteria to spread infection (20–22).

In Ireland, a test and slaughter policy for BTB was introduced in the early 1950s as part of the national BTB eradication scheme (23, 24). This policy includes compulsory screening of all animals using the single intradermal comparative tuberculin test alone or in conjunction with an *in vitro* enzyme-linked immunosorbent assay–based interferon γ (IFN-γ) release assay (IGRA) that increases the sensitivity of diagnosis (25). However, limitations of current diagnostic methods prevent early and accurate detection and subsequent removal of all infected animals from a herd, thereby contributing to the ongoing persistence of BTB, which continues to impact cattle production in Ireland, the United Kingdom, and other countries (24, 26). Therefore, the most important objective of an effective BTB control strategy—to identify and remove all infected cattle from a herd regardless of the stage of infection—is substantially hindered by current diagnostic technologies. Novel methods of BTB diagnosis are urgently required to augment current test procedures in conjunction with appropriate wildlife reservoir control measures (27).

In recent years, the availability of a well-annotated bovine genome sequence combined with high-throughput functional genomics technologies has provided an unprecedented opportunity to gain a deeper understanding of host–pathogen interaction, identify blood-derived RNA-based biomarkers, and develop new diagnostic methods for BTB caused by infection with *M. bovis* (28–33).

Previous transcriptomics studies of the host immune response to *M. bovis* have been performed using blood-derived RNA obtained from both naturally and experimentally infected animals, as it has been shown that host immune responses occurring in peripheral blood reflect those at the primary site of disease in BTB (34). In this regard, the dynamic transcriptome of circulating blood, which contains a large pool of "biosensors" in the form of RNA transcripts, can reflect physiological and pathological events occurring elsewhere in different tissues and organs, thereby providing a comprehensive overview of the status of the immune system (35, 36). In addition, peripheral blood has provided information on the pathobiology of many diseases; it is accessible and easily collected, making it ideally suited for the development of diagnostic biomarker tests based on transcriptional profiling (37–39).

For the experimental work described here, RNA sequencing (RNA-seq) was used to study the bovine whole peripheral blood transcriptome in response to infection with *M. bovis* across a large-scale 14-week animal infection time course. The main objectives of the study were to examine the host peripheral blood transcriptional responses across the early stages of *M. bovis* infection and identify differentially expressed (DE) genes across the infection time course that represent promising candidate biomarkers for BTB. In addition, we aimed to identify host canonical pathways and interaction networks enriched for DE genes, which may shed light on the immunobiology of *M. bovis* infection in cattle. We also used time-series analysis and Gene Ontology (GO) information to identify functionally important groups of DE genes across the infection time course.

MATERIALS AND METHODS

Overview of Animal Infection Time Course Experiment

Animal resources for the present study were obtained from a 26-week vaccination and challenge experiment of age- and sex-matched cattle infected with *M. bovis* (40–44). Ten male age-matched Holstein–Friesian calves (4–6 months old) were sourced from farms known to be free of BTB disease. The animals used for the experimental work described here were the naive control group (non-vaccinated) for a vaccine efficacy study (40). **Figure 1** shows the experimental schedule used by Dean et al. (40) and details the sampling time points for the 10 non-vaccinated control cattle used for the research work described here.

Inoculation With *M. bovis* Strain AF2122/97

The challenge strain, *M. bovis* AF2122/97 (2, 45), was delivered endobronchially at 2×10^3 colony-forming units per animal using the following procedure described by Whelan et al. (46).

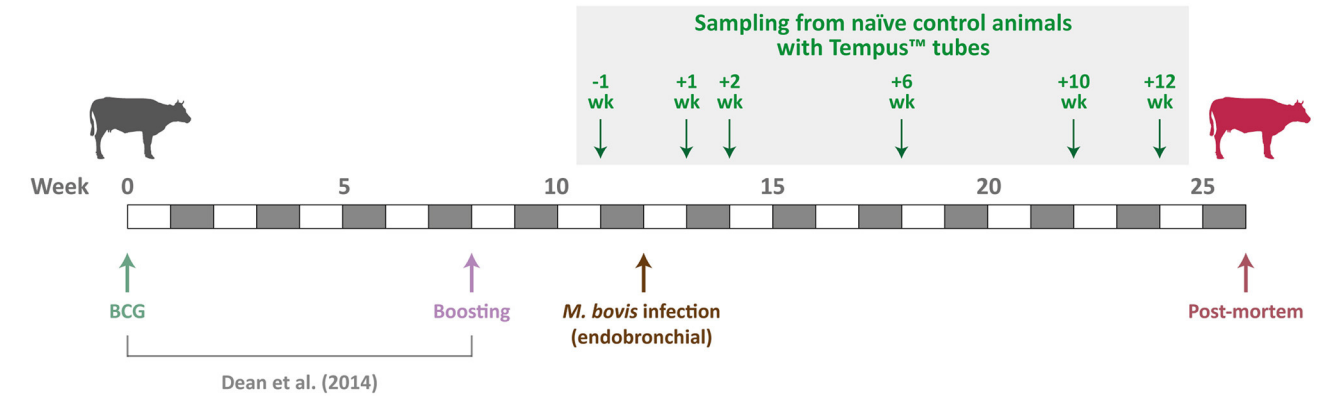

FIGURE 1 | Schedule for the *M. bovis* infection time-course experiment. Sampling time points for the 10 non-vaccinated control cattle used are indicated by green arrows.

Prior to endobronchial inoculation animals were sedated with Rompun® (Bayer Animal Health, Newbury, UK) according to the manufacturer's instructions. Following this, an LSVP 22 VGS89x14 endoscope (Veterinary Endoscopy Services, Welshpool, UK) lubricated with Vet-Lubigel (Millpledge Veterinary, Clarborough, UK) was inserted through a nostril into the trachea and placed above the bronchial opening to the cardiac lobe and the main bifurcation between left and right lobes. A cannula of 1.8-mm internal diameter (Veterinary Endoscopy Services) was inserted through the endoscope and used to deliver the *M. bovis* AF2122/97 inoculum in 2 mL of phosphate-buffered saline (PBS). Following this, an additional 2 mL of PBS was then used to wash the cannula, and the cannula and endoscope were withdrawn. For each individual animal, a new sterile cannula was used, the internal channel of the endoscope, through which the cannula was inserted, was rinsed with 20 mL of PBS, and the outside of the endoscope was cleaned thoroughly with sterilising tissue wipes (Medichem International, Sevenoaks, UK).

Individual responses to infection across the time course and disease pathology for the animals used in this study have been described in detail previously and include whole-blood IFN-γ assay, evaluation of peripheral blood mononuclear cell (PBMC) cytokine responses by intracellular cytokine staining, gross (visible) pathology and histopathology, and evaluation of bacterial load in lymph nodes (40, 41).

Peripheral Blood Collection and Total RNA Extraction

Approximately 3 mL of *ex vivo* peripheral blood was sampled from all 10 naive control animals at −1 week pre-infection and then at +1, +2, +6, +10, and +12 weeks post-infection (**Figure 1**). All blood samples were obtained during the morning (between 7:00 and 10:00 A.M.) of each collection day and directly collected into Tempus™ blood RNA tubes (Applied Biosystems®/Thermo Fisher Scientific, Warrington, UK). Immediately after blood collection at each time point, Tempus™ tube samples for each animal were vortexed for ∼10 s to ensure complete red blood cell lysis. Tempus™ tube blood lysate samples for animals at each of the nine time points were then stored at −80°C until they were used for total RNA extraction and purification.

The Tempus™ Spin RNA Isolation Kit (Applied Biosystems®/Thermo Fisher Scientific) was used for total RNA extraction and purification using the following protocol provided by the manufacturer. Tempus™ tube blood lysate samples were thawed at room temperature prior to RNA extraction and purification. Once thawed, for each sample, ∼3 mL of blood lysate was transferred to a 50-mL plastic centrifuge tube, and PBS was added to a final volume of 12 mL. Each sample was then mixed by vortexing for 30 s and then centrifuged at 3,000 × *g* for 30 min at 4°C. The supernatant was then removed, and the remaining RNA-containing pellet was resuspended with a brief vortex in 400 μL of the proprietary RNA Purification Resuspension Solution. Following this, the resuspended RNA sample was pipetted into the RNA purification filter inserted into a 1.5-mL microcentrifuge tube for waste collection. The RNA purification filter/microcentrifuge tube was then centrifuged at 16,000 × *g* for 30 s, and the liquid waste and microcentrifuge tube discarded. The RNA purification filter was then placed in a clean microcentrifuge tube, 500 μL of proprietary RNA Purification Wash Solution 1 was added, followed by another centrifugation step at 16,000 × *g* for 30 s and disposal of the liquid waste and microcentrifuge tube. This step was then repeated using 500 μL of proprietary RNA Purification Wash Solution 2 with a centrifugation step at 16,000 × *g* for 30 s. A final wash step was then performed with 500 μL of RNA Purification Wash Solution 2 and centrifugation at 16,000 × *g* for 30 s followed by disposal of the liquid waste and microcentrifuge tube. The RNA purification filter was then placed in a clean microcentrifuge tube and centrifuged at 16,000 × *g* for 30 s to dry the membrane. The RNA purification filter was then inserted into a clean RNase-free collection microcentrifuge tube and 100 μL of Nucleic Acid Purification Elution Solution was added and incubated for 2 min followed by centrifugation at

16,000 × *g* for 30 s; the RNA eluate was then pipetted back onto the filter membrane, and the centrifugation step was repeated. Approximately 90 μL of the final RNA eluate was then pipetted (avoiding particulate material) into a new labelled RNase-free collection microcentrifuge for long-term storage at −80°C.

RNA Quality Checking and Quantification

RNA quantity and quality checking were performed using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer using an RNA 6000 Nano LabChip kit (Agilent Technologies, Cork, Ireland). The majority of samples displayed a 260/280 ratio >1.8 and RNA integrity numbers >8.0 (**Supplementary Table 1** in **Supplementary Material 1**). RNA quality and quantity checking revealed that three samples did not have measurable quantities of RNA, and these were excluded from downstream RNA-seq library preparation (15, ID 6520, +2 weeks; 21, ID 6522, +2 weeks; and 27, ID 6526, + 2 weeks).

Strand-Specific RNA-Seq Library Preparation and Sequencing

For RNA-seq library preparation, 1 μg of total RNA from each sample was used to prepare individually barcoded strand-specific RNA-seq libraries. Two rounds of poly(A)$^+$ RNA purification were performed for all RNA samples using the Dynabeads® mRNA DIRECT™ Micro Kit (Ambion®/Thermo Fisher Scientific, Austin, TX, USA) according to the manufacturer's instructions. The purified poly(A)$^+$ RNA was then used to generate strand-specific RNA-seq libraries using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit, the ScriptSeq™ Index PCR Primers (sets 1–4), and the FailSafe™ PCR enzyme system (all sourced from Epicentre®/Illumina® Inc., Madison, WI, USA) according to the manufacturer's instructions. RNA-seq libraries were purified using the Agencourt® AMPure® XP system (Beckman Coulter Genomics, Danvers, MA, USA) according to the manufacturer's instructions for double size selection (0.75× followed by 1.0× ratio). RNA-seq libraries were quantified using a Qubit® fluorometer and Qubit® dsDNA HS Assay Kit (Invitrogen™/Thermo Fisher Scientific, Carlsbad, CA, USA), whereas library quality cheques were performed using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent Technologies Ltd.). Individually barcoded RNA-seq libraries were pooled in equimolar quantities, and the quantity and quality of the final pooled libraries (three pools in total) were assessed as described previously. RNA-seq library sample barcode index sequences are detailed in **Supplementary Table 1** (**Supplementary Material 1**).

Prior to high-throughput sequencing, the content of several RNA-seq libraries was validated using conventional Sanger dideoxy sequencing. Library inserts from 16 libraries were cloned using the Zero Blunt® TOPO® PCR Cloning Kit according to the manufacturer's instructions (Invitrogen™/Thermo Fisher Scientific). Sanger sequencing of 36 plasmid inserts from these selected libraries confirmed that the RNA-seq libraries contained inserts derived from bovine mRNA. Plasmid sequencing was outsourced (Source Bioscience Ltd., Dublin, Ireland), and sequences generated were validated using BLAST searching of the DNA sequence database (47). Cluster generation and high-throughput sequencing of the pooled RNA-seq libraries were performed using an Illumina® HiSeq™ 2000 Sequencing System at the MSU Research Technology Support Facility (RTSF) Genomics Core (https://rtsf.natsci.msu.edu/genomics; Michigan State University, MI, USA). Each of the three pooled libraries was sequenced independently on five lanes split across multiple Illumina® flow cells. The pooled libraries were sequenced as paired-end 2 × 100 nucleotide reads using Illumina® version 5.0 sequencing kits. Additionally, after exploratory data analysis (**Supplementary Figures 1, 2**), it was decided to remove animal ID 6522 completely from the analysis and proceed with 52 RNA-seq sample data sets (**Supplementary Table 1** in **Supplementary Material 1**). All RNA-seq data generated for this study have been deposited in the European Nucleotide database with experiment series accession numbers (PRJEB27764 and PRJEB44470).

Bioinformatics Analyses of RNA-Seq Data

Except where indicated, bioinformatics procedures and analyses were performed on a 32-core Compute Server running Linux Ubuntu (version 12.04.2) hosted at the UCD Research IT Data Centre (stampede.ucd.ie) and administered by the UCD Animal Genomics Group. All of the bioinformatics workflow/pipeline components including Linux Bash, Perl, and R scripts used were deposited in a GitHub repository (https://github.com/kmcloughlin1/RNA-sequencing) and were modified from published methods described by our group (48). **Supplementary Figure 3** shows a schematic of the complete RNA-seq bioinformatics workflow and the downstream tools used for time-series analysis and various systems biology methods.

Deconvolution (filtering and segregation of sequence reads based on the unique RNA-seq library barcode index sequences; **Supplementary Table 1** in **Supplementary Material 1**) was performed by the MSU RTSF Genomics Core using a pipeline that simultaneously demultiplexed and converted pooled sequence reads to discrete FASTQ files for each RNA-seq sample with no barcode index mismatches permitted. The RNA-seq FASTQ sequence read data were then downloaded from the MSU RTSF Genomics Core FTP server, and a custom Perl script was used to filter out paired-end reads containing adapter sequence contamination (with up to three mismatches allowed) and to remove poor quality paired-end reads (i.e., one or both reads containing 25% of bases with a Phred quality score <20). The quality of individual RNA-seq sample library files was then reassessed postfiltering using the FastQC software package (version 0.10.1) (49).

Paired-end reads, from each filtered individual library, were aligned to the *Bos taurus* reference genome (UMD3.1.73) (50) using the STAR aligner software package (version 2.3.0) (51). For each library, raw counts for each gene based on the sense strand data were obtained using the featureCounts software from the Subread package (version 1.3.5-p4) (52). The featureCounts parameters were set to unambiguously

assign uniquely aligned paired-end reads in a stranded manner to the exons of genes within the UMD3.1.73 *B. taurus* reference genome annotation. The gene count outputs were then used to perform differential gene expression analysis using the edgeR Bioconductor package (version 3.2.4) (53) within an R-based pipeline that was customised to perform the following functions:

1. Use biomaRt (54) to generate a detailed bovine gene annotation for downstream analyses, then filter out all bovine rRNA genes.
2. Filter out genes displaying expression levels below a minimal detection threshold of one count per million in at least $n = 9$ individual libraries (where n = smallest group of biological replicates).
3. Calculate normalisation factors for each library using the trimmed mean of M values method (55).
4. Identify DE genes between the pre-infection animal group (−1 week) and each of the post-infection animal groups (+1, +2, +6, +10, and +12 weeks) using a paired-sample approach with the edgeR package. Differential expression was evaluated by fitting a negative binomial generalised linear model for each gene.
5. Correct for multiple testing using the Benjamini–Hochberg method (56) with a false discovery rate (FDR) threshold of ≤0.05.

Systems Analyses of RNA-Seq Gene Expression Data

The Ingenuity® Pathway Analysis (IPA) software package (57) with the Ingenuity® Knowledge Base (Qiagen Corp., Redwood City, CA, USA; release date July 2014; www.ingenuity.com) was used to identify overrepresented (enriched) canonical pathways and construction of biological interaction networks for sets of DE genes at each post-infection time point (+1, +2, +6, +10, and +12 weeks) compared to the pre-infection time point (−1 week). For identification of overrepresented canonical pathways, a multiple testing correction (Benjamini–Hochberg method) was applied with an FDR threshold ≤0.05. The IPA Biomarker Filter tool was also used to identify and prioritise molecular biomarker candidates such that only experimentally observed and high-confidence predicted biological relationships were included. All the IPA data sources were used for three mammalian species in the IPA Knowledge Base (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*). Biological interaction networks were ranked according to Network Score values generated algorithmically by IPA and based on the hypergeometric distribution and calculated with the right-tailed Fisher exact test (58).

Time-Series Analysis of RNA-Seq Gene Expression Data

Time-series analysis of gene expression data from the animal infection time-course experiment was performed using the Short Time-series Expression Miner (STEM) software package (59). The computational procedure for selecting model profiles that are representative and distinct is described by Ernst et al. (60). The software package implements a method for clustering short time-series expression data that can differentiate between real and random patterns of temporal gene expression changes and assigns each gene to the model profile that most closely matches the temporal gene expression profile for that gene as determined by the correlation coefficient. A permutation test is then used to determine which model profiles have a statistically significant number of genes assigned compared to random expectations from the mean number assigned to each profile based on the permuted data (59). STEM also incorporates GO enrichment functionality for biological interpretation of time-series gene expression data.

RESULTS AND DISCUSSION

RNA-Seq Summary Statistics

Deconvolution and filtering of sequence reads to remove adaptor-dimer contamination yielded a mean of 20.6 ± 2.0 million reads per individual barcoded RNA-seq sample library ($n = 52$ libraries and ± SD); this corresponded to a mean of 82% reads that passed this filtering step. These filtered reads were then aligned to the *B. taurus* UMD3.1.73 genome build. This yielded a mean of 15.4 ± 1.7 million filtered reads (91.5%) that uniquely mapped to this bovine genome build with a mean mapped length of 195.6 ± 0.6 bp; a mean of 0.77 ± 0.19 million reads (4.6%) that mapped to multiple genomic locations and 0.67 ± 0.17 million reads (3.9%) that did not map to any genomic location. Further analysis demonstrated that a mean of 63.1% of the filtered uniquely mapping reads (9.7 ± 1.1 million reads) were assigned to Ensembl gene IDs for the UMD3.1.73 genome build and 36.9% (5.7 ± 0.86 million reads) were ambiguous or could not be assigned to an annotated genomic region. **Supplementary Table 2** (**Supplementary Material 1**) and **Supplementary Figure 4** show the RNA-seq summary statistics. Filtering of the RNA-seq data using 52 samples (60 minus the three technical dropouts and the animal ID 6522 samples at −1, +1, +6, +10, and +12 weeks) produced 12,406 genes suitable for downstream differential expression analysis.

Multidimensional scaling plots (**Supplementary Figure 5**) demonstrated that it was not possible to differentiate the infected animals from non-infected animals during the early stages of the animal infection time course (+1 week post-infection vs. −1 week pre-infection, and +2 weeks vs. −1 week pre-infection). Conversely, discrimination of infected and non-infected animals was partially observed at +6 weeks post-infection and was clearly evident at +10 weeks post-infection, but this pattern of discrimination effectively disappeared by +12 weeks post-infection. Previous work by our group has shown that microarray and RNA-seq gene expression data sets from peripheral blood leukocytes (PBLs) can be used to unambiguously discriminate *M. bovis*–infected from non-infected control cattle (28, 31). However, it is important to note that the *M. bovis*–infected animals used for these earlier studies were heavily infected animals, which were maintained for ongoing disease surveillance and potency testing of diagnostic reagents.

Differentiation of *M. bovis*–Infected and Non-infected Control Groups: Toward the Development of Transcriptomics-Based Biomarkers

The concept and the implications regarding biomarker identification and biosignature development for infectious disease have been explored thoroughly by Chaussabel et al. (36, 61, 62). In particular, these researchers emphasise that leukocytes present in peripheral blood convey valuable information about the status of the immune system that can be translated to biomarkers and onward to sensitive and specific biosignatures of infection. In addition, peripheral blood is easily accessible and can be stabilised and processed for high-throughput transcriptomics analyses using RNA-seq and other massively parallel gene expression technologies such as microarrays. It is also notable that development of transcriptional biomarkers in cattle is relatively straightforward because very low levels of globin gene transcripts (*HBA* and *HBB*) are observed in bovine peripheral blood compared to other mammalian species (63, 64).

Statistical analysis of the RNA-seq gene expression data with a B-H FDR adjusted $P \leq 0.05$ demonstrated that differential gene expression was evident between each of the five post-infection time points (+1, +2, +6, +10, and +12 weeks) and the −1 week pre-infection time point (**Figure 2A**). Relatively small numbers of DE genes were detected at +1 week (37 exhibited increased and 20 exhibited

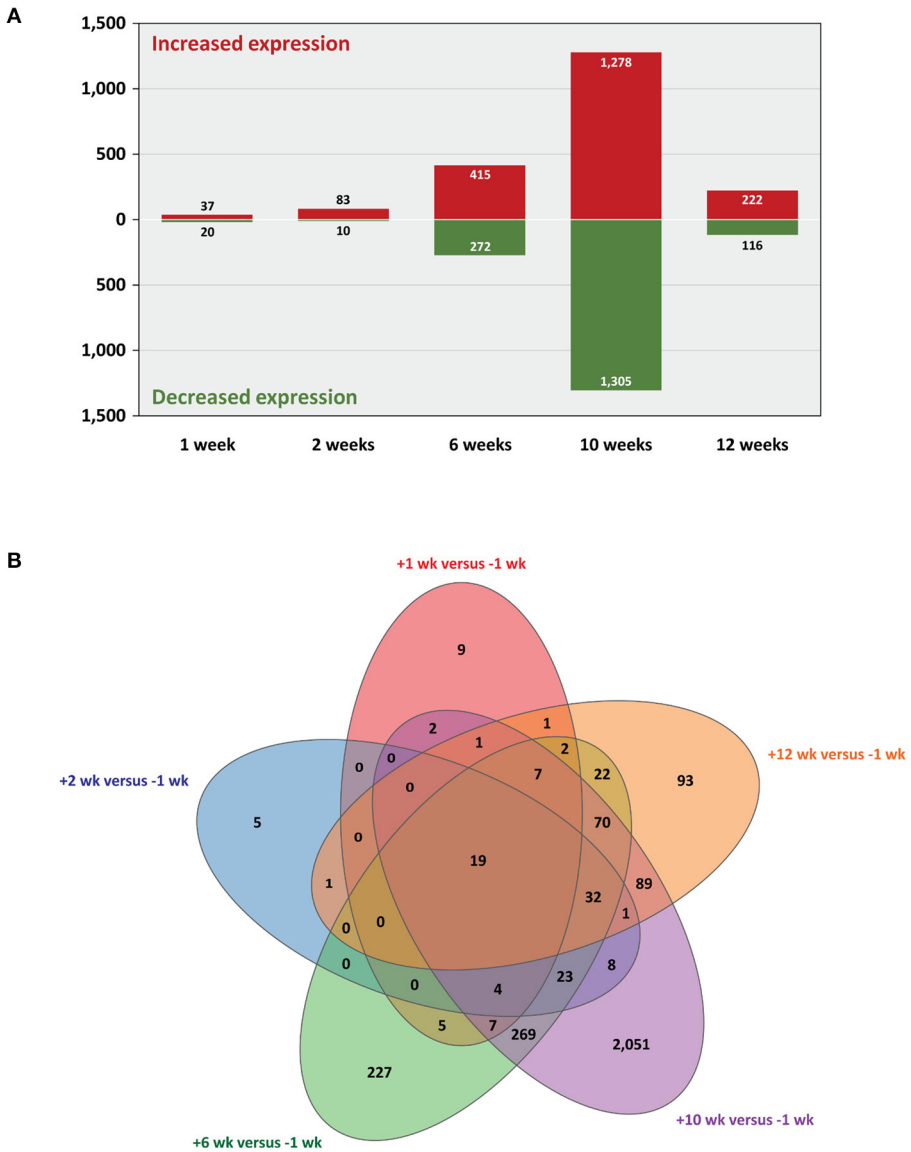

FIGURE 2 | Statistically significant differentially expressed genes. Five post-infection time points are shown relative to the −1 week pre-infection time point (B-H FDR adjusted $P \leq 0.05$). **(A)** Bar graph showing numbers of genes with increased and decreased expression and **(B)** Venn diagram showing the overlaps of DE genes for every multiple-time-point comparison.

decreased expression) and +2 weeks (83 increased and 10 decreased); however, the numbers of DE genes were substantially greater at +6 weeks (415 increased and 272 decreased), +10 weeks (1,278 increased and 1,305 decreased), and +12 weeks (222 increased and 116 decreased). **Supplementary Tables 3–7** (**Supplementary Material 2**) provide detailed information on the differential expression analysis results at each of the five post-infection time points relative to the −1 week pre-infection time point for the complete set of 12,406 filtered genes. **Figure 2B** also shows a Venn diagram for the significant DE genes at each of the five post-infection time points (relative to the −1 week pre-infection control time point). In addition, **Table 1** provides detailed information for 19 genes shown in **Figure 3** that were significantly DE across all the five post-infection time points compared to the −1 week pre-infection control time point.

As shown in **Figure 3**, there is a striking concordance between the patterns of expression for these 19 genes across the infection time course and earlier PBL microarray and RNA-seq studies published by our group (28, 31). These results provide good support for the hypothesis that a biosignature of *M. bovis* infection can be generated using transcriptomics data from cattle with early- and later-stage BTB. It also provides evidence that putative transcriptional biomarkers, identified using an experimental challenge with a relatively high *M. bovis* infectious dose, can be translated as diagnostic tools for use in naturally infected animals. Diagnostic biosignature development focusing on smaller panels of transcriptomics-based biomarkers has been used with notable success for human TB. In this case, research work has focused on specificity and differentiating active TB from latent TB and also TB disease from non-infected controls and diseases with similar pathology but distinct aetiology such as sarcoidosis, pneumonia, and lung cancer (35, 39, 65–71).

Previous work on *M. tuberculosis* and human immunodeficiency virus infection in humans has shown that *CXCR4* is upregulated in blood monocytes and bronchoalveolar lavage cells from human patients with pulmonary TB (72–74). In addition, a loss-of-function mutation in the murine *Thbd* gene that impairs activated protein C production results in uncontrolled lung inflammation in mice infected with *M. tuberculosis*, highlighting the importance of the *THBD* gene in mammalian TB disease (75). Galietti et al. have also shown that *M. tuberculosis*– and *M. bovis*–infected, but not *M. avium*–infected, human monocytes showed increased expression of the CDKN1A protein encoded by *CDKN1A* (76). A range of studies have shown that levels of the protein product of the *PLAUR* gene are elevated in serum from human patients infected with *M. tuberculosis* (77–81). Increased expression of the *OSM* gene and induction of matrix metalloproteinases, which contribute to tissue damage characteristic of TB, have been demonstrated in *M. tuberculosis*–infected human monocytes (82). High expression of *OSM* was also observed in the blood transcriptome of patients presenting with high mycobacterial load sputum (83). In addition, the *OSM* gene is located within a candidate QTL region for TB susceptibility identified using admixture mapping in humans (84).

Ten of the 19 genes that showed consistently increased expression across all post-infection time points were observed to overlap with results from RNA-seq of an *in vitro* infection time-course experiment using bovine AMs stimulated with *M. bovis* (48). *FOSB* and *NR4A1* were upregulated in AMs at 2 hpi; *CXCL8*, *NR4A1*, *PLAUR*, and *RGS16* were upregulated at 6 hpi; *EVI2A*, *CXCL8*, *FOSB*, *HBEGF*, *OSM*, *PLAUR*, *RGS16*, and *THBD* were upregulated at 24 hpi; the eight genes observed at 24 hpi plus the *CDKN1A* gene were also upregulated at 48 hpi.

One of the most notable putative transcriptional biomarkers represented in the panel of 19 genes and in the independent PBL studies is *CXCL8* (previously known as *IL8*). CXCL8 is a chemokine encoded by the *CXCL8* gene, which is a strong neutrophil chemoattractant and also chemotactic for monocytes and T cells (85, 86); it has been observed to exhibit increased expression for different mycobacterial infections in a range of mammalian systems (87–95). CXCL8 enhances killing of mycobacteria by neutrophils and macrophages (96, 97), and these immune cells also secrete CXCL8 when stimulated by *M. tuberculosis* (98). In this regard, Godaly and Young showed that *M. bovis* bacillus Calmette–Guérin (BCG) induces CXCL8 secretion by human neutrophils via MyD88-dependent signalling through TLR2 and TLR4 (99). Also, stimulation of human lung fibroblasts *in vitro* using conditioned medium from *M. tuberculosis*–infected monocytes caused prolonged expression of *CXCL8* mRNA and >10-fold increase in CXCL8 secretion (88).

With regard to the *CXCL8* mRNA transcript as a biomarker of infection, Alessandri et al. were able to detect significantly elevated levels of the CXCL8 cytokine in plasma from patients with pulmonary TB (87). More recently, based on reversion of IGRA test results in a Chinese cohort, it has been proposed that decreased serum levels of CXCL8 are associated with clearance of *M. tuberculosis* infection (100). In addition, using microarray and reverse transcriptase–qualitative polymerase chain reaction technologies, Widdison et al. have shown that *M. tuberculosis*– and *M. bovis*–infected bovine AMs express high levels of *CXCL8* transcripts compared to non-infected control cells (90). In support of this, using RNA-seq, we have shown that *CXCL8* increases in expression in bovine AMs infected with either *M. tuberculosis* or *M. bovis* across a 48-h time course (101). *CXCL8* has also been shown to be significantly increased in expression after *in vitro* PPD-b stimulation of PBMCs from cattle infected with *M. bovis* (102) and bovine monocyte-derived macrophages (103). *CXCL8* also exhibited increased expression in PBL (28) but decreased expression in non-stimulated PBMCs from *M. bovis*–infected cattle (104). Also, Almeida de Souza et al. have shown that antimycobacterial treatment reduces high plasma levels of CXCL8 and other CXC chemokines detected in plasma from human patients with active TB (91), and Huang et al. have also demonstrated that AMs and PBMCs from TB patients express CXCL8 at significantly higher levels than healthy controls (94). Interestingly, the potential specificity of increased *CXCL8* gene expression as a biomarker for *M. bovis* infection in cattle is illustrated by recent results obtained by Alonso-Hearn et al. (105). Using similar RNA-seq methodology, they observed significantly decreased expression of *CXCL8* in peripheral blood from cattle infected with *M. avium* subsp. *paratuberculosis*, the causative agent of Johne disease. Finally, it is important to note that several primary studies and meta-analyses

TABLE 1 | Nineteen genes that exhibited statistically significant differential expression for each of the five post-infection time points vs. the −1 week pre-infection control time point.

Ensembl ID	Gene symbol	Gene name	+1 week post-infection		+2 weeks post-infection		+6 weeks post-infection		+10 weeks post-infection		+12 weeks post-infection	
			Fold-change	B-H FDR *P*-value	Fold-change	B-H FDR *P*-value	Fold-change	B-H FDR *P*-value	Fold-change	B-H FDR *P*-value	Fold-change	B-H FDR *P*-value
ENSBTAG00000000306	*ITK*	IL-2–inducible T-cell kinase	+1.28	0.006848	+1.36	0.000235	+1.23	0.009551	+1.37	0.000001	+1.35	0.000044
ENSBTAG00000000507	*NR4A1*	Nuclear receptor subfamily 4, group A, member 1	+2.51	0.026721	+3.52	0.000214	+4.49	0.000000	+11.04	0.000000	+2.51	0.007555
ENSBTAG00000001060	*CXCR4*	Chemokine (C-X-C motif) receptor 4	+1.99	0.004449	+2.05	0.003150	+2.13	0.000126	+3.09	0.000000	+2.30	0.000037
ENSBTAG00000002758	*THBD*	Thrombomodulin	+1.89	0.033131	+2.01	0.015038	+2.76	0.000001	+3.10	0.000000	+1.86	0.012975
ENSBTAG00000003553	*ZFP36L2*	ZFP36 ring finger protein-like 2	+1.52	0.035631	+1.58	0.020932	+1.69	0.000259	+1.87	0.000001	+1.52	0.011716
ENSBTAG00000003650	*NR4A2*	Nuclear receptor subfamily 4, group A, member 2	+2.49	0.029283	+3.49	0.000308	+4.32	0.000001	+9.96	0.000000	+2.59	0.006347
ENSBTAG00000004305	*RGS16*	Regulator of G-protein signalling 16	+2.42	0.037004	+3.09	0.000829	+2.84	0.000682	+4.84	0.000000	+2.27	0.028004
ENSBTAG00000006806	*KRT17*	Keratin 17	+5.57	0.003229	+7.94	0.000098	+10.26	0.000001	+14.58	0.000000	+4.16	0.010868
ENSBTAG00000008182	*FOSB*	FBJ murine osteosarcoma viral oncogene homologue B	+2.50	0.004449	+4.02	0.000001	+5.05	0.000000	+6.27	0.000000	+2.93	0.000079
ENSBTAG00000008353	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	+1.71	0.049537	+2.24	0.000142	+2.59	0.000000	+3.73	0.000000	+2.03	0.000391
ENSBTAG00000009354	*EVI2A*	Ecotropic viral integration site 2A	+1.34	0.002116	+1.26	0.047028	+1.43	0.000003	+1.57	0.000000	+1.37	0.000092
ENSBTAG00000013125	*PLAUR*	Plasminogen activator, urokinase receptor	+3.52	0.001379	+5.30	0.000001	+5.96	0.000000	+13.03	0.000000	+3.81	0.000049
ENSBTAG00000016163	*OSM*	Oncostatin M	+2.44	0.029283	+4.03	0.000023	+3.83	0.000003	+6.79	0.000000	+2.61	0.004204
ENSBTAG00000019716	*CXCL8*	Chemokine (C-X-C motif) ligand 8	+3.09	0.001379	+6.56	0.000000	+5.93	0.000000	+14.19	0.000000	+4.87	0.000000
ENSBTAG00000021766	*HBEGF*	Heparin-binding EGF-like growth factor	+3.94	0.001379	+4.18	0.000308	+5.45	0.000001	+9.77	0.000000	+3.00	0.007067
ENSBTAG00000031707	*FRMD6*	FERM domain containing 6	+1.29	0.013896	+1.28	0.022194	+1.36	0.000059	+1.41	0.000001	+1.44	0.000001
ENSBTAG00000035224	—	Uncharacterized protein	+6.24	0.002116	+5.88	0.001857	+7.06	0.000071	+22.21	0.000000	+6.64	0.000304
ENSBTAG00000037608	—	Uncharacterized protein	+2.94	0.030981	+3.43	0.005467	+4.08	0.000066	+9.59	0.000000	+3.07	0.006033
ENSBTAG00000039037	*SERPINB4*	Serpin peptidase inhibitor, clade B	+3.50	0.017337	+4.90	0.000308	+5.58	0.000006	+12.68	0.000000	+4.24	0.000868

Linear mean fold-change values are shown for each gene at each post-infection time point vs. the −1 week pre-infection control time point.

			Animal infection time course - RNA-seq (versus -1 wk)					PBL study	PBL study
			+1 wk	+2 wk	+6 wk	+10 wk	+12 wk	RNA-seq	Microarray
ENSBTAG00000000306	*ITK*	IL2-inducible T-cell kinase	1.28	1.36	1.23	1.37	1.35	1.68	
ENSBTAG00000000507	*NR4A1*	Nuclear receptor subfamily 4, group A, member 1	2.51	3.52	4.49	11.04	2.51	4.04	1.56
ENSBTAG00000001060	*CXCR4*	Chemokine (C-X-C motif) receptor 4	1.99	2.05	2.13	3.09	2.30	3.23	2.48
ENSBTAG00000002758	*THBD*	Thrombomodulin	1.89	2.01	2.76	3.10	1.86	1.50	1.29
ENSBTAG00000003553	*ZFP36L2*	ZFP36 ring finger protein-like 2	1.52	1.58	1.69	1.87	1.52	4.06	
ENSBTAG00000003650	*NR4A2*	Nuclear receptor subfamily 4, group A, member 2	2.49	3.49	4.32	9.96	2.59	7.32	3.22
ENSBTAG00000004305	*RGS16*	Regulator of G-protein signaling 16	2.42	3.09	2.84	4.84	2.27	2.84	1.55
ENSBTAG00000006806	*KRT17*	Keratin 17	5.57	7.94	10.26	14.58	4.16	40.23	5.39
ENSBTAG00000008182	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	2.50	4.02	5.05	6.27	2.93		
ENSBTAG00000008353	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.71	2.24	2.59	3.73	2.03	3.01	2.14
ENSBTAG00000009354	*EVI2A*	Ecotropic viral integration site 2A	1.34	1.26	1.43	1.57	1.37		
ENSBTAG00000013125	*PLAUR*	Plasminogen activator, urokinase receptor	3.52	5.30	5.96	13.03	3.81	6.91	4.52
ENSBTAG00000016163	*OSM*	Oncostatin M	2.44	4.03	3.83	6.79	2.61	4.37	
ENSBTAG00000019716	*CXCL8*	chemokine (C-X-C motif) ligand 8	3.09	6.56	5.93	14.19	4.87	5.41	1.70
ENSBTAG00000021766	*HBEGF*	Heparin-binding EGF-like growth factor	3.94	4.18	5.45	9.77	3.00	10.02	
ENSBTAG00000031707	*FRMD6*	FERM domain containing 6	1.29	1.28	1.36	1.41	1.44	2.48	1.30
ENSBTAG00000035224	---	Uncharacterized protein	6.24	5.88	7.06	22.21	6.64		
ENSBTAG00000037608	---	Uncharacterized protein	2.94	3.43	4.08	9.59	3.07	7.83	
ENSBTAG00000039037	*SERPINB4*	Serpin peptidase inhibitor, clade B	3.50	4.90	5.58	12.68	4.24	10.13	

FIGURE 3 | Heat map showing linear fold-change values for the panel of 19 consistently DE genes across the *M. bovis* infection course. Linear fold-change values for the post-infection time points are shown relative to the −1 week pre-infection time point. Also shown are the equivalent results obtained using RNA-seq and Affymetrix® GeneChip® Bovine Genome Array technologies by McLoughlin et al. (31).

have provided evidence that a single-nucleotide polymorphism (rs4073) at the human *CXCL8* gene locus is associated with resistance/susceptibility to *M. tuberculosis* infection (106–109).

Functional Biology of Peripheral Blood Gene Expression Across the Infection Time Course

Of the 12,406 genes (50.40% of total *B. taurus* reference genes) that were suitable for differential expression analysis, 10,703 genes (86.27%) were mapped to molecules in the IPA Knowledge Base. IPA was used to identify overrepresented canonical pathways and construct biological interaction networks for sets of DE genes at each post-infection time point (+1, +2, +6, +10, and +12 weeks) compared to the pre-infection control time point (−1 week). Only DE genes that were significant after a multiple testing correction was applied (Benjamini–Hochberg method, FDR threshold $\leq$0.05) were used. The gene expression data for the panel of 19 genes (**Table 1**) were also analysed using IPA and to identify enriched canonical pathways and biological interaction networks. However, these analyses did not reveal any notable functionally relevant pathways or networks (results not shown).

+1 Week Post-infection Time Point

Forty-eight of the 57 DE genes detected between sample groups at +1 week post-infection and −1 week pre-infection were mapped to the IPA Knowledge Base (84.21%); however, no statistically significant canonical pathways were detected for this gene expression contrast. Four biological interaction networks were generated from this 57-DE-gene set using the IPA Knowledge Base. **Supplementary Figure 6** shows the highest-ranked network, which is associated with embryonic development, organismal development, and reproductive system development and function, and has the ubiquitin C protein encoded by the *UBC* gene as a central hub.

+2 Weeks Post-infection Time Point

Eighty-one of the 93 DE genes detected between sample groups at +2 weeks post-infection and −1 week pre-infection were mapped to the IPA Knowledge Base (87.10%). **Supplementary Table 8** (**Supplementary Material 3**) details the overrepresented IPA canonical pathways for this DE gene set. The top-ranked canonical pathway at +2 weeks post-infection was the *Glucocorticoid Receptor Signalling* pathway with eight genes displaying increased expression (*CDKN1A*, *CXCL8*, *DUSP1*, *FOS*, *IL10*, *PLAUR*, *PTGS2*, and *SGK1*) out of a total of 275 members of this pathway ($P = 1.05 \times 10^{-5}$). The main effects of glucocorticoid steroid hormones signalling through the cytosolic nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1) protein on the immune system are to upregulate expression of anti-inflammatory genes and downregulate expression of proinflammatory genes

(110, 111). Therefore, glucocorticoid receptor signalling activity evident in the peripheral blood transcriptome during the early stages of *M. bovis* infection may reflect perturbation of homeostasis (112) and possible modulation of host cellular mechanisms at the site of infection in the lungs.

Nine biological interaction networks were generated from this 81-DE-gene set using the IPA Knowledge Base. **Figure 4** shows the highest-ranked network, which was centred on increased expression of the *IL10*, *CXCL2*, *CXCR4*, and *CXCR2* genes with *Cellular Movement*, *Haematological System Development and Function*, and *Immune Cell Trafficking* as the top IPA disease and function categories. In this regard, IL-10, an inhibitory and anti-inflammatory pleiotropic cytokine with a major role in suppression of macrophage and dendritic cell functions, has been hypothesised as a target for modulation and manipulation by mycobacterial pathogens (113, 114). Also, IL-10 is linked to chronic mycobacterial infection in the mouse model (115–117). It has been shown that mycobacterial RNA induces IL-10 production in infected cells through TLR3-mediated activation of the PI3K/AKT signalling pathway (118) and that *M. tuberculosis* infection of THP-1 cells induces *IL10* expression through perturbation of the histone deacetylases HDAC6 and HDAC11 (119). In addition, it has been observed that increased levels of IL-10 cytokine in TB patients lead to impaired T-cell function, thereby contributing to an inefficient host immune response (120).

+6 Weeks Post-infection Time Point

Six hundred twenty-six of the 687 DE genes detected between sample groups at +6 weeks post-infection and −1 week pre-infection were mapped to the IPA Knowledge Base (91.12%). **Supplementary Table 9** (**Supplementary Material 3**) details the overrepresented IPA canonical pathways for this DE gene set. Twenty-five biological interaction networks were generated from this 626-DE-gene set using the IPA Knowledge Base, and **Supplementary Figure 7** shows the highest-ranked network, which contains mostly down-regulated focus molecules associated with DNA replication, recombination and repair, and control of gene expression and the cell cycle.

+10 Weeks Post-infection Time Point

For the +10 weeks post-infection vs. −1 week pre-infection contrast, 2,247 of the 2,583 DE genes were mapped to the IPA Knowledge Base (86.99%). **Supplementary Table 10** (**Supplementary Material 3**) details the overrepresented IPA canonical pathways for this DE gene set. The top-ranked canonical pathway was the *Protein Ubiquitination Pathway*, with 40 of 64 entities present in the pathway containing 255 members ($P = 1.36 \times 10^{-11}$) exhibiting decreased expression relative to the −1 week pre-infection group. In this regard, it is noteworthy that *M. tuberculosis* has recently been demonstrated to suppress innate immunity by exploiting the host ubiquitination system (121–124).

Twenty-five biological interaction networks were generated from this 2,247-DE-gene set using the IPA Knowledge Base. **Figure 5** shows the highest-ranked network, which was centred on the amyloid β (A4) precursor protein encoded by the *APP* gene (previously known as *ABPP*) as a central hub, and the top IPA disease and function categories represented were *Antigen Presentation*, *Carbohydrate Metabolism*, and *Cardiovascular Disease*. Currently, there are few published research works demonstrating differential expression of the *APP* gene during TB in vertebrates (125, 126); however, the presence of this network at +10 weeks post-infection suggests that the represented genes and gene products may have roles in BTB development and host–pathogen interactions. In addition, using RNA-seq, the *APP* gene was also significantly increased in expression in PBL from *M. bovis*–infected cattle compared to non-infected controls (31).

+12 Weeks Post-infection Time Point

There was a marked decrease in the number of DE genes at +12 weeks compared to +10 weeks post-infection, which may reflect control of the infection by the immune system at this stage of the time course. Two hundred ninety-three of the 338 DE genes detected between sample groups at +12 weeks post-infection and −1 week pre-infection were mapped to the IPA Knowledge Base (86.69%). **Supplementary Table 11** (**Supplementary Material 3**) details the overrepresented IPA canonical pathways for this DE gene set. The top-ranked canonical pathway was *T Cell Receptor Signalling* with 12 molecules detected from a total of 97 pathway members ($P = 8.96 \times 10^{-9}$). These 12 genes (*CAMK4*, *CD247*, *CD3D*, *CD3E*, *CD3G*, *CD8A*, *CD8B*, *FOS*, *ITK*, *LCK*, *PRKCQ*, and *ZAP70*) all displayed increased expression relative to −1 week pre-infection, pointing towards the presence of mycobacterial antigen presentation and T-cell activation via T-cell receptor (TCR) signal transduction (12, 127, 128).

Nineteen biological interaction networks were generated from this 293-DE-gene set using the IPA Knowledge Base, and **Figure 6** shows the highest-ranked network, which was centred on increased expression of the CXCR4, PTGS2, and KLF4 proteins, and the top IPA disease and function categories represented were *Cellular Development*, *Haematological System Development and Function*, and *Cell-mediated Immune Response*. As described above, the *CXCR4* gene is known to be upregulated in blood monocytes and bronchoalveolar lavage cells from human patients with pulmonary TB (74). In addition, the *PTGS2* gene (previously known as *COX-2*) encodes prostaglandin-endoperoxide synthase 2, a key enzyme in prostaglandin biosynthesis, which is known to be triggered in macrophages—via a TLR2-dependent mechanism—by ESAT-6 proteins secreted by virulent *M. tuberculosis* and *M. bovis* (129). In this regard, it has been hypothesised that induction of PTGS2 may facilitate intracellular mycobacterial survival through inhibition of p53-dependent apoptosis (130). Conversely, it has also been shown that PTGS2 enhances bactericidal activity in *M. tuberculosis*–infected macrophages through promotion of autophagy (131).

The *KLF4* gene encodes a zinc finger–containing transcription factor that regulates macrophage polarisation, displaying increased expression in M2 macrophages and strongly decreased expression in M1 macrophages (132). Integrative network analyses of transcriptome, protein–protein interaction, and transcription factor–binding site data have shown that KLF4 is an important regulator of lung cell gene expression during the early events of *M. tuberculosis* infection in mice (133). It has

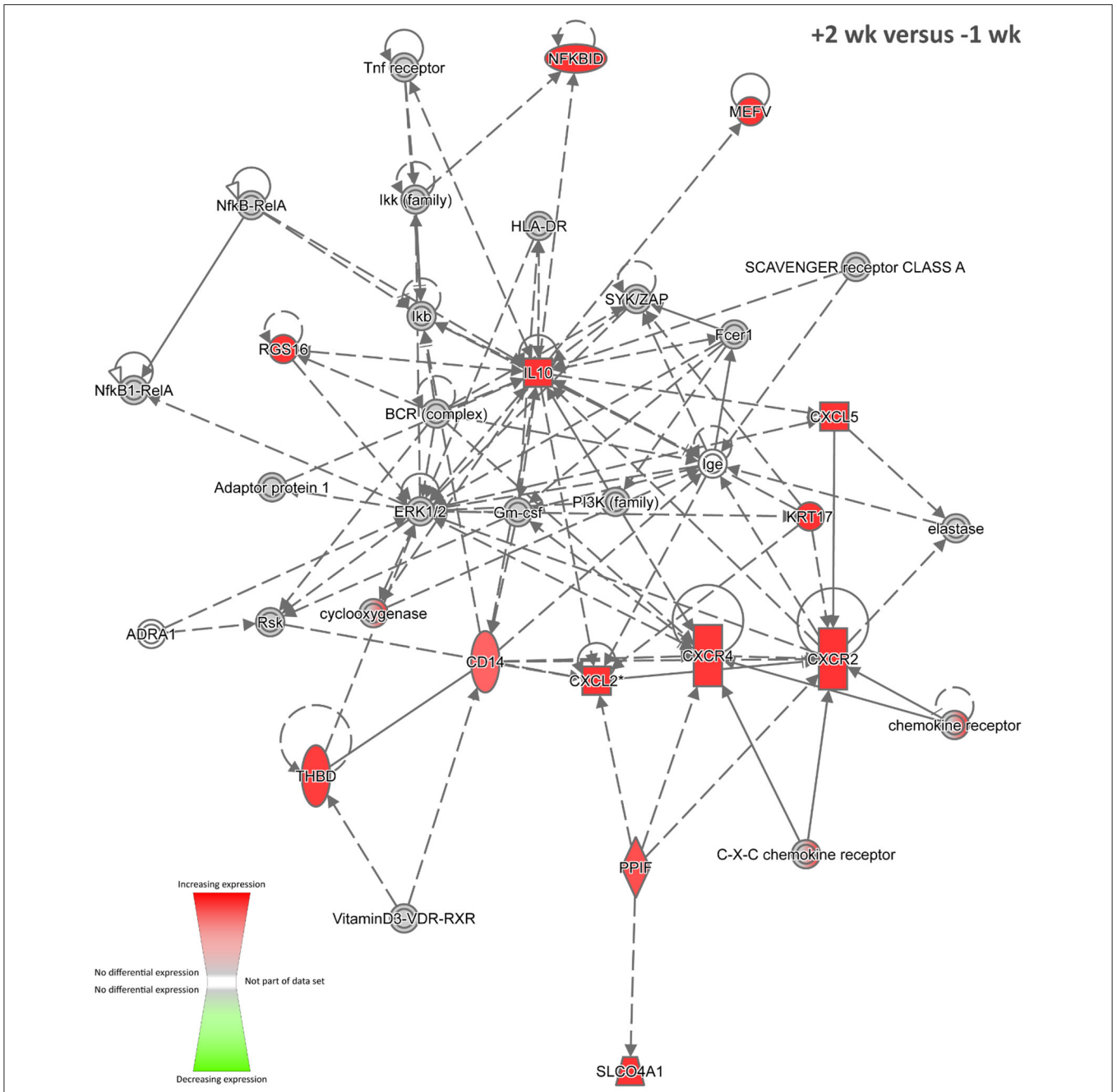

FIGURE 4 | The top-ranked biological interaction network generated using IPA for +2 weeks post-infection. Differential gene expression is represented with a red–green colour scale. This network consisted of 13 focus molecules (IPA Network Score = 24), and the top IPA Disease and Function categories represented were *Cellular Movement*, *Haematological System Development and Function*, and *Immune Cell Trafficking*. A detailed legend for IPA biological interaction networks including a key for node shapes and edge classifications is available at the following link: https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend.

also been shown that nitric oxide (NO) and KLF4 epigenetically modify class II transactivator protein causing repression of major histocompatibility complex (MHC) class II expression during *M. bovis* BCG infection of murine macrophages (134). Furthermore, downregulation of microRNA-26a during *M. tuberculosis* infection of murine macrophages upregulates KLF4, in turn promoting increased arginase and decreased activity of inducible NO synthase, as well as preventing trafficking of *M. tuberculosis* to lysosomes (135). Taken together, these results support the hypothesis that increased expression of *KLF4* facilitates mycobacterial evasion of host immune surveillance.

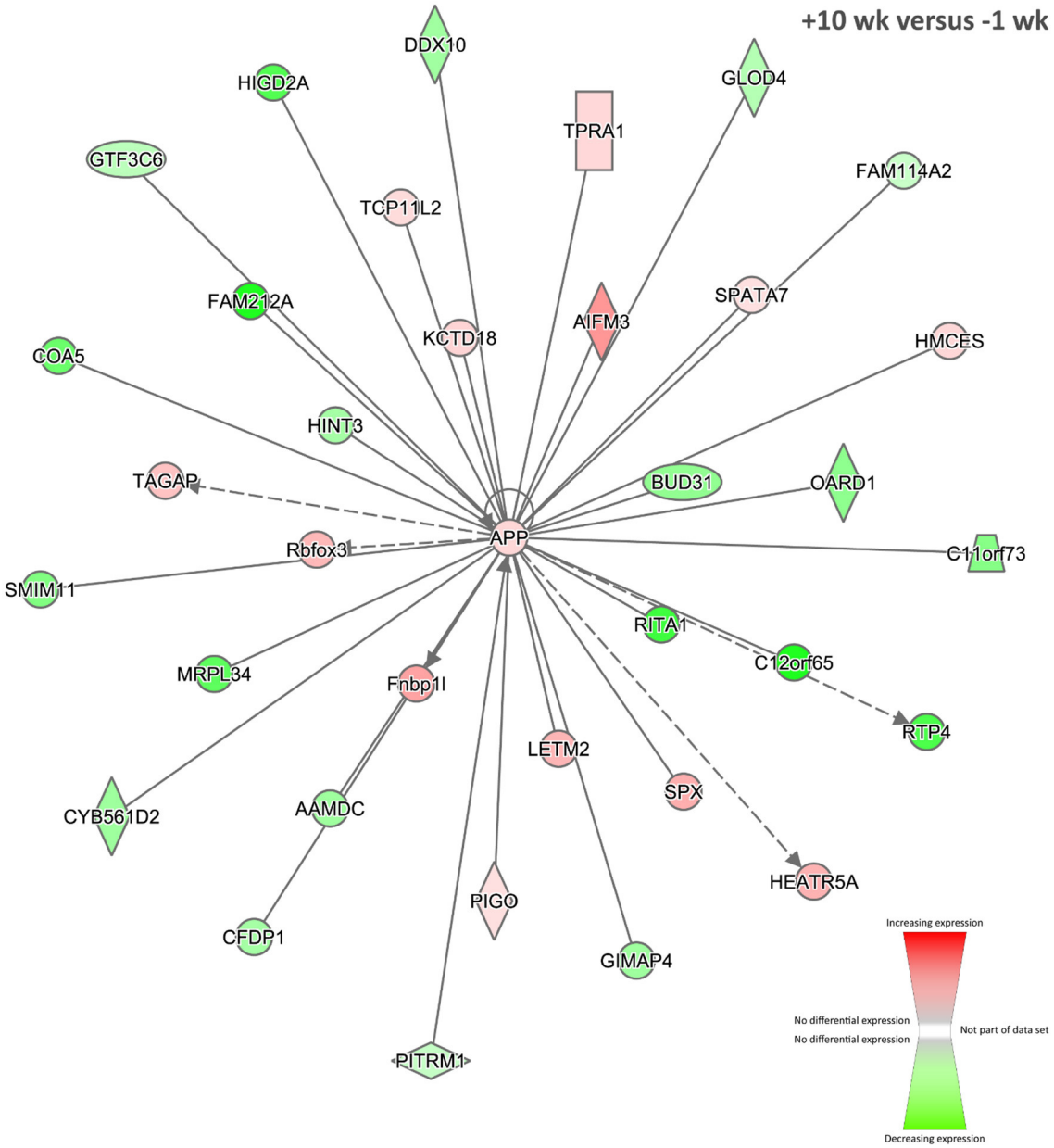

FIGURE 5 | The top-ranked biological interaction network generated using IPA for +10 weeks post-infection. Differential gene expression is represented with a red–green colour scale. This network consisted of 35 focus molecules (IPA Network Score = 39), and the top IPA Disease and Function categories represented were *Antigen Presentation*, *Carbohydrate Metabolism*, and *Cardiovascular Disease*. A detailed legend for IPA biological interaction networks including a key for node shapes and edge classifications is available at the following link: https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend.

Time-Series Analysis

The STEM tool was designed specifically for analyses of short time-series data sets (3–8 time points) (59, 60) similar to the RNA-gene expression data set obtained from the *M. bovis* animal infection time-course experiment described here. Time-series analysis can be a powerful technique for uncovering networks of coregulated genes in longitudinal time-course experiments (136–138), particularly for gene expression data associated with host immunobiological responses to infection (139–142).

For the present study, STEM time-series analyses of differential gene expression across the *M. bovis* infection time course demonstrated that large groups of genes exhibited comparable patterns of gene expression across the five post-infection time points (+1, +2, +6, +10, and +12 weeks post-infection) relative to the −1 week pre-infection time point. Two different STEM analyses were performed based on (a) expression data for all detectable expressed genes across the infection time course, which corresponded to 4,103 genes (*STEM analysis 1*)

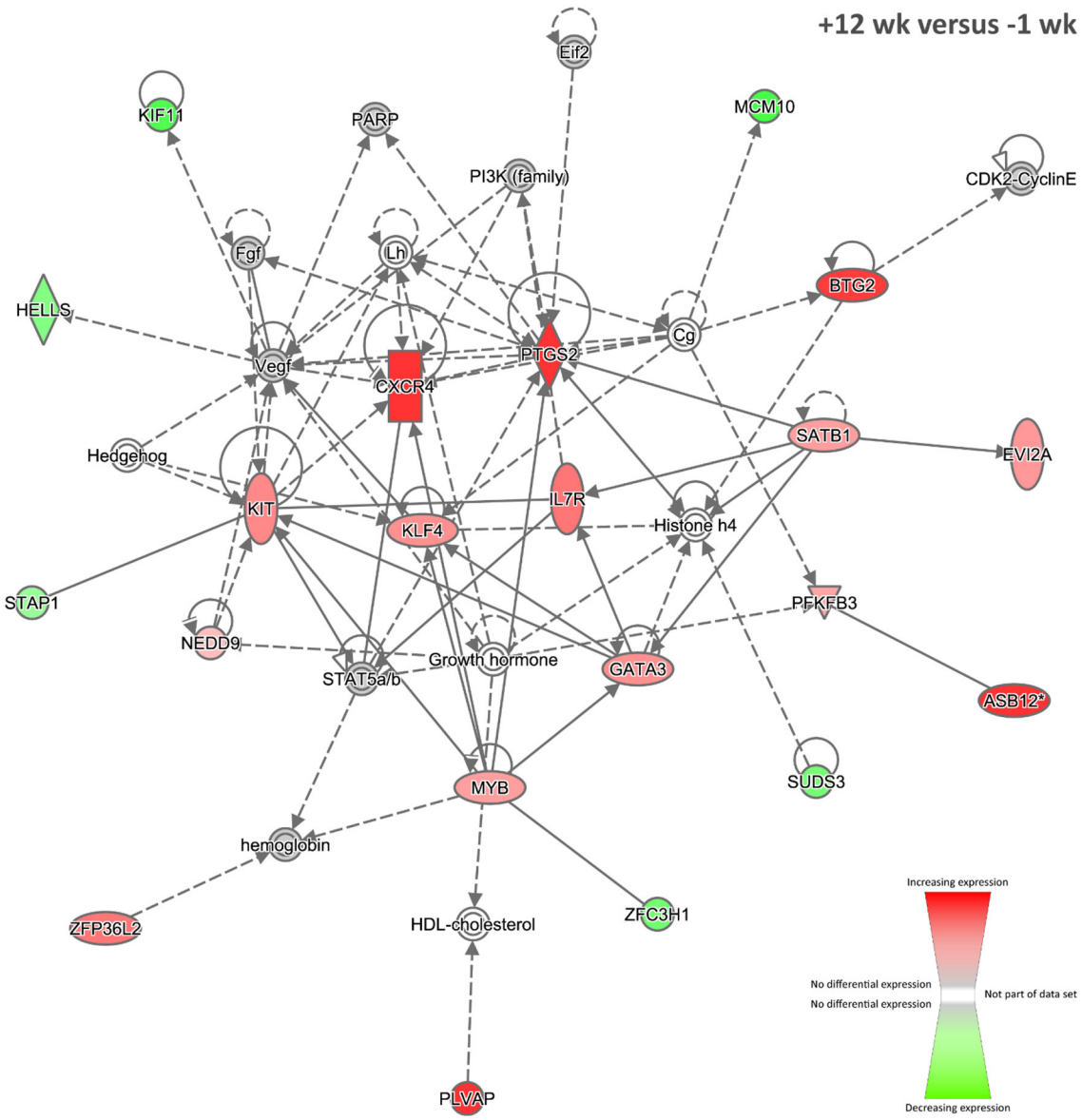

FIGURE 6 | The top-ranked biological interaction network generated using IPA for +12 weeks post-infection. Differential gene expression is represented with a red–green colour scale. This network consisted of 21 focus molecules (IPA Network Score = 33), and the top IPA Disease and Function categories represented were *Cellular Development*, *Haematological System Development and Function*, and *Cell-mediated Immune Response*. A detailed legend for IPA biological interaction networks including a key for node shapes and edge classifications is available at the following link: https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend.

and (b) the union set of DE genes from all post-infection time points vs. −1 week pre-infection, which corresponded to 2,935 genes (*STEM analysis 2*). **Supplementary Figure 8** shows the top 50 time-series model profiles obtained for *STEM analysis 1* and *STEM analysis 2*.

The top-ranked time-series profiles (by *P*-value) obtained using the two different STEM analyses were very similar. For example, as shown in **Supplementary Table 12** (**Supplementary Material 4**), the first-ranked STEM model profile for *STEM analysis 1* (profile 40; **Figure 7**) was enriched for the *signal transduction*, *single organism signalling*, *cell communication*, *regulation of multicellular organismal process*, and *cellular response to stimulus* GO terms. This profile was also similar to the second-ranked model profile for *STEM analysis 2* (profile 40; **Supplementary Figure 9**), which was enriched for many of the same GO terms (*signal transduction*, *single organism signalling*, *cell communication*; see **Supplementary Table 13**, **Supplementary Material 4**). The third-ranked model profile for *STEM analysis 1* (profile 23; **Supplementary Figure 9**) was highly similar to the first-ranked model profile for *STEM analysis*

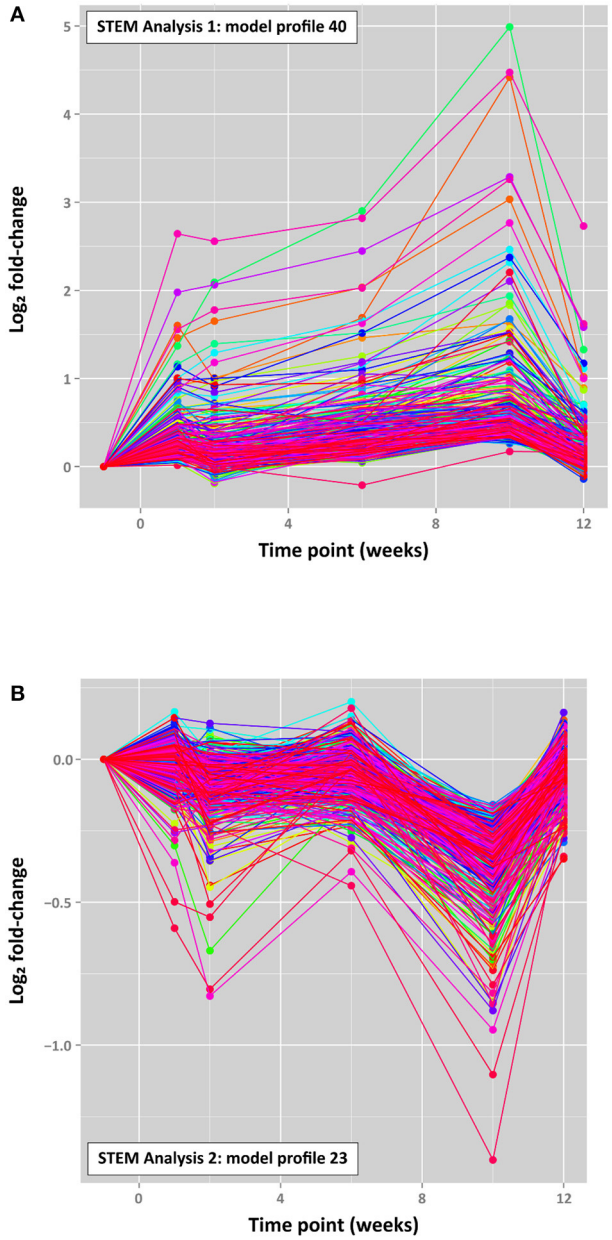

FIGURE 7 | Top-ranked STEM time series profiles. **(A)** The first-ranked model profile (profile 40) for *STEM analysis 1* (4,103 filtered genes) (303 genes assigned; 80.7 genes expected; FDR-adjusted $P = 3.2 \times 10^{-99}$) and **(B)** the first-ranked model profile (profile 23) for *STEM analysis 2* (2,935 filtered genes) (516 genes assigned; 108.9 genes expected; FDR-adjusted $P = 1.6 \times 10^{-187}$). The coloured lines in each model profile represent individual genes.

2 (profile 23; **Figure 7**) with exactly the same overrepresented GO terms (*mitochondrial inner membrane*, *organelle inner membrane*, *mitochondrial part*, *mitochondrial membrane*, *mitochondrial envelope*; **Supplementary Tables 14, 15**, **Supplementary Material 4**).

It is interesting to note that STEM profile 40 in each analysis is characterised by a cluster of ∼300 genes associated with cell signalling and cellular response to stimuli, which exhibited increasing expression across the four time points with a peak at +10 weeks, followed by a substantial decrease at +12 weeks (**Figure 7**). Conversely, STEM profile 23 (516 genes for *STEM analysis 2*) is characterised by genes associated with mitochondrial components, particularly the mitochondrial membrane, which displayed an oscillating pattern of expression with a marked decrease at +1 week 10 post-infection (**Figure 7**).

These time-dependent patterns of gene expression in peripheral blood may reflect pathogenesis of early BTB disease during the infection time course with concomitant host cellular responses to *M. bovis* infection, disruption of homeostasis, and changing cellular, tissue, and organismal energy requirements (143–145). In addition, it is important to note that although these longitudinal patterns of gene expression may be due to coregulation of genes in the same cluster, they are likely to also reflect fluctuations in peripheral blood cell type populations comparable to those previously observed for comparisons of *M. bovis*–infected and control non-infected cattle (28, 104).

CONCLUSIONS

The results presented here provide good support for the hypothesis that the peripheral blood transcriptome constitutes a source of gene expression biomarkers for BTB caused by *M. bovis* infection in cattle. This is particularly apparent for the panel of 19 genes exhibiting consistently, statistically significantly increased expression across the infection time course, the majority of which (16 genes) were also significantly increased in PBL harvested from an independent cohort of field-infected cattle. However, the sensitivity and specificity of putative transcriptional biosignatures of *M. bovis* infection will need to be verified and validated using larger panels of cattle naturally infected with *M. bovis* and also populations of animals infected with a range of viral and bacterial pathogens.

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 at: https://www.ebi.ac.uk/ena, PRJEB27764 and PRJEB44470.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal & Plant Health Agency (APHA), Animal Use Ethics Committee (UK Home Office PCD number 70/6905).

AUTHOR CONTRIBUTIONS

DMac, SG, EG, KM, AW, BV-R, and HV conceived and designed the project and organised bovine sample collection. KM, NN, DMag, and JB performed RNA extraction and RNA-seq library generation. KM, NN, KR-A, CC, and DMac performed the

analyses. KM, CC, and DMac wrote the manuscript. All authors reviewed 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.2021.662002/full#supplementary-material

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Evaluation of Three Commercial Interferon-γ Assays in a Bovine Tuberculosis Free Population

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The interferon-γ assay has been used worldwide as an ancillary test for the diagnosis of bovine tuberculosis (bTB). This study aimed to describe, based on the bTB-free status in Switzerland, the difference of applying a more stringent cutoff point of 0.05 compared with 0.1 for bTB surveillance. Moreover, the effect of time between blood collection and stimulation, culture results, optical density values, and the influence of testing different breeds were evaluated. Blood samples from a total of 118 healthy cows older than 6 months were tested with three commercial interferon-gamma assays. To confirm the bTB-free status of the tested animals and to investigate potential cross-reactions with nontuberculous mycobacteria, pulmonary and abdominal lymph nodes in addition to ileal mucosa from each cattle were used for the detection of viable *Mycobacteria* spp. by specific culture. Significant differences regarding the proportion of false-positive results between the two Bovigam tests and between Bovigam 2G and ID Screen were found. Samples analyzed with Bovigam 2G were 2.5 [95% confidence interval (CI) 1.6–3.9] times more likely to yield a false-positive test result than samples analyzed with Bovigam TB. Similarly, the odds ratio (OR) for testing samples false-positive with ID Screen compared with Bovigam TB was 1.9 (95% CI 1.21–2.9). The OR for testing false-positive with ID Screen compared with Bovigam 2G was less to equally likely with an OR of 0.75 (95% CI 0.5–1.1). When using a cutoff of 0.05 instead of 0.1, the OR for a false-positive test result was 2.2 (95% CI 1.6–3.1). Samples tested after 6 h compared with a delayed stimulation time of 22–24 h were more likely to yield a false-positive test result with an OR of 3.9 (95% CI 2.7–5.6). In conclusion, applying a more stringent cutoff of 0.05 with the Bovigam 2G kit generates a questionable high number of false-positive results of one of three tested animals. Furthermore, specific breeds might show an increased risk to result false-positive in the Bovigam 2G and the ID Screen assays.

Keywords: cattle, bovine tuberculosis, diagnosis, interferon-gamma assay, *Mycobacterium bovis*, *Mycobacterium avium* subsp *paratuberculosis*, *Mycobacterium avium* subsp *hominissuis*, *Mycobacterium persicum*

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic zoonotic disease caused by *Mycobacterium bovis* and *Mycobacterium caprae* (1). Cattle (*Bos Taurus*) are considered to be the main reservoir of bTB, and eradication programs worldwide focus primarily on this domestic species (2). Although such programs were able to significantly reduce the prevalence of the disease and some industrialized countries are considered to have official bTB-free status, specific geographical areas are still faced with this burden. Despite remarkable public and private efforts, the causes of this failure are multiple, including the varying clinical signs, intrinsic features of these bacteria such as the broad host spectrum and environmental resilience, the transmission modalities, and the absence of accurate *antemortem* diagnostic test applicable in the field (3–5).

The standard method for *antemortem* bTB detection and international trade of cattle is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPDB) in the cervical area or the caudal fold of the tail (6). Skinfold thickness at the injection site is measured before and 72 h after PPDB is injected to calculate any increase. In addition, cutaneous reactions such as induration and swelling are evaluated. A more specific variant of the single intradermal test [single intradermal cervical comparative test (SICCT)] involves the additional injection of avian tuberculin (PPDA) into different sites, theoretically enabling distinction between animals infected with bTB and those responding to PPDA as a result of exposure to mycobacteria other than *Mycobacterium tuberculosis* complex (MTBC) (6). There are numerous known weaknesses associated with tuberculin skin testing, e.g., variations in specificity (Sp) and sensitivity (Se) related to different PPD products or even from batch-to-batch, the subjective injection and measurement ability of the test performer, and the necessity of restraining the animals twice (7, 8).

To overcome some of the mentioned drawbacks, a whole-blood cellular assay using a sandwich enzyme immunoassay (EIA) for the bovine cytokine interferon-gamma (IFN-γ) has been developed and used as a standalone or ancillary test to the intradermal tuberculin test for diagnostic purposes (9, 10). There are numerous advantages of the IFN-γ assay over the skin tests, including that the animals are captured only once, inconclusive tests can be readily repeated, quantification of the lymphocytes reaction to various stimulants is based on optical densities (ODs), and stimulation controls are included. Moreover, the IFN-γ assay may detect bTB-infected animals up to 60–120 days earlier than the single cervical tuberculin test (11–13).

The first IFN-γ assay (Bovigam TB, Thermo Fisher Scientific, Reinach, Switzerland) is now Office International des Epizooties-certified as an ancillary assay to the tuberculin test and may be authorized to maximize detection of infected cattle (Council Directive 64/432/ECC), including bTB-freedom certification for animals or products movement purposes and prevalence estimation (14). Switzerland was faced with bTB with two distinct outbreaks in 2013–2014 (15). At the time of writing, these events represent the last detection of *M. bovis* and *M. caprae* in domestic or wild animals in Switzerland. Although the first event was caused by the reemergence of an undetected *M. bovis* strain that circulated in the Swiss cattle population over at least 15 years, the second outbreak originated from cattle infected during summer pasturing in an Austrian endemic area (16, 17). Consequent test and cull measures based primarily on SICCT and comprehensive epidemiological contact-tracing investigation enabled the preservation of the official bTB-free status. Under these circumstances, the IFN-γ assay was used in the late stage of the epidemiological investigations as an ancillary test for non-negative SICCT animals.

In addition to the ongoing slaughterhouse surveillance through *postmortem* meat inspection, a nationwide monitoring program (LyMON) for early detection of bTB was started in 2013 (18). Meat inspectors were encouraged to submit altered bovine lymph nodes to the bTB reference laboratory for macroscopic inspection and culture for mycobacteria. Over an 8-year period, lymph nodes originated from 793 cows were sliced into thin sections (1–2 mm) and investigated for the presence of lesions compatible with bTB. Suspicious samples ($n = 121$) were homogenized and tested for the presence of MTBC DNA by real-time polymerase chain reaction (PCR) and cultured in mycobacterial selective media (15). Moreover, in 2014, a red deer monitoring program involving two bordering Swiss Cantons and the Principality of Liechtenstein was implemented (19). Over a 7-year period, retropharyngeal and mediastinal lymph nodes of 1,382 randomly and risk-based selected red deer, such as emaciated individuals or animals killed by motor vehicles, and 33 other wild animals (roe deer, fallow deer, fox, chamois, and Alpine ibex) were investigated macroscopically by trained personnel. Of these, a total of 412 wild animals were additionally tested for the presence of MTBC DNA by real-time PCR and cultured in mycobacterial selective media (15). No further bTB cases were detected after the two mentioned outbreaks in cattle, and although *M. caprae* is still endemic in the Austrian border region, none of the investigated red deer and other wild animals resulted positive.

Although the described surveillance measures testified that the Swiss population is currently free of bTB, punctual reintroductions through illegal imports or wildlife movements are possible. Therefore, in accordance with the European Food Safety Authority's scientific opinion, EIA cutoff thresholds should be evaluated in each country based on the local epidemiological conditions to ameliorate the accuracy of the IFN-γ assay (20). Hence, these modifications of the laboratory evaluation criteria may affect the Sp and Se of the assay. An estimated median Se of 87.6% (73–100%) and Sp of 96.6% (85–99.6%) were reported for the Bovigam TB kit based on 15 field studies conducted over a 15-year period (7). However, the median Sp value (96.6%) published by de la Rua-Domenech and colleagues (7) differs from the Sp values (6.9–74%) observed in further studies (21–23). These discrepancies may be the result of different testing conditions, laboratory procedures, and evaluation criteria and, most of all, may depend on the TB prevalence and the presence of nontuberculous mycobacteria (NTM), leading to cross-reactivity in the tested populations (24). Moreover, the IFN-γ production of specific breeds such as French bullfight cattle has been shown to be significantly lower than classical diary animals (4, 24).

Recently, a new IFN-γ assay has become commercially available (ID Screen Bovine Tuberculosis IFN-γ, IDvet, Grabels, France), and it has already been extensively used in different countries including Switzerland. However, independent evaluations on the test accuracy under field conditions are either not publicly available or scarce, showing low Se (36.7%) when applying the cutoff recommended by the manufacturer or slightly better values (49.0–56.0%) using more stringent cutoff thresholds (25).

In their scientific opinion, the expert panel convened on the use of the IFN-γ test for the diagnosis of bTB highlighted the necessity to harmonize the assay protocol (20). In this regard, various critical points need to be evaluated, such as the time between blood collection and stimulation, antigens used and their concentrations, interpretation criteria including cutoff values, and finally, the inclusion of stimulation control and EIA control. Nevertheless, specific epidemiological conditions, such as exposure to environmental mycobacteria in certain geographical areas, possibly negatively influence the test accuracy (26–28).

Besides the members of the MTBC, over 190 species of NTM have been described (www.bacterio.net/mycobacterium.html). Several NTMs are commonly encountered in the environment and have been isolated from a variety of sources, such as water, feed, soil, dust, aerosol, protozoa, and animals, including cattle (29, 30). Of these, more than 60 species are known to be opportunistic pathogenic to humans and other mammals, and human infections with these emerging pathogens are now more common than tuberculosis in industrialized countries (31–33).

To avoid cross-reactive immune responses in cattle exposed to NTM, PPDA is injected in the SICCT or included in first-generation IFN-γ assays. However, in geographical areas where the dominant NTM shares epitopes with MTBC members, more specific diagnostic markers have been identified (34–36) and implemented in second-generation IFN-γ assays and subsequent versions (37, 38). Among these, antigen cocktails containing the 6-kDa early secretory antigenic target- and 10-kDa culture filtrate protein-derived peptides have gained great importance for their presumptive MTBC specificity (39). Despite earlier evidence to the contrary, orthologs of 6-kDa early secretory antigenic target and 10-kDa culture filtrate protein have now been shown to be present in frequently isolated NTM, such *Mycobacterium smegmatis*, *Mycobacterium kansasii*, *Mycobacterium persicum*, *Mycobacterium marinum*, *Mycobacterium szulgai*, *Mycobacterium gastri*, and *Mycobacterium flavescens* (34, 40–43). The specificity of the available commercial IFN-γ assays is therefore questionable in geographical areas where cattle may be exposed to NTM, influencing the test accuracy. An increased number of false-positive (FP) tested animals can result in reduced stakeholders' acceptance of control measurements, undermining the credibility of the involved authorities.

This study aimed to describe, based on the epidemiological situation (bTB-free status) in Switzerland, the difference of applying a cutoff point of 0.05 compared with 0.1 and considering the ethical implications, i.e., if a more stringent cutoff is ethically acceptable for bTB surveillance and import/export of livestock. Additionally, the effect of time between blood collection and stimulation, culture results, and OD values were evaluated.

MATERIALS AND METHODS

Selection Criteria and Collection of Blood Samples

From a total of 118 randomly selected healthy cows older than 6 months originating from 101 different premises and 14 Swiss Cantons, two blood samples were collected *antemortem*. No more than two cows originating from the same premise were included in the study. Three different breeds covered ∼90% of the animals, with Holstein/Red Holstein ($n = 36$) being the most represented group, followed by Swiss Fleckvieh/Simmental ($n = 35$) and Swiss Brown ($n = 32$). The remaining animals were Montbéliard ($n = 8$) Normande ($n = 2$), one Charolais, one Limousin, and three mixed breeds. Of these, 90% were regularly moved to pastures in mountain regions during the summer months. Before sampling, animals were stabled in the proximity of the abattoir during the morning of the day before being killed, and heparinized blood was collected by trained personnel from the caudal vein $\geq$3 h after delivering. Each sample was immediately transported unchilled to the laboratory.

Culture and Identification of Mycobacteria

To confirm the bTB-free status of the tested 118 animals and to detect potential cross-reactions with NTM species, a pool of pulmonary lymph nodes (left bronchial and caudal mediastinal) and a pool of intestinal tissues (ileal mucosa and jejunal/cecal lymph nodes) from each cattle were collected immediately after killing. The pulmonary lymph nodes were cultured at 37°C for 8 weeks as described elsewhere (30). The pool of intestinal tissues was cultured at 37°C for 16 weeks on Herrold's Egg Yolk Agar with mycobactin J and ANV (BD, Basel, Switzerland), on BBL Stonebrink agar slants (BD) and on liquid MGIT supplemented with PANTA (BD) and mycobactin J (IDvet) as culture media.

All tubes showing growth of presumptive mycobacterial colonies were investigated for acid-fast bacilli after Ziehl–Neelsen staining. The colonies were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry, and presumptive positive *Mycobacterium avium* subsp. *paratuberculosis* (MAH) colonies were confirmed by the ID Gene Paratuberculosis Duplex PCR (IDvet). For three isolates, sequencing of two housekeeping genes (*hsp65* and 16S rRNA) was performed (30). DNA sequencing was performed at Microsynth (Balgach, Switzerland). The resulting sequences were assembled using CLC Genomics Workbench 7.5.1 (Qiagen), and BLAST similarity searching for multiple sequence alignment was performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Interferon-γ Assays

The commercial IFN-γ kits Bovigam TB (Thermo Fisher Scientific), Bovigam 2G (Thermo Fisher Scientific), and ID Screen Bovine Tuberculosis IFN-γ (ID Screen) were tested for each blood sample according to the manufacturers' instructions. The blood samples were divided into four aliquots and incubated with PPDB, PPDA, both in addition to pokeweed mitogen

(PWM) (Prionics, Lelystad, The Netherlands for Thermo Fisher Scientific; CZ Veterinaria, Porriño, Spain for IDvet) and phosphate-buffered saline (NIL) as positive and negative stimulation controls, respectively. Two different stimulations times for each blood sample were performed, and these occurred within 6 and 22–24 h after collection, respectively. Released IFN-γ was measured using the enzyme-linked immunosorbent assay kit provided by the respective manufacturer.

Interpretation of the three kits was performed using the following criteria, with the mentioned ODs representing mean values:

- Criterion 1: positive test outcome = $OD_{PPDB} - OD_{NIL} \geq 0.05$ and $OD_{PPDB} - OD_{PPDA} \geq 0.05$
- Criterion 2: positive test outcome = $OD_{PPDB} - OD_{NIL} \geq 0.1$ and $OD_{PPDB} - OD_{PPDA} \geq 0.1$

The results of the ID Screen kit were additionally evaluated using the S/P ratio, as recommended by the manufacturer.

- Positive test outcome: $\frac{S}{P} = \left(\frac{OD_{PPDB} - OD_{PPDA}}{OD_{ELISA\ positive\ control} - OD_{ELISA\ negative\ control}}\right) * 100 \geq 35\%$.

To assess the viability of T cells, stimulation with PWM was included for each test, and OD values of samples that not fulfilled the following criteria were interpreted as invalid. For the ID Screen kit, the following interpretation was adopted: $\frac{OD_{PWM}}{OD_{ELISA\ positive\ control} - OD_{ELISA\ negative\ control}} \geq 0.5$, whereas for both Bovigam variants, mean values of $OD_{PWM} - OD_{NIL}$ were supposed to be ≥0.5.

Moreover, animals responding to unspecific stimulation where excluded if their mean OD_{NIL} was ≥0.3.

Statistical Methods

With the aim to assess if the proportion of FP test results (i) differed between the three IFN-γ assays, (ii) was affected by the time elapsed between sampling and stimulation (6 and 22–24 h), (iii) and accounting for two different cutoffs (0.05 and 0.1), a model was fit with generalized estimating equations with the function geeglm from the package geepack in R (44). To account for potential within-animal clustering, an exchangeable correlation was chosen in the marginal model. Adjustment for multiple comparisons between the three IFN-γ assays was performed with Tukey's approach from the multcomp package (45). The resulting effect sizes are presented in the form of odds ratios (ORs) with their corresponding 95% confidence intervals (CIs) and *p*-values, adjusted for multiple comparisons.

Based on culture outcome, three different groups were defined as follows: MAP ($n = 6$), MAH ($n = 14$), and culture-negative animals ($n = 94$). Responses to PPDA (OD_{PPDA}) of the three groups were investigated for the different kits and stimulation within 6 h using a linear mixed-effects model with animals as a random effect (nlme-package) (46). The differences of the mean ODs PPDA-NIL of the three groups were compared for each kit. In addition, the effect of time between blood collection and stimulation (6 and 22–24 h) on the mean OD_{PWM} values was assessed for the three kits using the same model as for PPDA. Fisher's exact test using GraphPad Prism 9.1.0 (GraphPad Software) was performed to evaluate a possible association between positive test outcome and animal breed for the three major breed groups after 6 h stimulation.

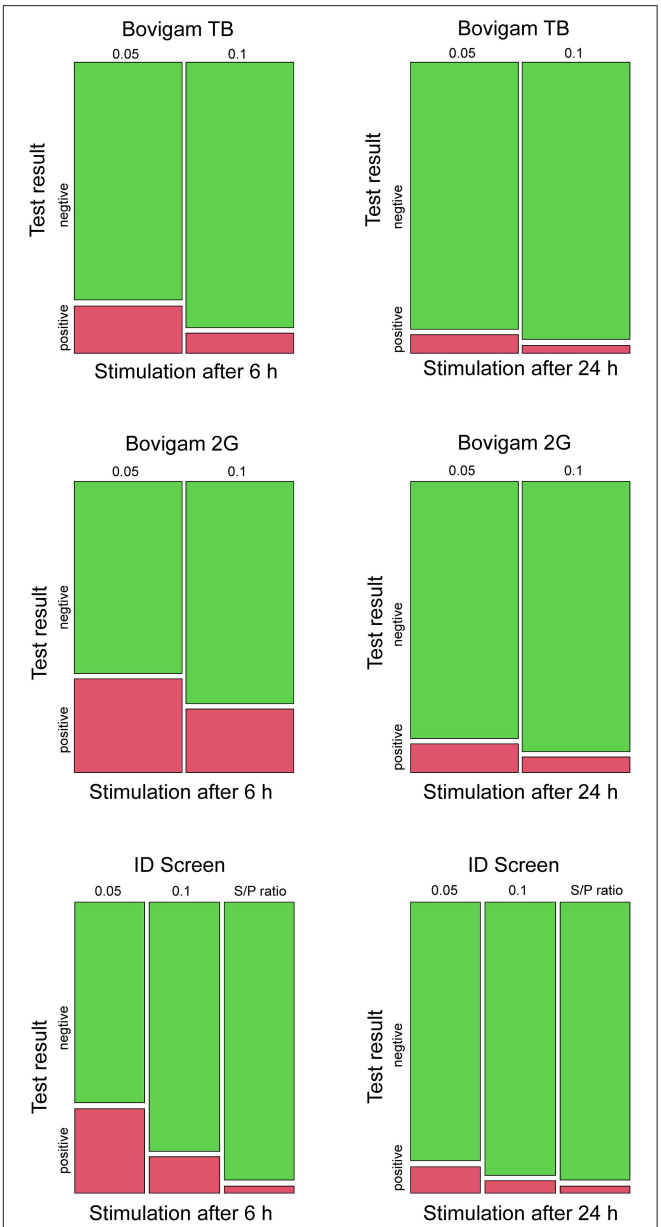

FIGURE 1 | Mosaic plots for the test outcomes using three different kits (Bovigam TB, Bovigam 2G, ID Screen), stimulation times (6 and 24 h), and cutoffs (0.05, 0.1, S/P ratio). Results are displayed in green and red for negative and positive responses, respectively.

RESULTS

Culture and Identification of Mycobacteria

All the tissue samples were negative for MTBC, whereas NTM was found in 17 of the 118 cultured pulmonary lymph node pools with MAH as the predominant species ($n = 14$). In the

remaining three pools, *M. persicum*, *Mycobacterium lentiflavum*, and a member of the *Mycobacterium chimaera/intracellulare* group were isolated. In six of the 118 intestine tissue pools, MAP was cultured, and one animal was positive for *Mycobacterium engbaekii.*

Interferon-γ Assays

The test outcomes for each kit, stimulation time, and cutoffs are resumed in **Figure 1**. The animal positive for *M. persicum* showed a clear positive result with all three kits, stimulation times, and cutoffs. Cattle tested positive for MAP were negative with all kits and test conditions except for two FP results with the lower cutoff of 0.05 (all three kits). The majority of the MAH-positive animals showed negative results with the IFN-γ assay; only two animals were positive using the stricter cutoff of 0.05 (Bovigam 2G and ID Screen), whereas one animal was also positive using the cutoff of 0.1 and the Bovigam 2G kit.

Taken the culture as a gold standard and assuming the likelihood of an MTBC infection in Swiss cattle as very unlikely, every positive test result was assumed to be an FP result. The false-positive rate (FPR) for the three kits, stimulation times, and cutoff were evaluated. The ID Screen kit with the S/P ratio evaluation showed the best performance with an FPR of 2.5% (95% CI 0.8–7.2), independently from the stimulation times. The other two kits showed high amounts of FP results, especially when applying the more stringent cutoff (criterion 1) and the stimulation time of 6 h after blood collection, e.g., FPR 32.7% (95% CI 24.2–41.3) for the Bovigam 2G (**Table 1**).

Comparison of Kits, Cutoffs, and Stimulation Time

The applied statistical models enabled a comparison of the three kits, taking into account the two different cutoffs and stimulation times. Based on the results of the generalized equation estimates, significant differences regarding the proportion of FP between the two Bovigam tests and between Bovigam 2G and ID Screen were found. Samples analyzed with Bovigam 2G were 2.5 (95% CI 1.6–3.9) times more likely to yield an FP test result than samples analyzed with Bovigam TB. Similarly, the OR for testing samples FP with ID Screen compared with Bovigam TB was 1.9 (95% CI 1.21–2.9). The OR for testing with ID Screen compared with Bovigam 2G was 0.75 (95% CI 0.5–1.1). When using a cutoff of 0.05 instead of 0.1, the OR for an FP test result was 2.2 (95% 1.6–3.1). For samples with a stimulation time of 6 h compared with 22–24 h, the OR of testing FP was 3.9 (95% CI 2.7–5.6).

Culture Outcome and OD_{PPDA} Values

The OD values in response to PPDA of the MAP-($n = 6$) and MAH-($n = 14$) infected animals were compared with the corresponding values of the culture-negative animals. No significant variation could be observed between the three groups ($p = 0.166$). Because the number of animals with MAP is low, this result should be considered with caution due to power.

Stimulation Time and OD_{PWM} Values

Delay in blood stimulation (22–24 h) caused a significant reduction of the mean OD_{PWM} obtained. This phenomenon was particularly evident for the ID Screen kit (overall mean OD_{PWM6h} 2.94 and $OD_{PWM22-24h}$ 2.36). The smallest effect of time stimulation was observed for the Bovigam TB kit (overall mean OD_{PWM6h} 1.40 and $OD_{PWM22-24h}$ 1.29); this kit had the lowest mean OD values and also the most invalid results ($n = 10$; 8.5%) compared with the other two kits (**Table 1**). The Bovigam 2G kit had two invalid results because of low mean $OD_{PWM22-24h}$ values (overall mean OD_{PWM6h} 2.58 and $OD_{PWM22-24h}$ 2.17), by contrast, five invalid result for this kit were observed due to high mean $OD_{NIL6h/22-24h}$ values ($\geq$0.3). Using the described interpretation criteria, the ID Screen kit showed one invalid result due to a high mean OD_{NIL6h} value ($\geq$0.3) in one animal.

Association Between Positive Test Outcome and Animal Breed Groups

Three major animal breed groups were investigated, with Holstein/Red Holstein ($n = 36$) being the most represented group, followed by Swiss Fleckvieh/Simmental ($n = 35$) and Swiss Brown ($n = 32$). As shown in **Figure 2**, by using a 0.1 cutoff and the Bovigam 2G kit, animals classified as Swiss Brown were more likely to test positive compared with Swiss Fleckvieh/Simmental (37.50 and 14.29%, respectively, $P < 0.05$). This difference was even more evident by applying the more stringent 0.05 cutoff, with 43.75 and 17.14% positive Swiss Brown and Swiss Fleckvieh/Simmental, respectively ($P < 0.05$). The opposite effect was observed by using the ID Screen kit (0.1 cutoff), with 25.71% of the Swiss Fleckvieh/Simmental animals resulting positive compared with 3.13% of the Swiss Brown. This phenomenon was exacerbated by applying the more stringent 0.05 cutoff, with 45.71 and 6.25% positive Swiss Fleckvieh/Simmental and Swiss Brown, respectively ($P < 0.001$). No statistically significant difference was observed between the test outcome and the three breed groups for the Bovigam TB kit.

DISCUSSION

According to the Swiss technical instruction for bTB, the IFN-γ assay is applied in specific epidemiologically relevant situations where the intradermal tuberculin skin test leads to inconclusive results. Cattle are the major livestock species in Switzerland, with roughly 1.5 million animals (Federal Statistical Office, census 2019). Approximately two-thirds of the Swiss cattle industry is dedicated to dairy production, and the average lifespan of a dairy cow in Switzerland is 6.2 years, giving birth on average 3.7 calves in a lifetime (47).

The agents causing bTB have been isolated from numerous different domestic and wild animal species, with the latter possibly covering long distances and spreading the disease (48, 49). This results in continuous interspecies transmissions from wild animals to livestock and *vice versa*, hindering national and international eradication programs (16, 50–52). Badger (*Meles meles*), free-ranging red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*) are the most relevant known wild animals acting as a reservoir of bTB in Europe.

Accuracy of the IFN-γ assay for diagnosis of bTB varies considerably according to the literature and depends on the

TABLE 1 | Specificity of the three commercial kits assessed with two different times after stimulation and OD cutoffs. Specificity (95% CI), time after stimulation shown in hours after blood collection, and OD cutoffs including criterion 1 (0.05), criterion 2 (0.1), and S/P ratio for the ID Screen kit were evaluated.

Kit	Stimulation time	Criterion 1	Criterion 2	S/P ratio
Bovigam TB	6 h	83.33 (75.20–89.66) [4][†)]	92.98 (86.64–96.92) [4]	/
	22–24 h	93.52 (87.10–97.35) [10]	97.22 (92.10–99.42) [10]	/
Bovigam 2G	6 h	67.26 (57.79–75.79) [5]	77.88 (69.10–85.14) [5]	/
	22–24 h	90.09 (82.96–94.95) [7]	94.59 (88.61–97.99) [7]	/
ID screen	6 h	70.34 (61.23–78.39) [1]	87.29 (79.90–92.71) [1]	97.46 (92.75–99.47) [1]
	22-24 h	90.68 (83.93–95.25) [0]	95.76 (90.39–98.61) [0]	97.46 (92.75–99.47) [0]

[†)] Number of invalid tests results excluded from the analysis according to the interpretation criteria described in the Materials and Methods section are displayed in square brackets.

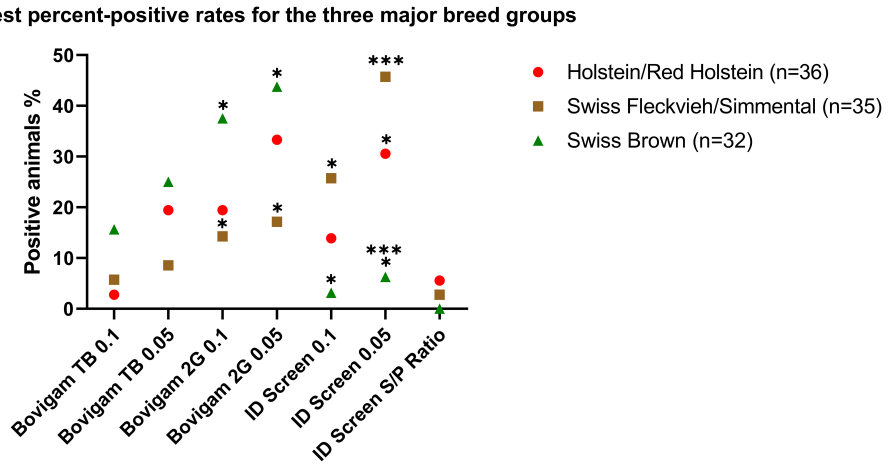

FIGURE 2 | Test percent-positive rates for the three major breed groups. Data are shown as a positive percentage of each breed group for the tested kits at 6 h stimulation. Fisher's exact test was used to evaluate the different groups. *$P < 0.05$; ***$P < 0.001$.

epidemiological settings of the tested population, the laboratory procedure, and the evaluation criteria adopted. The use of more stringent cutoff thresholds can increase the Se of the assay but may negatively influence the Sp. To date, different evaluation criteria, including thresholds, are currently used by European Member States (20). In our hands, statistically significant differences were observed for the different kits concerning the test outcome (Bovigam TB *vs.* Bovigam 2G and ID Screen *vs.* Bovigam TB), whereas the mean OD values in response to PPDA of the three culture groups (MAP, MAH, and culture-negative animals) showed no statistically significant differences.

Delay in the stimulation of the blood samples >6–8 h has been described to negatively affect IFN-γ assay performance, significantly decreasing ODs of tuberculin skin-test reactor and non-reactor animals (9, 53). The mentioned delay was deliberately included in the project to simulate a sample delivery overnight in comparison with immediate blood stimulation. Statistically significant reduction of the OD values obtained from samples PWM-stimulated within 6 h and after 22–24 h was observed for all three kits tested in the present study. This is in line with previous observations in cattle and goats (53, 54) and could negatively affect the Se of the assay due to the reduction of viable T cells in the sample.

As previously shown for *M. kansasii* (55), exposure to *M. persicum* may lead to FP results, independently from the assay used, whereas, based on the present findings, MAH and MAP seem to play a minor role in the cross-reaction. Hence, contrary to our expectations, no significant effect on the PPDA median OD values was observed between the three groups (MAP, MAH, and culture-negative) analyzed. This may be due to previous contacts of the negative culture group with mycobacteria sharing common antigens with those included in the PPDA cocktail. Among these, MAH, a ubiquitous environmental saprophyte frequently isolated from water, soil, and various animal species, including cattle, is to be mentioned and possibly plays a crucial role (30, 56).

Similar to *M. kansasii, M. persicum* shows marked homologies in surface protein expression, e.g., CFP-10 and ESAT-6, to MTBC members (42). Based on these findings, the inclusion of more specific antigens as proposed by second-generation assays may not overcome cross-reactivity issues due to NTM.

Alarming high amounts of FP results were observed using the two Bovigam assays, especially when applying the more stringent cutoff (criterion 1) and the stimulation time of 6 h after collection. For instance, using the Bovigam 2G kit, an FPR of 32.7% (95% CI 24.2–41.3) was determined, meaning that one-third of the tested

animals are supposed to be classified as positive, although MTBC was not cultured in the tested population.

According to the manufacturer's recommendations, 8.5% ($n = 10$) and 5.9% ($n = 7$) of the cattle showed an invalid result when blood samples were stimulated 22–24 h after collection and tested with the Bovigam TB and the Bovigam 2G, respectively. For the ID Screen kit, one invalid result was observed. Under realistic conditions, an invalid result would require an additional farm visit for blood sample collection, resulting in increased time and cost efforts. Previous reports showed that the collection of blood samples before stunning or even at the commencement of exsanguination is a reliable method for accessing bTB infection using the IFN-γ assay (57). A negative effect on the IFN production due to holding and handling procedures in the present study, however, cannot be excluded (24). Thus, the number of invalid results should be interpreted with caution.

Genetic influence of the breed and the outcome of the SICCT test have been reported (58). A similar impact on the Bovigam TB test result, however, has only been demonstrated in particular cases (4). Although the present findings need further confirmation with larger animal numbers, specific breeds might show an increased risk to result in FP in the Bovigam 2G and the ID Screen assays, whereas, in accordance with previous observations (59), this was not seen for the Bovigam TB kit.

Epidemiological aspects, such as farming conditions, have been shown to play a pivotal role in the *antemortem* bTB diagnostic (4, 24). Swiss cattle spend the summer months grazing on Alpine and pre-Alpine pastures, possibly resulting in prolonged close contact with environmental NTM. Considering the particular epidemiological context of the present study, similar low individual Sp values of the IFN-γ assay were observed in neighboring countries such as France and Germany (22, 23). This suggests that the observed local cross-reactivity of NTM and possibly the negative effect on the assay due to specific breeds may not be restricted to Switzerland.

In conclusion, the general application of the IFN-γ assay and, in particular, the use of more stringent interpretation criteria should be carefully evaluated. Under specific settings such as testing animals originating from herds with bTB-positive cases or import from endemic areas, the assay ensures the detection of additional infected animals and, in some cases, permits their earlier recognition compared with the tuberculin test (12). In the authors' opinion, despite the unsatisfactory Sp observed in the present study, interpretation criteria should be adapted depending on the epidemiological context. For bTB-epidemiologically linked herds, Se should be prioritized over Sp, applying the more stringent cutoff. Conversely, for surveillance purposes within a cattle population with low bTB prevalence, however, Sp should be prioritized using a more suitable cutoff or an alternative test. Within the context of a notifiable and zoonotic disease such as bTB, however, culling of infected animals to stop the spread of the causative pathogen and to reduce animal suffering is considered necessary for disease control. Preemptive culling and culling due to compromised welfare due to transport restrictions raised ethical concerns. These concerns are resumed in the EU directive 2003/85/EC: "One of the Community's tasks in the veterinary field is to improve the state of health of livestock, thereby increasing the profitability of livestock farming and facilitating trade in animals and animal products. At the same time the Community is also a Community of values, and its policies to combat animal diseases must not be based purely on commercial interests but must also take genuine account of ethical principles" (46). Still, it is not clear what according to the EU legislation is meant by "taking ethical principles into genuine account." One possible approach to gain clarity is to consider three standard ethical principles, which are respect for well-being, autonomy, and fairness or justice (47). Thus, the ethical question here is to assess if applying a more stringent cutoff for IFN-γ tests negatively affects the well-being, autonomy, and fairness of cows, farmers, and other potential stakeholders. Although the arbitrary value of the culled animals is currently reimbursed by the Swiss Government, the individual value of single animals for their farmers is often higher and results from decades of meticulous genetic selection. Briefly sketched, when applying a more stringent cutoff threshold, the well-being of the cows and the farmers will be affected because—based on the present OR of 2.2 (95% CI 1.6–3.1)—the odds or chance of an FP test result is two times more likely when reducing the cutoff from 0.1 to 0.05. A harmonized or generally prescribed cutoff of 0.05—irrespective of the local epidemiological situation, which is determined by the local prevalence of bovine TB, the occurrence of other cross-reacting NTM, and the tested cattle breed—might affect the principle of autonomy, presumably mostly relevant for the local veterinary authorities. As cows living in geographical areas with a higher occurrence of certain NTM species, they might be more likely to obtain an FP test result, which would affect the principle of fairness and justice. Briefly summarized, following the principles approach, the general application of a stringent cutoff of 0.05 compared with 0.1 is ethically questionable. Still, based on the results of our study, with a cutoff of 0.1 and the stimulation time of 6 h, the point estimates of the specificities of ID Screen and Bovigam 2G are below 90%. For the purpose of illustration, in an epidemiological setting with a true TB prevalence of 10%, assumed test sensitivity and specificity of 95 and 90%, the probability that a cow with a positive test result is truly infected is 51%. If the true prevalence is 5%, this probability is reduced to 33.3%.

Modifying the cutoff with the aim to increase the specificity is also not a feasible solution, as this would potentially lead to a decrease in sensitivity. A potential TB outbreak might be detected later, affecting subsequently more cows, causing more welfare losses in both cows and farmers. The application of a more stringent cutoff during the clarification of an outbreak may result in a significantly high number of animals with negative *postmortem* tests, such as RT-PCR or culture, greatly diminishing the reliability of positive IFN-γ results and consequently the long-term compliance of farmers. Based on our results regarding the proportion of FP and the presence of NTM potentially causing FP test results, we suggest evaluating the IFN-γ assay in light of the local epidemiological situation (20). Thus, a better understanding of the local epidemiological situation, including the identification of breeds that are more likely to react FP with specific test assay, is crucial. Monitoring programs such as LyMON provide essential data on the possible

reoccurrence of bTB, and the present findings highlight an alarmingly high number of FP reactors. Moreover, transparency of data generated from surveillance studies is essential for the establishment of an international standard based on OD-values.

Within the context of One Health, the well-being, autonomy, and fairness of the involved parties such as dairy farmers, consumers, and cows should be considered. Among others, single aspects including food safety, animal welfare, and the intrinsic value of animals are some of the interests to be evaluated before more stringent diagnostic tests are officially approved.

CONCLUSION

The application of a more stringent threshold leads to a questionable high number of FP results. Depending on the epidemiological context, including cross-reactive NTM in specific geographic areas, expected bTB prevalence, consequences of a positive test, the tested cattle breed, and the assay threshold should be carefully selected. If necessary, the inclusion of more specific antigens is to be considered.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All animals included in this study were sampled in accordance with the Swiss Act SR 455. The animal testing was approved by the Animal Welfare Committee of the Canton of Zurich under license number ZH185/18.

AUTHOR CONTRIBUTIONS

GG, SS, and RS designed and coordinated the study. PL, MM, GG, and UF performed the experiments. SH and GG conceived and carried out the statistical analyses. GG, SS, and SH drafted the manuscript. All authors read and approved the final manuscript.

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frontiers in Veterinary Science

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Mycobacterial Infection of Precision-Cut Lung Slices Reveals Type 1 Interferon Pathway Is Locally Induced by *Mycobacterium bovis* but Not *M. tuberculosis* in a Cattle Breed

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Tuberculosis exacts a terrible toll on human and animal health. While *Mycobacterium tuberculosis* (Mtb) is restricted to humans, *Mycobacterium bovis* (Mb) is present in a large range of mammalian hosts. In cattle, bovine TB (bTB) is a noticeable disease responsible for important economic losses in developed countries and underestimated zoonosis in the developing world. Early interactions that take place between mycobacteria and the lung tissue early after aerosol infection govern the outcome of the disease. In cattle, these early steps remain poorly characterized. The precision-cut lung slice (PCLS) model preserves the structure and cell diversity of the lung. We developed this model in cattle in order to study the early lung response to mycobacterial infection. *In situ* imaging of PCLS infected with fluorescent Mb revealed bacilli in the alveolar compartment, in adjacent or inside alveolar macrophages, and in close contact with pneumocytes. We analyzed the global transcriptional lung inflammation signature following infection of PCLS with Mb and Mtb in two French beef breeds: Blonde d'Aquitaine and Charolaise. Whereas, lungs from the Blonde d'Aquitaine produced high levels of mediators of neutrophil and monocyte recruitment in response to infection, such signatures were not observed in the Charolaise in our study. In the Blonde d'Aquitaine lung, whereas the inflammatory response was highly induced by two Mb strains, AF2122 isolated from cattle in the UK and Mb3601 circulating in France, the response against two Mtb strains, H37Rv, the reference laboratory strain, and BTB1558, isolated from zebu in Ethiopia, was very low. Strikingly, the type I interferon pathway was only induced by Mb but not Mtb strains, indicating that this pathway may be involved in mycobacterial virulence and host tropism. Hence, the PCLS model in cattle is a valuable tool to deepen our understanding of early interactions between lung host cells and mycobacteria. It revealed striking differences

between cattle breeds and mycobacterial strains. This model could help in deciphering biomarkers of resistance vs. susceptibility to bTB in cattle as such information is still critically needed for bovine genetic selection programs and would greatly help the global effort to eradicate bTB.

Keywords: cattle, *Mycobacterium bovis*, *ex vivo*, precision cut lung slices, alveolar macrophages, type I interferon

INTRODUCTION

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (Mb) remains one of the most challenging infections to control in cattle. Because of its zoonotic nature, this pathogen and its associated noticeable disease in cattle are under strict surveillance and regulation in the European Union. When bTB cases are detected through surveillance, culling of these reactor cattle is mandatory. In spite of intensive eradication campaigns, bTB is still prevalent in European cattle (1, 2) and has significant economic, social, and environmental implications. Since 2001, France is an officially bTB-free country, a status that was achieved through costly surveillance programs. However, each year, around 100 Mb foci of infection are identified (3), with certain geographical areas showing a constant rise in disease prevalence since 2004.

bTB eradication is an unmet priority that faces two major difficulties: the persistence of undetected infected animals in herds because of the lack of diagnostic sensitivity and the risk of transmission from infected sources (4). Moreover, the poor understanding of bTB pathophysiology in cattle and the lack of correlates of protection are substantial knowledge gaps that must be resolved so as to better tackle the disease (DISCONTOOLS, https://www.discontools.eu/).

Both Mb and *Mycobacterium tuberculosis* (Mtb) belong to the same genetic complex. Mtb is responsible for tuberculosis (TB) in humans, which displays similar features with bTB. It is estimated that one-third of the global human population are latently infected with Mtb, which kills 1.4 million people each year (5). Despite the high degree of identity that Mtb and Mb share both at the genetic level as well as during the infection process, the two pathogens display distinct tropism and virulence depending on the host. While Mb is highly virulent and pathogenic for cattle and a range of other mammals, Mtb is restricted to sustain in humans. An experimental infection of cattle with the widely used Mtb laboratory strain H37Rv, which was genome-sequenced in 1998 (6), shows a strong attenuation compared to Mb (7, 8). However, the natural infection of cattle with Mtb has been reported, and the strain Mtb BTB1558 was once such a case, isolated from a zebu bull in Ethiopia (9, 10). In comparison to the original UK Mb strain AF2122/97, the first genome-sequenced Mb isolate (11, 12), the Mtb strain BTB1558 displayed a much lower virulence in European cattle (13).

The Mb strains that circulate in France today are phylogenetically distant from the UK Mb reference strain. While AF2122 belongs to the European 1 clonal complex (14), the European 3 clonal complex is widespread in France, (15). The Eu3 genetic cluster is composed of field strains that share the SB0120 spoligotype with the attenuated Bacillus-Calmette-Guerin (BCG) vaccine strain (16, 17). In our study, we used Mb3601 as the representative strain of this widespread French cluster. Originally, Mb3601 was isolated from the tracheobronchial lymph node of an infected bovine in a bTB highly enzoonotic area in France (16). However, despite the widespread circulation in its original area, nothing is known today of the pathophysiology of Mb3601 infection.

Indeed greater knowledge is available on Mtb infection process and disease development both in humans and mouse models compared to Mb infection in cattle. With both mycobacteria, the alveolar macrophage (AMP) is the frontline cell that first presents the first niche for mycobacteria entering the lung, and the role of the AMP in early-stage infection is well established (8). Both Mtb and Mb have established their lifestyle in AMPs: they can escape its bactericidal mechanisms and multiply within this niche. During the infection process, bacilli disseminate to different anatomical sites and establish new infection foci both in the lungs and secondary lymphoid organs (18, 19). During Mtb infection, lung epithelial cells also play key roles in host defense [reviewed in (20–22)]. Type II pneumocytes are infected by Mtb (23) and produce pro-inflammatory cytokines which augment the AMP innate resistance mechanisms (24). The role of type II pneumocytes during Mb infection in cattle is not well known. Most of the available knowledge on the role of bovine macrophages (MPs) during Mb infection also comes from studies conducted with monocytes sampled from blood and derived as MPs during *in vitro* culture (25, 26).

In our study, we wanted to investigate the bovine innate response following Mb or Mtb infection in a preserved lung environment to allow the resident lung cells to interact with bacilli and crosstalk. Precision cut lung slices (PCLS) are an experimental model in which resident lung cell types are preserved and remain alive for at least 1 week (27). The tissue architecture and the interactions between the different cells are maintained. PCLS have already been validated for the study of various respiratory pathogens (27–29). In chicken PCLS, mononuclear cells are highly motile and actively phagocytic (30). This model is well designed to study complex interactions taking place early after the host–pathogen encounter. During Mb infection in cattle, important differences in the production of key proinflammatory cytokines such as IFNγ or TNFα by peripheral blood mononuclear cells are observed, depending on the clinical status of the animal. Interestingly, such differences are observed at early time points (31), indicating that the innate phase of the host response is key to the establishment of the pathological outcome of the infection.

Therefore, the PCLS model is ideally suited to investigate early host–pathogen interactions in the bovine lung during Mb infection and may help to find clues to the impact of the innate response on the outcome of infection. This model, which fully mimics the early environment of the bacillus entering the lung (compared to monocyte-derived MPs), may also aid in understanding the molecular basis of mycobacterial host preference (32). To this end, we decided to compare four mycobacterial strains: two Mtb species—namely, the Mtb H37Rv reference strain for human TB and the cattle derived Mtb BTB1558—and two Mb species—namely, Mb AF2122 as representative of the EU1 clonal complex and Mb3601 as the hallmark EU3 strain. Since the host genetic background also has a profound impact on the outcome of bTB disease (33), we decided to compare PCLS from two prevalent beef breeds in France—Charolaise and Blonde d'Aquitaine—and conducted a thorough characterization of the lung responses to Mb and Mtb during *ex vivo* infection. The PCLS allowed us to decipher important differences in the transcriptomic and cytokine profile during the innate response to infection, depending both on the breed, i.e., between Blonde d'Aquitaine and Charolaise cows, and on the mycobacterial species, i.e., between Mtb and Mb.

MATERIALS AND METHODS

Animal Tissue Sampling

Lungs from 15 Blonde d'Aquitaine and nine Charolaise cows were collected post-mortem at a commercial abattoir. The animals were between 3 and 11 years old and originated from eight different French departments where no recent bTB outbreak had been noticed (**Supplementary Figure 1**). No ethical committee approval was necessary as no animal underwent any experimental procedure. After slaughter by professionals following the regulatory guidelines from the abattoir, the lungs from each cow were systematically inspected by veterinary services at the abattoir. The origin of each animal was controlled, and its sanitary status was recorded on its individual passport: the animals were certified to be free of bTB, leucosis, brucellosis, and infectious bovine rhinotracheitis.

Bacterial Strains and Growth Conditions

Strains Mb AF2122/97 and Mb MB3601 had previously been isolated from infected cows in Great Britain and France, respectively (12, 15). The Mb3601-EGFP fluorescent strain was derived by electroporation with an integrative plasmid expressing EGFP and selected with Hygromycin B (50 μg/ml) (Sigma, USA) as described previously (34). Mtb BTB1558 had been previously isolated from a zebu bull in Ethiopia (13). Bacteria were grown in Middlebrook 7H9 broth (Difco, UK) supplemented with 10% BBLTM Middlebrook albumin–dextrose–catalase (BD, USA) and 0.05% Tween 80 (Sigma-Aldrich, St Louis, USA). At mid-log phase, the bacteria were harvested, aliquoted, and stored at −80°C. Batch titers were determined by plating serial dilutions on Middlebrook 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase (BD, USA), with 0.5% glycerol or 4.16 g/L sodium pyruvate (Sigma, USA) added for Mtb or Mb strains, respectively. The plates were incubated at 37°C for 3–4 weeks (H37Rv, BTB558, and AF2122) and up to 6 weeks for Mb3601 before colony-forming unit (CFU) numeration. The inocula were prepared from one frozen aliquot (titer determined by CFU numeration) that was thawed in 7H9 medium without glycerol and incubated overnight at 37°C. After centrifugation for 10 min at 3,000 × *g*, the concentration was adjusted to 10^6 CFU/ml in RPMI medium.

Obtention and Infection of Precision-Cut Lung Slices

PCLS were obtained from fresh lungs using a tissue slicer, MD 6000 (Alabama Research and Development). For each animal, the right accessory lobe was filled *via* the bronchus with RPMI containing 1.5% low-melting-point (LMP) agarose (Invitrogen) warmed at 39°C. After 20 min at 4°C, the solidified lung tissue was cut in 1.5-cm slices with a scalpel. A 0.8-mm diameter-punch was used to obtain biopsies that were placed in the microtome device of the Krumdieck apparatus, filled with cold phosphate-buffered saline (PBS), and 100-μm-thick PCLS were cut. One PCLS was introduced in each well of a 24-well plate (Nunc); 1 ml of RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), and PANTA™ antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton Dickinson) was added to the well, and the plate was incubated at 37°C with 5% CO_2. The medium was changed every 30 min during the first 2 h to remove all traces of LMP agarose. At 24 h later, after the last medium change, ciliary activity was observed under a microscope to ensure tissue viability.

The PCLS were infected for 2 days with 10^5 CFU of Mb or Mtb strains. As indicated, the PCLS were either fixed in formalin for imaging or lysed with a Precellys in lysing matrix D tubes in 800 μl Tri-reagent for RNA extraction. The bacillary load of each strain present in the PCLS was compared after the transfer of the PCLS to a new plate at 1 day after infection (dpi), two washes in 1 ml of PBS, and homogenization in 1 ml of PBS in lysing matrix D tubes (MP Biomedicals) with a Precellys (Ozyme). To determine CFUs, serial dilutions were plated as described above.

Alveolar Macrophages

To harvest alveolar macrophages (AMPs) from Blonde d'Aquitaine cows, broncho-alveolar lavages (BAL) were performed on the left basilar lobe of the lung at a local abattoir after culling the animal. The lobe was filled with 2 × 500 ml of cold PBS containing 2 mM EDTA (Sigma-Aldrich). After the massage, the BAL was collected and transported at 4°C to the laboratory. BAL was filtered with a 100-μm cell strainer (Falcon) and centrifuged for 10 min at 300 × *g*. The cells were washed in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), and PANTA™ Antibiotic Mixture. Then, 10^7 BAL cells per milliliter were suspended in 90% FCS and 10% dimethyl sulfoxide (Sigma-Aldrich) and cryopreserved in liquid nitrogen. At 1 day before infection, the BAL cells were thawed at 37°C, washed in complete RPMI medium, and transferred to a 75-cm^2 culture flask with a ventilated cap. After 2 h at 37°C and 5% CO_2, non-adherent cells were removed, and adherent AMPs were incubated 2 × 10 min

at 4°C with 10 ml of cold PBS to detach and enumerate them in a Malassez chamber. Then, 5×10^5 AMPs/well were distributed in a 24-well plate and incubated overnight at 37°C and 5% CO_2. The medium was changed once, and AMPs were infected with Mb3601 or Mtb H37Rv at a multiplicity of infection (MOI) of 1. At 6 and 24 h post-infection, the supernatants were filtered through a 0.2-μm filter, and the cells were lysed in 800 μl of Tri-reagent for RNA extraction. The MOI was checked by CFU determination at 24 h after infection.

Cell Supernatant Collection and Lactate Dehydrogenase Assay

In order to evaluate cytotoxicity, supernatants from infected PCLS or AMPs were passed through a 0.2-μm filter at indicated time points, and cells were lysed in 1 ml of lysis buffer (5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, Triton 1%, pH 7.4), containing anti-proteases (Roche), in a lysing matrix D tube, with a Precellys apparatus. The homogenates were clarified by centrifugation for 10 min at 10,000 × *g*, filtered through 0.2 μm, and collected on microplates. The cytotoxicity of infection in PCLS was assessed using the Non-radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. The cytotoxicity was calculated as cytotoxicity (%) = [OD_{490} of lactate dehydrogenase (LDH) in the supernatant]/(OD_{490} of LDH in the supernatant + OD_{490} of LDH in the PCLS homogenates) × 100.

Immunohistochemistry on PCLS

The infected PCLS were fixed 24 h at 4°C with 4% formalin and then transferred to a 48-well culture plate in PBS. All steps that will be described below were done under gentle agitation at room temperature (RT). The PCLS were incubated for 2 h with 100 μl of PBS, 0.25% Triton X-100, and 10% horse serum for permeabilization and saturation (saturation buffer). They were incubated overnight at 4°C with primary Ab (anti-bovine MHCII clone MCA5655 from BioRad and anti-bovine pancytokeratine clone BM4068 from Acris) diluted in saturation buffer. The PCLS were washed four times with 300 μl of PBS (two times for 5 min and then two times for 10 min) and then incubated for 3 h with fluorescent-conjugated secondary antibodies diluted in saturation buffer (goat anti-mouse IgG1-APC and goat anti-mouse IgG2a A555 from Invitrogen). The PCLS were washed four times with 300 μl of PBS (two times for 5 min and then two times for 10 min), transferred on cover slides which were mounted with Fluoromount-G™ mounting medium containing DAPI (Invitrogen), and sealed with a transparent nail polish. Z-stack imaging was performed at ×63 enlargement with a confocal microscope (LEICA) and analyzed with LAS software. The presence/absence of Mb and number of macrophages per alveoli were numerated by eye at the confocal microscope, with one person counting and the other confirming and reporting the data.

Quantification of Cytokines and Chemokines Released by PCLS and AMPs

The cytokine and chemokine levels produced by PCLS after 2 dpi were assessed in a Multiplex assay in supernatants (dilution 1:2) with MILLIPLEX® Bovine cytokine/chemokine panel 1 (BCYT1-33K-PX15, Merck) according to the manufacturer's instructions. IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα, and VEGF-A were measured. Data were acquired using a MagPix instrument (Luminex) and analyzed with Bio-Plex Manager software (Bio-Rad). IL-8 was out of range in the Multiplex, so we performed a sandwich ELISA with the following references: goat anti-bovine interleukin-8 Ab AHP2817, recombinant bovine interleukin-8 PBP039, and goat anti-bovine interleukin-8 Ab conjugated to biotin AHP2817B (all from Bio-Rad), following the protocol according to the manufacturer's instructions.

RNA Extraction and Gene Expression Analysis

The total RNA from two pooled PCLS was extracted using a MagMAX™-96 Total RNA isolation kit (ThermoFisher). For AMPs, we used the Nucleospin RNA isolation kit (Macherey Nagel). After DNase treatment (ThermoFisher or Macherey Nagel), the mRNAs were reverse-transcribed with iScript™ Reverse Transcriptase mix (Biorad) according to the manufacturer's instructions. The primers (Eurogenetec; **Supplementary Table 1**) were validated, using a serially diluted pool of cDNA mix obtained from bovine lung, lymph nodes, blood, and bone marrow, with a LightCycler® 480 Real-Time PCR System (Roche). Gene expression was then assessed with the BioMark HD (Fluidigm) in 96 × 96-well integrated fluidic circuit plate according to the manufacturer's instructions. The annealing temperature was 60°C. The data were analyzed with Fluidigm RealTime PCR software to determine the cycle threshold (Ct) values. The messenger RNA (mRNA) expression was normalized to the mean expression of three housekeeping genes (*PPIA*, *GAPDH*, and *ACTB*) to obtain the ΔCt value. For each animal, values from infected PCLS were normalized to the uninfected PCLS gene expression (ΔΔCt value and relative quantity = $2^{-\Delta\Delta Ct}$). Principal component analysis (PCA) was performed using ΔΔCt values in R studio (version 1.1.456, ©2009–2018 RStudio, PBC) using the FactoMineR packages (version R 3.5.3).

Statistical Analysis

The individual data and the median and interquartile range are presented in the figures, except for **Figure 2** where the mean and standard error of the mean (SEM) are presented. Statistical analyses were performed with Prism 6.0 software (GraphPad). Analyses were performed on data from two to six independent experiments, with two-way ANOVA or Wilcoxon non-parametric tests for paired samples used. The represented *p*-values were $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Ex vivo Infection With Mycobacteria of Live Bovine Lung Tissue in PCLS Allows Bacilli Uptake by AMPs and Their Recruitment to the Alveoli

The early events of bTB pathophysiology in the bovine lung remain poorly defined due to the complexity of biocontained experimental infection in large animals. Since PCLS have been

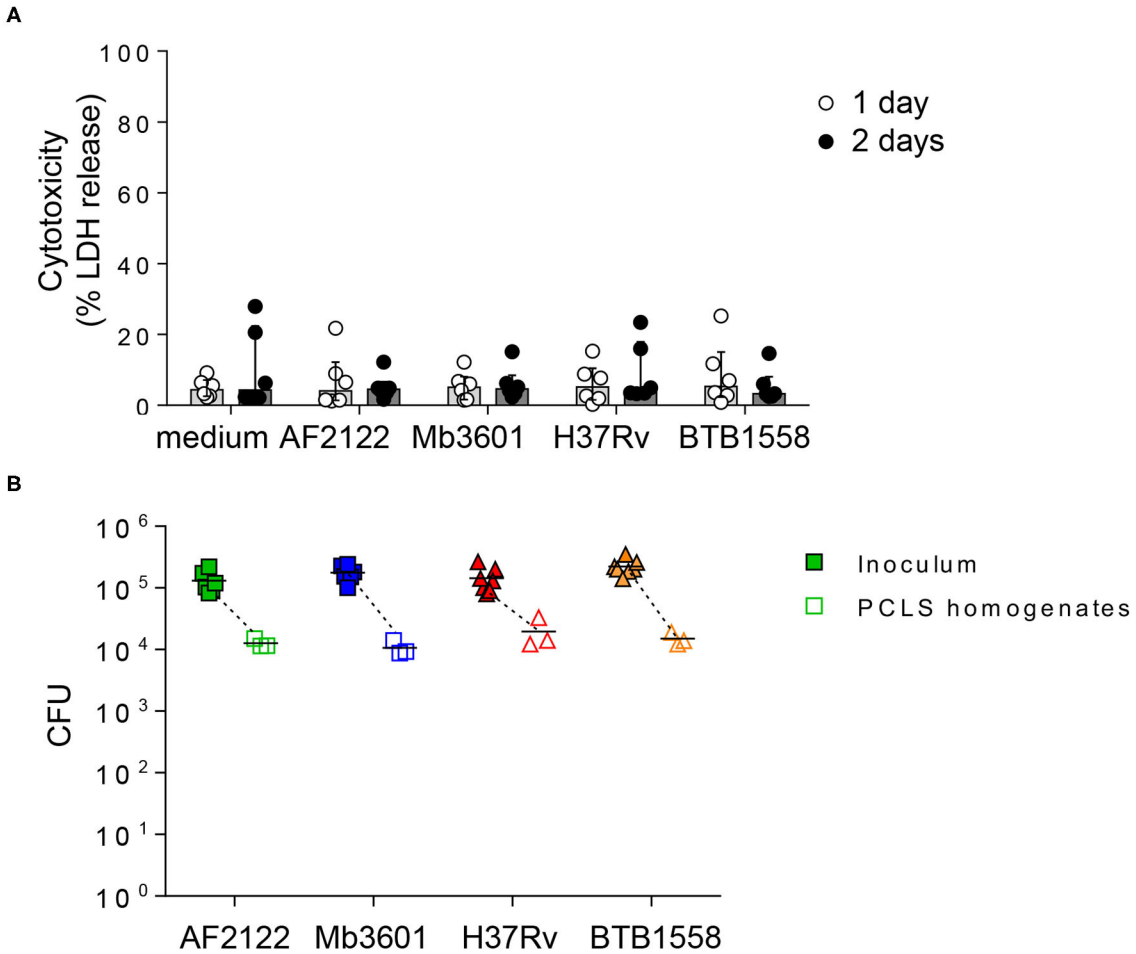


FIGURE 1 | Precision-cut lung slice (PCLS) infection with four different Mb or Mtb strains does not induce lung tissue cytotoxicity, and equivalent numbers of bacilli are recovered 24 h post-infection. **(A)** PCLS prepared from Blonde d'Aquitaine lungs post-mortem were infected with 10^5 CFU of two Mb strains (AF2122 or Mb3601) or two Mtb strains (H37Rv or BTB1558). After 1 and 2 days post-infection, the PCLS supernatants were harvested, and tissue was homogenized. Lactate dehydrogenase (LDH) was measured in both compartments using the "non-radioactive cytotoxicity assay" kit. Cytotoxicity was determined as (%) = (O.D. 490 nm LDH in supernatant)/(O.D. 490 nm LDH in supernatant + O.D. 490 nm LDH in PCLS homogenates) × 100. Individual data and the median and interquartile range in each group are presented ($n = 6$ animals from six independent experiments). **(B)** At 24 h post-infection, the PCLS were washed and homogenized to recover bacilli. The inoculum and PCLS homogenates were serially diluted and plated with colony-forming units numerated after 3–6 weeks of incubation. Individual data and the mean in each group are presented ($n = 6$ independent inocula prepared; PCLS homogenates data represent the mean of technical duplicates from $n = 3$ animals from three independent experiments).

used to study viral respiratory infections in the bovine (27), we decided to use this model to assess early events taking place following entry of Mb into the lung. We infected bovine PCLS obtained *ex vivo* with the four mycobacterial strains: Mb AF2122, Mb3601, Mtb H37Rv, or BTB1558.

We first monitored tissue cytotoxicity at 1 and 2 dpi using a LDH release assay. The mean percentage of cytotoxicity remained below 10%, and no difference was observed between infected and non-infected PCLS (**Figure 1A**). The ciliary activity from the PCLS bronchial cells monitored every day under a light microscope remained vigorous and stable after infection (data not shown). We calibrated our model and inocula to use 10^5 CFUs for each of the four different strains. We analyzed CFUs still present in PCLS at 24 h later and observed an equivalent 1 log decrease for all strains (**Figure 1B**). This indicated an equivalent infection by all strains, allowing them to be directly compared. Therefore, with a similar bacterial load and excellent tissue viability in all experimental conditions, we validated PCLS as a model to study the early events taking place in the bovine lung after infection with mycobacteria.

In order to visualize the interactions taking place between bacilli and lung cells, we infected the PCLS with a fluorescent version of the Mb3601 strain, and at 1 and 2 dpi, we analyzed the cells by *in situ* immunohistochemistry. The lung structure was visualized by DAPI and pancytokeratine staining, and we used confocal microscopy to image 10–15-μm sections and localize Mb3601-EGFP (**Figure 2A**). We observed Mb in 27 ± 3% of PCLS alveoli (**Figures 2A,B**) and almost always in

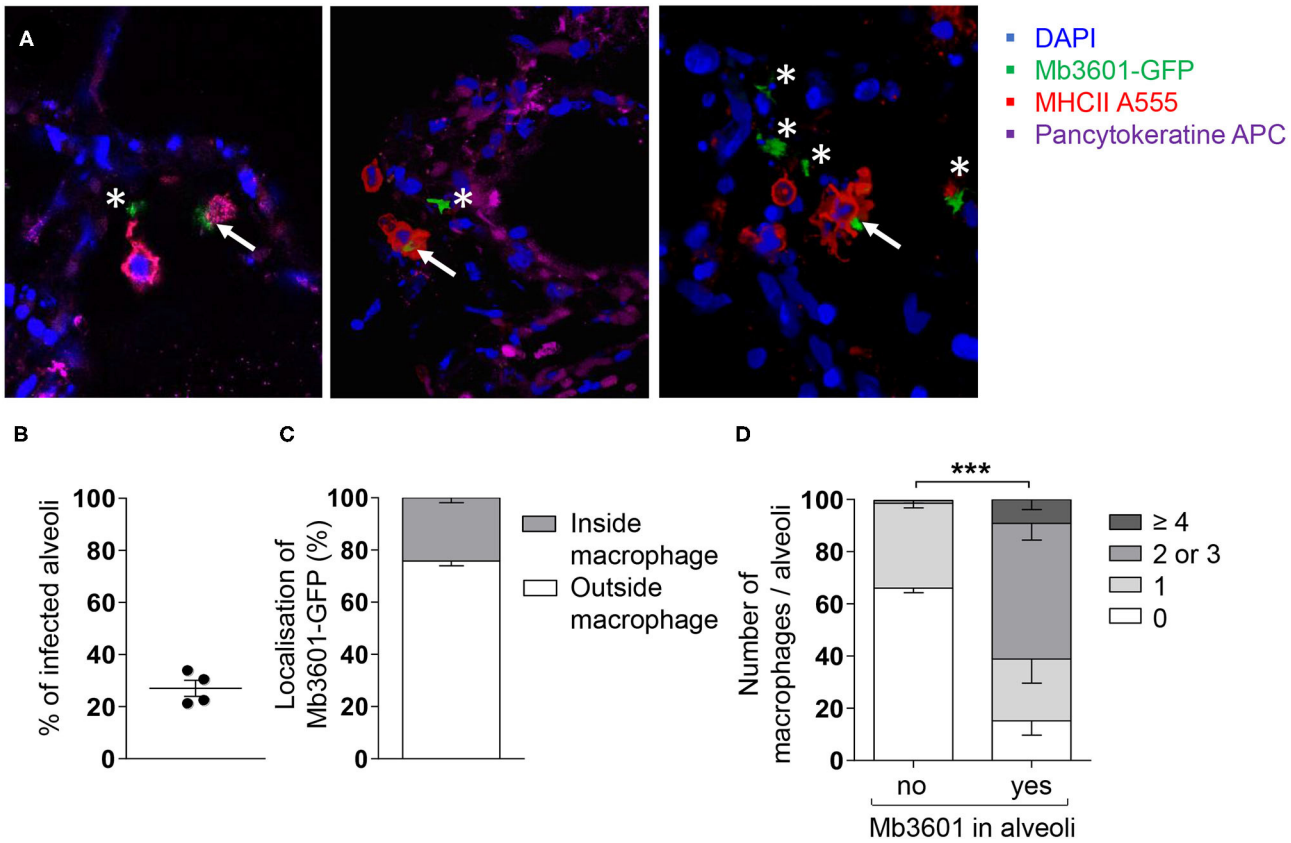


FIGURE 2 | Mb3601 is internalized by alveolar macrophages (AMPs) in the preserved lung structure from precision-cut lung slice (PCLS), and the infected alveoli contain higher numbers of AMPs compared to non-infected alveoli. The PCLS were infected with 10^5 colony-forming units of the green fluorescent protein Mb3601-GFP recombinant strain and fixed 2 days later. After labeling with anti-pancytokeratine (magenta) and anti-MHCII antibodies (Alexa 555, red), the PCLS were mounted with Fluoromount-G™ mounting medium containing DAPI (blue) and analyzed under a Leica confocal microscope **(A)**; 3D images were analyzed with Leica LAS software. Z-stack imaging was performed at ×63 enlargement (10–15 μm in thickness, step size of 0.5–1 μm). The white asterisks indicate extracellular bacilli, and the white arrows indicate bacilli inside MHC-II^{pos} AMPs. **(B)** The graph represents the percentage of infected alveoli per PCLS among the 55–80 alveoli that were observed under the microscope ($n = 4$ PCLS from two different Blonde d'Aquitaine cattle). **(C)** Stack histogram of the mean percentage ± SEM of intra- or extracellular bacilli among a minimum of 15 infected alveoli that were observed ($N = 4$ PCLS). **(D)** The number of MHC-II^{pos} AMPs per alveoli was counted in infected or non-infected alveoli. The data presented as percent are the mean ± SEM of $n = 4$ PCLS from two different Blonde d'Aquitaine cattle. Between 55 and 80 alveoli were observed to obtain these data (two-way ANOVA, $^{***}p < 0.001$).

close contact with large MHC-II-positive AMPs. The bacilli were localized outside AMPs in 76 ± 2% observations and resided intracellularly in AMPs in 24 ± 2% (**Figures 2A,C** and **Supplementary Video 1**). Interestingly, the number of AMPs per alveoli differed upon bacilli presence or absence (**Figure 2D**). In uninfected PCLS, lung alveoli generally contained one AMP (data not shown). However, in Mb-infected PCLS, we either observed no AMPs in 66 ± 2% of alveoli or one AMP in 33 ± 2% of alveoli in the absence of any Mb. On the contrary, the number of AMPs significantly increased in alveoli where at least one Mb was observed (**Figure 2D**, $p < 0.001$). The number of AMPs varied among infected alveoli, with 24 ± 9% containing one AMP, 52 ± 6% containing two or three AMPs, and 9 ± 4% containing more than four AMPs. Such observations indicated that, during the 2 days of infection, AMPs were recruited from one alveolus to another in response to signals linked to Mb infection. In conclusion, even though Mb infection was performed *ex vivo*, bacilli were observed in the alveoli, close or inside their target host cell, i.e., the AMP. Moreover, the PCLS model was physiological enough to allow AMPs to crawl in response to signals linked to bacilli entry.

The Lung Response to Mycobacterial Infection Vastly Differs Between Blonde d'Aquitaine and Charolaise Cows

Two bovine beef breeds are widely used in France: Blonde d'Aquitaine and Charolaise. We decided to compare how these two breeds respond to mycobacterial infection, using our PCLS system. We measured 15 cytokines and chemokines secreted by the lung tissue at 2 dpi with the four mycobacterial strains and performed a PCA. As depicted in **Figure 3A**, the PCA revealed important differences in the immune response of the lung tissue between the two breeds. The group samples clearly

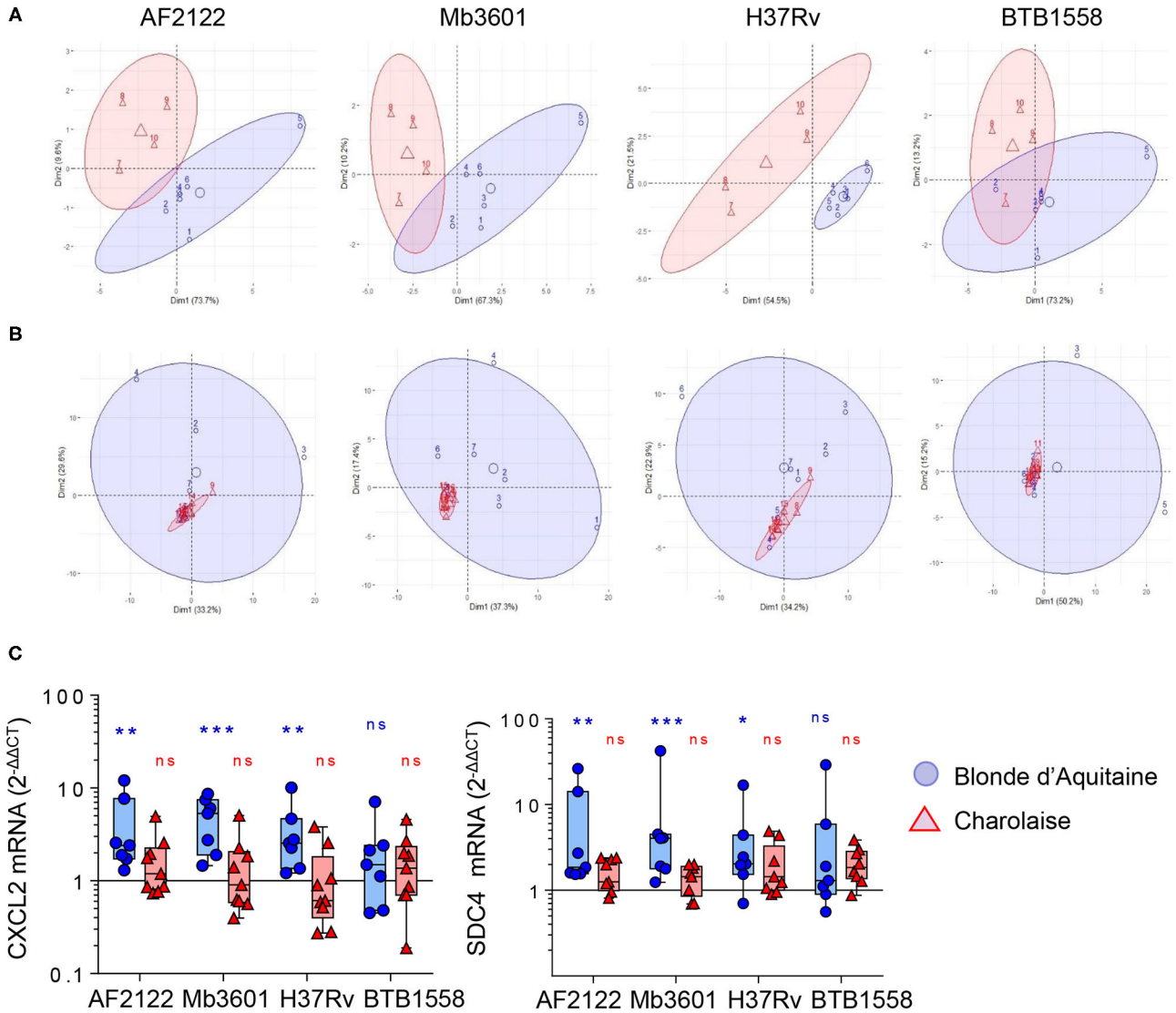
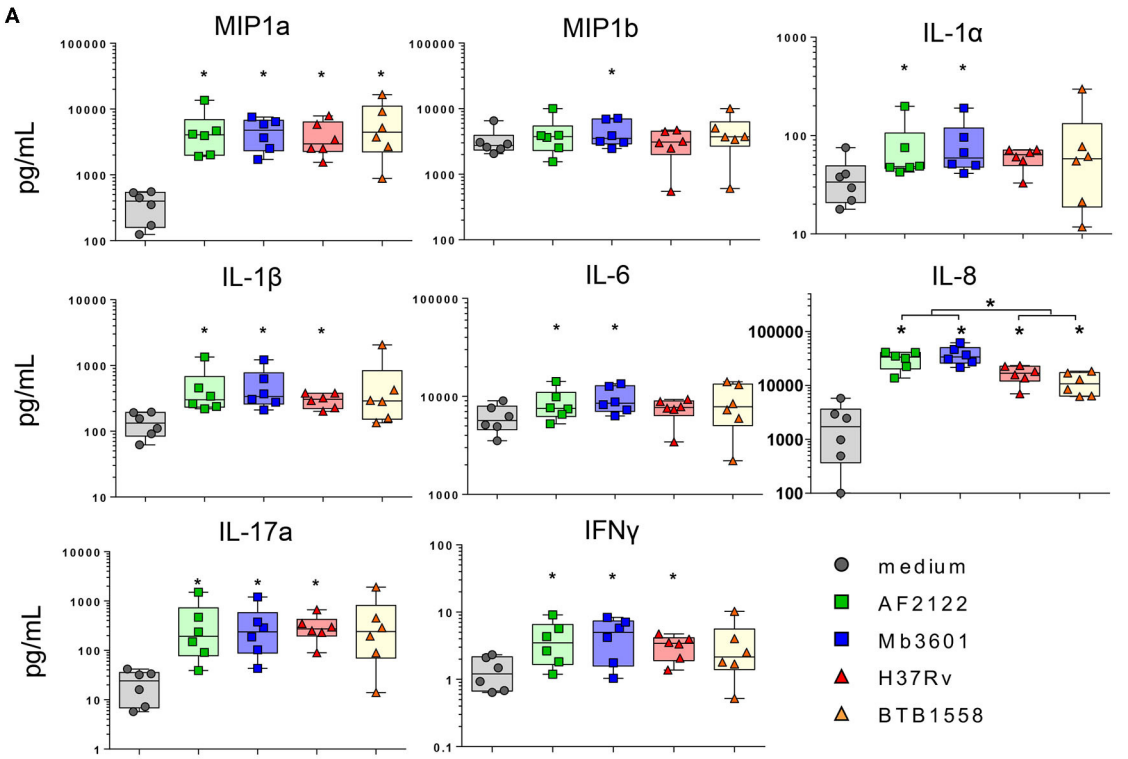


FIGURE 3 | Principal component analysis (PCA) of inflammatory lung tissue signature reveals differences between two beef cattle breeds after 2 days of infection by Mb or Mtb. **(A)** Fifteen cytokines and chemokines were measured in PCLS supernatants from Blonde d'Aquitaine or Charolaise cows 2 days after infection with four different mycobacterial strains. Raw data were used to run PCA in R studio. Individual data are shown ($n = 4$ for Charolaise, red; $n = 6$ for Blonde d'Aquitaine, blue). The ellipses represent a confidence range of 90%. **(B)** PCA were built from the expression data of 96 genes ($2^{-\Delta\Delta Ct}$) obtained from precision-cut lung slice total RNA extracted 2 days after infection. Individual data are shown ($n = 9$ for Charolaise, red; $n = 7$ for Blonde d'Aquitaine, blue). The ellipses represent a confidence range of 90%. **(C)** Two examples of differentially expressed genes. Individual data and the median and interquartile range in each group are presented ($n = 7$ Blonde d'Aquitaine and $n = 9$ Charolaise) $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Two-way ANOVA test.

plotted apart, and their ellipses showed either a small overlay (AF2122 and Mb360A) or no overlay at all (H37Rv). The results for the BTB1558 group showed less clustering of samples due to higher individual variations. We then extracted total RNA from PCLS after 1 or 2 dpi and analyzed the expression of 96 genes related to innate immunity and inflammation (see the full list in **Supplementary Table 1**). The RT-qPCR data were normalized and expressed as fold change compared to uninfected PCLS control for each cow. Gene expression was higher at 2 days after infection compared to that at 1 dpi (data not shown). We therefore decided to focus our analysis on this 2-dpi time point. Remarkably, the transcriptomic signature induced by infection was very low for the Charolaise breed, whichever mycobacterial strain was used, which explains the clustering of Charolaise samples (**Figure 3B**). Increasing the inoculum in the Charolaise PCLS up to 5×10^6 CFU did not induce gene expression (**Supplementary Figure 2**). The response of the lung tissue to mycobacterial infection in Blonde d'Aquitaine was very different compared to that in Charolaise as revealed by a PCA (**Figure 3B**). Whereas, in PCLS from Charolaise the gene expression from



B

Gene ID	AF2122	Mb3601	H37Rv	BTB1558
IL-1α	1.7	3.8	2.7	2.2
IL-1β	1.9	4.1	2.5	3.4
IL-6	4,2	**7.3 ***	4.2	4.5
IL-17a	4.5	10.9	2.3	2.1
CCL2	4.2	7.1	2.4	6.3
CCL5	3.9	5.5	6.4	5.5
CCL20	4.4	2.0	5.8	4.1
CXCL1	3.2	**4.2 ***	3.3	3.0
CXCL2	**4.2 ***	**3.6 ***	**4.8 ***	2.2
CXCL5	1.5	**2.1 ***	2.2	2.0
CXCL8	3.6	**4.8 ***	4.0	4.7
G-CSF	**21.1 ***	1.7	8.3	3.9
GM-CSF	3	1.5	4.3	7.5
MIF	3.2	2.8	3.9	3.3
S100A8	5.5	7.5	3.1	6.7
S100A9	1.7	2.4	1.8	1.5
TNFα	16.4	14.1	2.4	3.7

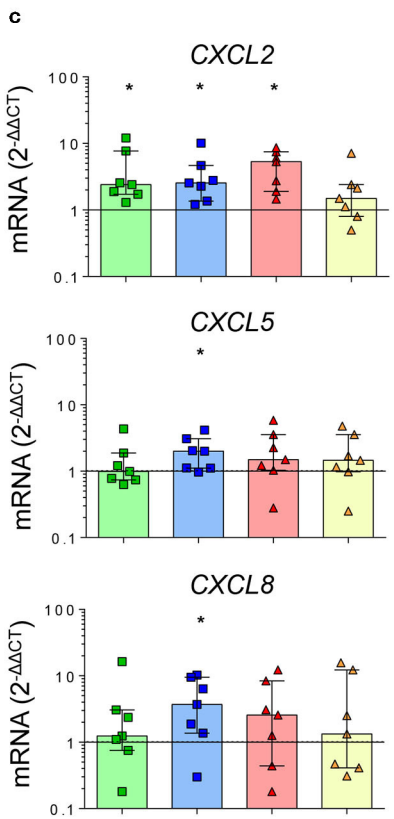


FIGURE 4 | The lung inflammatory neutrophil and monocyte recruitment signature induced by infection in precision-cut lung slice (PCLS) from Blonde d'Aquitaine cows is more efficiently triggered by *Mycobacterium bovis* than *Mycobacterium tuberculosis*. **(A)** The cytokine and chemokine levels were measured in PCLS

(Continued)

FIGURE 4 | supernatant by Multiplex ELISA 2 days after infection with two Mb or two Mtb strains. Individual data and the median and interquartile range in each group are presented ($n = 6$ cows). **(B)** Table of the mean of fold change ($2^{-\Delta\Delta CT}$) for each group ($n = 7$ cows) of 17 major genes involved in neutrophil and monocyte recruitment and inflammation. The graduated red box coloring represents levels of gene expression, and the asterisks mark significant differences compared to non-infected controls. **(C)** *CXCL2*, *CXCL5*, and *CXCL8* gene expression at 2 days post-infection. Individual data and the median and interquartile range in each group are presented ($n = 7$ cows). **(B,C)** $^*p < 0.05$ (Wilcoxon nonparametric test).

infected and non-infected controls clustered, in PCLS from Blonde d'Aquitaine, the gene expression levels were significantly more dispersed after infection compared to those of controls (**Figure 3B**). We compared the individual gene expression between the two breeds for a number of genes. For instance, both the *CXCL2* chemokine and the mycobacteria receptor syndecan 4 *SDC4* were significantly upregulated after PCLS infection with AF2122, Mb3601, or H37Rv in Blonde d'Aquitaine, but not in Charolaise (**Figure 3C**). Our data altogether revealed important differences in the early lung response to mycobacterial infection, depending on the breed of the animals, that could be measured both at the gene expression and protein production level in the PCLS system.

The Overall Inflammation Signature in the Lung Tissue Is Triggered More Efficiently by *M. bovis* Than *M. tuberculosis*

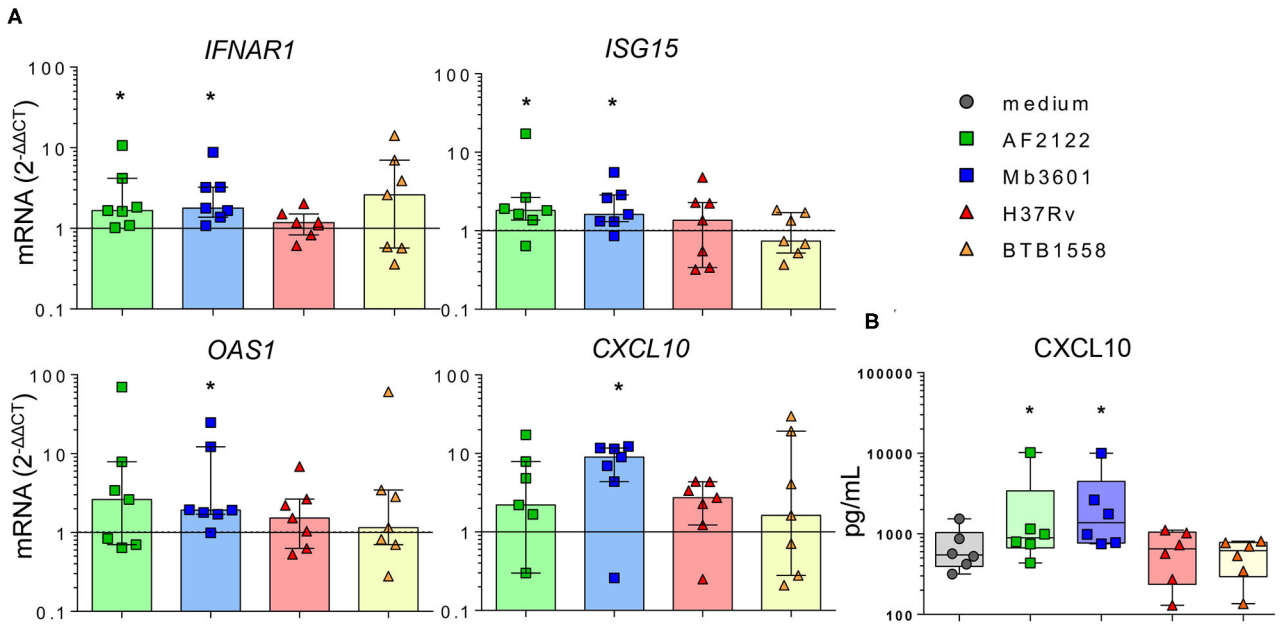
We then focused our analysis on Blonde d'Aquitaine to determine how the lung tissue responded to different mycobacterial strains. We analyzed 15 cytokines and chemokines produced in the PCLS supernatants 2 days following an infection. No IL-4 was detected, and the production of TNFα, IL-36RA, IL-10, VEGFA or MCP-1 was not different between infected PCLS and controls (**Supplementary Figure 3A**). We observed that *ex vivo* infection of PCLS with mycobacteria triggered an inflammatory response that contrasted between the strains (**Figure 4A**). At the protein level, the Mtb strain BTB1558 induced the most heterogenous response, and due to high individual variation, differences in chemokine/cytokine production between infected PCLS and controls only reached a statistical significance for MIP-1a (CCL3) and IL-8 (**Figure 4A**). These two inflammatory mediators were also strongly induced by all strains. IL-17A, IL-1β, and IFNγ were efficiently induced by mycobacterial infection, and no significant difference was observed between Mtb and Mb. By contrast, IL-6 and IL-1α were significantly induced after Mb, but not Mtb, infection, and IL-8 production was also significantly higher after Mb than Mtb infection (**Figure 4A**). The only strain able to induce a significant production of MIP-1b was Mb3601. We then analyzed the inflammatory transcriptomic signature using a panel of 17 genes involved in monocyte/macrophage and neutrophil recruitment (**Figure 4B**). A number of these genes was significantly upregulated upon PCLS infection even though significant differences were not always reached due to inter-individual variation. Remarkably, Mb3601 induced the strongest inflammatory response, with five out of 17 genes significantly upregulated compared to non-infected controls. Focusing on chemokines involved in neutrophil recruitment, we observed that *CXCL2* expression was induced by all strains—except BTB1558—whereas *CXCL1*, *CXCL5*, and *CXCL8* were only upregulated by Mb3601 (**Figures 4B,C**). *IL-6* expression was also high after Mb3601 infection. Therefore, the *ex vivo* infection of PCLS efficiently triggered signals involved in monocyte and neutrophil recruitment. Infection by Mb strains, more specifically the Mb3601 strain circulating in France, triggered inflammation in the bovine lung more efficiently than Mtb.

The Type I Interferon Pathway Is Induced in the Bovine Lung by Infection With *M. bovis*, but Not *M. tuberculosis*

Because in humans and mouse models susceptibility to mycobacterial infection and disease progression is driven by type I IFN (35–37), we decided to compare the induction of this pathway by Mtb and Mb strains in bovine lung tissue. We measured the expression of different genes involved in the type I IFN pathway in Blonde d'Aquitaine PCLS infected by the four mycobacterial strains (**Figure 5**). The gene expression of both *IFNβ* and the *IFNAR1* receptor was significantly increased after Mb but not Mtb infection (**Figures 5A,C**). Similarly, the major IFN-stimulated genes (ISG) *MX1*, *OAS1*, *ISG15*, and *CXCL10* were induced only after Mb infection (**Figures 5A,C**), and this difference was also detected at the protein level for CXCL10 (**Figures 5A,B**). Therefore, we observed the induction of a number of genes of the type I IFN pathway, recapitulated in **Figure 5D**, after infection with Mb, but not Mtb, strains. Strikingly, strain Mb3601 was the highest inducer of this pathway in the lung from Blonde d'Aquitaine cows.

Because AMPs are the most prominent host cells interacting with Mb (8), which we also observed in PCLS (**Figure 2**), we next decided to decipher if AMPs contributed to the induction of the type I IFN pathway after Mb3601 or H37Rv infection. At 1 day after the infection of AMPs with these two strains, similar bacterial levels were recovered (data not shown). At 6 h post-infection, no cell cytotoxicity was observed, and we analyzed the expression of genes from the type 1 IFN pathway at this early time point. While we did not observe differences in *IFNAR1*, *IRF3*, *STAT1*, nor *ISG15* expression induced by the two strains (**Figure 6A**), *IFNβ*, *LPG2*, *RIG1*, and *OAS1* were significantly induced after infection with Mb3601, but not H37Rv (**Figure 6B**). Regarding *MX1*, the same trend was observed, although statistical significance was not reached (**Figure 6B**, $p = 0.07$).

Interestingly, while CXCL10 was detected both at the mRNA and protein levels in PCLS infected with Mb (**Figure 5**), we did not detect the expression of this gene by AMPs in our analysis. These results altogether demonstrate that AMPs globally contribute to the type I IFN pathway in the lung after Mb infection, although other cells present in PCLS may also



C

	Gene ID	AF2122	Mb3601	H37Rv	BTB1558
	IFNβ	**13.1 ***	**3.5 ***	2.1	1.7
Innate sensors	IFNAR1	**3.2 ***	**3.0 ***	1.2	3.3
	LPG2	**15.8 ***	**40.7 ***	3.9	5.0
	RIG-I	11.8	**7.3 ***	1.4	4.9
	TLR3	4.8	3.7	1.4	5.3
	TLR7	8.6	3.7	1.9	4.4
Signaling molecules	IRF3	17.9	4.7	1.3	7.3
	IRF7	5.9	9.4	1.1	7.9
	IRF9	nd	nd	nd	nd
	MAVS	nd	nd	nd	nd
	STAT1	38.0 *	55.3 *	49.2 *	29.3 *
	STING	nd	nd	nd	nd
IFN-stimulated genes	IFIT3	nd	nd	nd	nd
	ISG15	**3.9 ***	**2.3 ***	1.7	1.0
	Mx1	13.0	**26.2 ***	4.3	5.2
	OAS1	12.3	**6.5 ***	2.2	9.9
	CXCL10	5,9	**9.0 ***	3.6	8,1

D

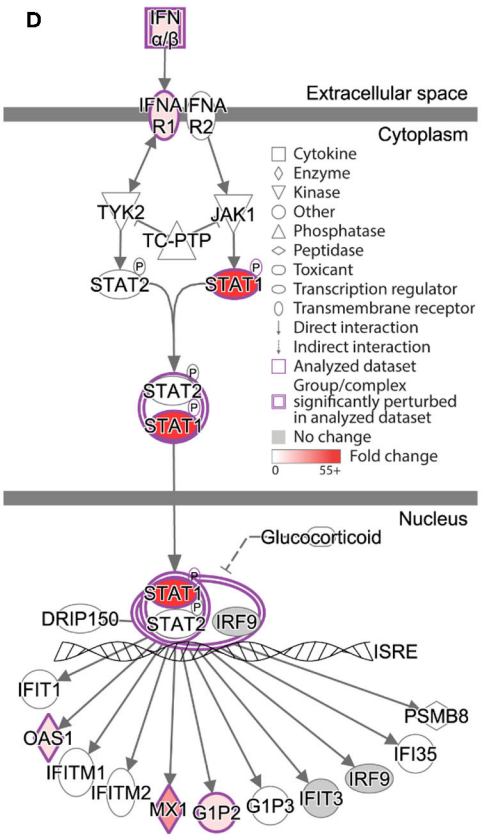


FIGURE 5 | Mb but not Mtb infection in the lung tissue from Blonde d'Aquitaine cows induces the type I interferon pathway. The precision-cut lung slice (PCLS) was infected as described in **Figure 1**. **(A)** *IFNAR*, ISG15, *CXCL10*, and *OAS1* gene expression at 2 dpi. Individual data and the median and interquartile range in each group are presented ($n = 7$). **(B)** CXCL10 protein level was measured in PCLS supernatant at 2 dpi. Individual data and the median and interquartile range in each group are presented ($n = 6$). **(C)** The table represents the mean of fold change (2^{-dCT}) for each group ($n = 7$) of major genes involved in type I interferon pathway. The graduated red box coloring is for higher gene expression, and the asterisks mark significant differences compared to uninfected PCLS. nd, not detected. **(D)** Ingenuity pathway analysis drawing of the type I interferon pathway under IFNAR in the Mb3601 group. The graduated red box coloring is for higher gene expression. **(A–C)** *$p < 0.05$ (Wilcoxon nonparametric test).

A

	Gene ID	Mb3601	H37Rv
	IFNβ	**3.1 ***	1.3
Innate sensors	IFNAR1	1.0	1.2
	LPG2	**2.9 ***	1.0
	RIG-I	**2.1 ***	1.6
Signalling molecules	IRF3	1.1	0.9
	IRF7	nd	nd
	STAT1	1.1	0.9
IFN-stimulated genes	ISG15	1.1	1.1
	MX1	2.3	1.4
	OAS1	**1.9 ***	0.9
	CXCL10	nd	nd

B

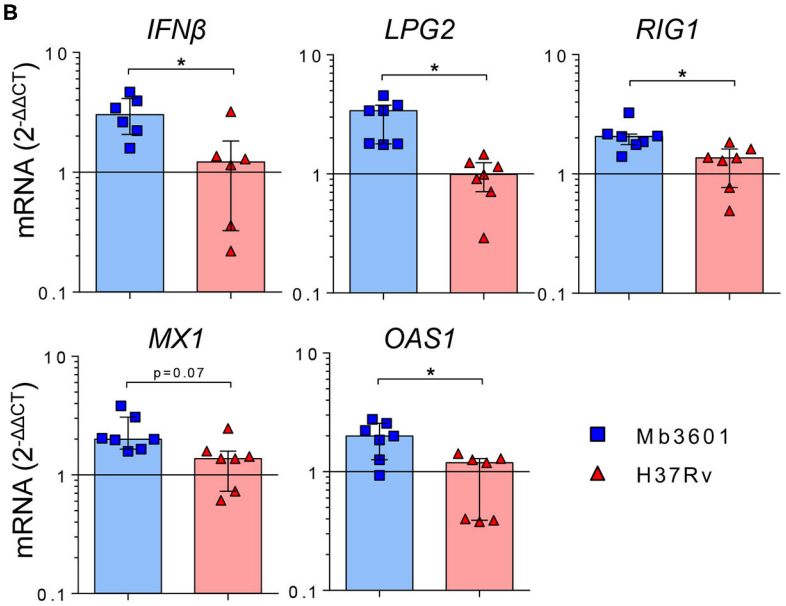


FIGURE 6 | Alveolar macrophages (AMP) from Blonde d'Aquitaine contribute to the type I IFN signature in the lung induced by Mb infection. AMPs from Blonde d'Aquitaine lungs were infected with 10^5 CFU of Mb3601 or Mtb H37Rv. At 6 h later, mRNA was extracted, and the expression of major genes from the type 1 IFN pathway was analyzed. **(A)** Mean fold change ($2^{-\Delta\Delta CT}$) of gene expression normalized to three housekeeping genes was calculated in each group ($n = 7$). The graduated red box coloring represents gene expression, and the asterisks mark significant differences compared to non-infected controls (nd, not detected). **(B)** *IFNβ*, *LPG2*, *RIG1*, *MX1*, and *OAS1* gene expression in AMPs was analyzed by RT-qPCR at 6 h post-infection. Individual data and the median and interquartile range in each group are presented ($n = 7$). **(A,B)** *$p < 0.05$ (Wilcoxon nonparametric test).

specifically induce some genes, such as *CXCL10* or *IRF7*, for example (**Figures 5C**, **6A**).

DISCUSSION

The lung is the main organ targeted by Mb infection in cattle (38), and early interactions between the different lung cell types and the bacillus that govern the pathophysiology of the disease need to be better understood. In this study, we used PCLS for the first time to monitor the early bovine lung response to Mb infection and validated this model as a means to measure the local innate response at the protein and mRNA level. A main advantage of PCLS is conservation of the complex lung tissue both in structure and diversity of cell types. After infection with mycobacteria, the ciliary activity of bronchial cells was maintained. The AMP main function is to patrol the lung, crawling in and between alveoli; they sensed, chemotaxed, and phagocytosed debris or inhaled bacteria (39). We observed increased numbers of AMPs in alveoli where Mb was present, indicating AMP mobility inside the tissue. In chicken, PCLS allowed the observation of the movement of macrophages and phagocytosis (30). The AMP is well established as the main host cell for Mtb infection in humans (40) and Mb infection in cattle (41). Accordingly, in PCLS, we observed Mb inside AMPs in 20% of infected alveoli. We sometimes observed several bacilli inside one AMP. Although Mb is able to replicate inside this hostile cell, it is difficult to know if this observation was due to bacillary multiplication or the phagocytosis of several bacilli. This issue would need live imaging of PCLS to follow the fate of fluorescent Mb, an approach which remains challenging under BSL3 conditions.

In uninfected PCLS, we observed generally one AMP for two to three alveoli [**Supplementary Figure 4**; in good correlation with the observations of Neupane et al. (39)]. After Mb infection, we observed several AMPs inside the same alveolus in 50% of cases. Moreover, when the alveoli contained more than four AMPs, they were in close contact. Multinucleated giant cells are formed by the fusion of several MPs and are a hallmark of TB pathophysiology. It has recently been demonstrated that, after infection of human or bovine blood-derived MPs by Mb or Mtb, only Mb was able to induce the formation of multinucleated cells (26). Although at 2 dpi we did not observe the formation of such cells in PCLS, it would be interesting to analyze if such events could be detected after longer infection periods. Goris et al. have maintained bovine PCLS during 1 week to study viral infections (27).

One other advantage of our model is the preserved diversity of lung cell composition. PCLS contain type I and II pneumocytes, endothelial cells, and bronchial cells (**Supplementary Figure 4**) and also produce key molecules like surfactant, which has an established role in Mtb uptake (42). Mtb is also capable of invading type II alveolar epithelial cells (23) that play important roles in host defense (20–22). In our study, we did not observe intraepithelial Mb, but specific labeling of bovine epithelial cells would be required to investigate interactions between bovine lung pneumocytes and Mb in more detail. However, as we have

observed that infected AMPs were in close contact with epithelial cells in PCLS, this model will allow a more refined analysis of the crosstalk between AMPs and pneumocytes during Mb infection (24).

One limitation of the PCLS model is the lack of recruitment of immune cells from circulating blood. During mycobacterial infection, in response to local signals, a variety of immune cells are recruited to the infection site to form the mature granuloma that constrains bacillary multiplication. How this response is orchestrated at the level of the lung tissue in cattle remains poorly established. Neutrophils, together with other innate cells, such as macrophages, γδ-T lymphocytes, and natural killer cells, were recently identified as key immune cells in the early containment of infection (43) and development of early lesions (44). Moreover, humans regularly exposed to Mtb or cattle exposed to Mb do not always develop signs of infection, i.e., remain negative in IFNg-release assay or skin testing. In humans, such resistance to infection through the successful elimination of bacilli could be mediated by neutrophils (45). Similarly, in cattle experimentally infected with Mb, some contact animals resist infection, while others develop lesions due to productive infection (46). It is possible that neutrophils could also play an important role in the early elimination of Mb in cattle (43). Immune signals involved in the early recruitment of neutrophils to the lung after the entry of Mb need to be better understood in cattle. It is known that epithelial cells secrete, among other cytokines and chemokines, MIP1 and CXCL8 that attract MPs and neutrophils to the site of infection. Interestingly, we measured important differences in the production of such mediators by PCLS in response to different strains of mycobacteria that could be linked to variable virulence. Although one cattle type II pneumocyte cell line has been described (47), such transformed cells are less physiologically relevant than primary cells. Recently, immortalized type II cells were co-cultured with endothelial cells as a model of the bovine alveolus to study mycobacterial interactions with BCG. In this study, the authors detected the production of IL-8, TNFα, IL-22, and IL-17a. One limitation of this model was epithelial cell death, which occurred shortly after infection (48). As a physiological model, PCLS could help in understanding the early orchestration of the local inflammatory response in the lung in response to mycobacterial infection.

Resistance to bTB is linked to the host genetics. Zebu breeds (*Bos indicus*) are more resistant to bTB disease than *Bos taurus*-derived breeds (49). Our results with PCLS, as a physiological model of the early lung response to infection, demonstrated striking differences between Blonde d'Aquitaine and Charolaise, emphasizing the importance of host genetics in response to Mb. It is not known whether the stronger inflammatory response of the Blonde d'Aquitaine tissue is associated with a greater sensitivity or resistance to Mb infection. While robust immunological responses are associated with an increased pathology at the level of the animal (31), at the cellular level, blood-derived MPs from animals with greater resistance to bTB (and that kill BCG more efficiently than cells from susceptible animals) produce higher levels of the pro-inflammatory mediators iNOS, IL-1β, TNFα, MIP1, and MIP3 (25). Although genetic selection of cattle would greatly complement bTB management and surveillance programs to control and ultimately eradicate the disease, especially in countries with the highest burden (50, 51), biomarkers to evaluate the resistance or susceptibility of cattle to Mb infection are critically missing. Some genomic regions and candidate genes have been identified in Holstein-Friesian cows, the most common dairy breed (52), and not surprisingly, these candidates are often involved in inflammation. A genomic region on chromosome 23, containing genes involved in the TNFα/NFκ-B signaling pathway, was strongly associated with host susceptibility to bTB infection (53). However, large within-breed analyses of Charolaise, Limousine, and Holstein-Friesian cattle identified 38 SNPs and 64 QTL regions associated with bTB susceptibility to infection (54). The genotyping of 1966 Holstein-Friesian dairy cows that were positive by skin test and either did or did not harbor visible bTB lesions, together with their skin test negative matched controls, led to the conclusion that these variable phenotypes following Mb exposure were governed by distinct and overlapping genetic variants (55). Thus, variation in the pathology of Mb seems to be controlled by a large number of loci and a combination of small effects. Similar conclusions were drawn from the genetic studies of human tuberculosis (56). In areas where Mb is highly prevalent, recurrent exposure to Mb may also imprint the bovine genome, and epigenetics could also contribute to the immune response in certain breeds. In France, the Nouvelle Aquitaine region accounted for 80% of Mb outbreaks last year. Interestingly, Blonde d'Aquitaine breed is very abundant in this area (**Figure 7**). Together with Limousine, another very abundant beef breed in this region, they contribute to most bTB outbreaks in Nouvelle Aquitaire (bovine tuberculosis national reference laboratory communication). In the future, comparisons between Blonde d'Aquitaine and Limousine would be interesting. In our study, Blonde d'Aquitaine or Charolaise cows were sampled from eight different French departments, none with recurrent Mb outbreaks, rendering previous exposure to Mb unlikely. Moreover, the breeding management was similar for the two breeds, as far as we could ascertain, suggesting that exposure to environment and possible wildlife sources would be comparable. We nevertheless observed striking differences in the early lung response to Mb infection between these two breeds, pointing to the possible control of Mb infection at the genetic or epigenetic level. Whether some cattle breeds are more susceptible to bTB than others remains an open question that deserves future studies with more consequent animal sampling. We furthermore believe that the PCLS model could greatly contribute to unraveling the role of tissue-level protective responses that would, in turn, reveal important biomarkers.

In addition to the cattle breed, our study pointed toward differences in the host response to distinct mycobacterial strains. The Mb strains were better inducers of a lung immune response than Mtb in cattle, which is in agreement with a previous work showing that Mtb H37Rv was attenuated *in vivo* in cattle compared to Mb AF2122 (13). *In vitro* studies with bovine AMPs infected with AF2122 or H37Rv revealed differences in the innate cytokine profiles: the CCL4, IL-1β, IL-6, and TNFα levels were more elevated in response to AF2122 than H37Rv (8), which is in agreement with our data. Interestingly, Mb3601,

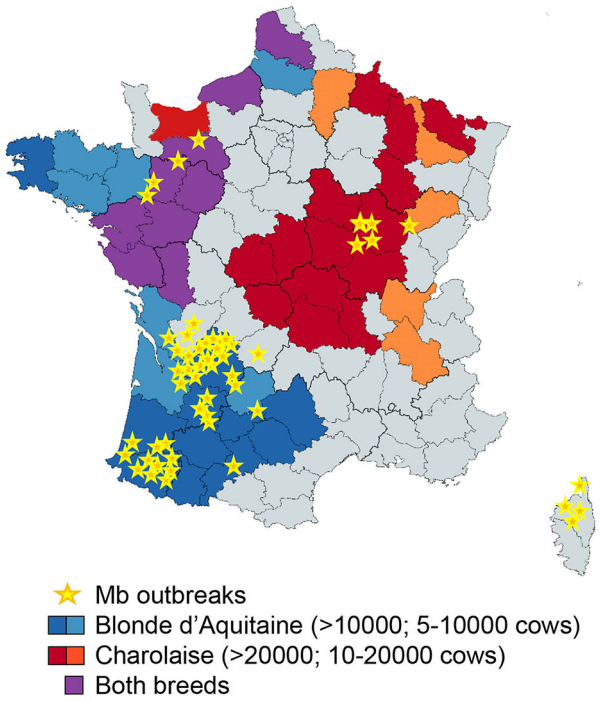


FIGURE 7 | Superposition of Blonde d'Aquitaine and Charolaise beef breeds in French counties where Mb outbreaks were declared between December 2019 and 2020. This map of France shows the counties where Mb outbreaks were declared between December 2019 and December 2020 (yellow stars) and was obtained with data extracted from https://www.plateforme-esa.fr/. Herd densities of Blonde d'Aquitaine (blue), Charolaise (red), or both breeds (violet) were extracted from data obtained from https://www.racesdefrance.fr/ (cows above 3 years old have been considered).

a representative strain of a highly successful genetic cluster that circulates both in cattle and wildlife in France (16), induced an inflammatory signature in the lung more efficiently than Mb AF2122. Whether this correlates with differences in Mb virulence in cattle or other mammals remains to be investigated; but, if this were the case, the PCLS model would be a practical tool to study and compare the virulence of Mb field strains compared to the *in vivo* experimental infection of cattle. Contrary to Mtb which is mostly restricted to humans, Mb is adapted to sustain across a large host range through repeated cycles of infection and transmission (57, 58). This remarkable trait is due to pathogen molecular genetic changes (59) that allow adapted bacilli to manipulate the host immune response to establish infection and disease and ultimately transmit infection to new, susceptible hosts (60, 61). We observed a weaker inflammation in the bovine lung after infection with Mtb compared to Mb, and it will be interesting to compare the ability of Mtb and Mb to induce inflammation in human PCLS obtained post-surgery. This latter comparative analysis could give clues on the links between lung innate inflammatory responses and host adaptation during TB.

Our most striking observation was the Mb-restricted induction of the type I IFN pathway in the bovine lung. This is in agreement with previous studies in bovine AMPs where cytosolic DNA-sensing pathways, in particular, RIG-I, were activated after 48 h of infection by Mb AF2122, but not Mtb H37Rv (32). In agreement with our data, these authors also demonstrated an induction of the RIG-I signaling pathway by Mb in AMPs (62). Therefore, AMPs contribute to type I IFN signaling in the lung. However, we also noticed differences between PCLS and AMPs in the induction of the IFN signature by Mb: for example, CXCL10 was detected in PCLS, but not in AMPs, in our study, which may be due to the time point used (63). However, it is also possible that other cells involved in crosstalk with AMPs contributed to CXCL10 production in response to Mb infection. Since CXCL10 has been proposed as a diagnostic biomarker of Mb infection in cattle (64), it will be interesting to better understand how this key mediator is regulated. Type I interferon favors Mb survival, and its induction may be a good manipulation strategy for the maintenance of infection. This manipulation mechanism, deciphered *in vitro* in murine bone marrow monocyte-derived MPs, involves the triggering of autophagy by cytosolic Mb DNA, in turn inducing IFNβ production. Autophagy antagonizes inflammasome activation to the benefit of Mb survival (65, 66). In C57BL/6 mice treated with IFNAR1 blocking Ab and infected with Mb, the recruitment of neutrophils was reduced, but the pro-inflammatory profile of MPs was increased, leading to a reduced bacillary burden (67). No impact on T-cells was observed in this *in vivo* model, revealing a role of type I IFN signaling during the innate phase of the host response to infection. Therefore, Mb exploits type I IFN signaling in many ways, and this pathway seems an important avenue to better understand Mb virulence. The PCLS model will greatly help to better dissect out this pathway in the lung during bTB. This could lead to new biomarkers to help genomic selection programs for cattle that are more resistant to bTB as well as new immunostimulation strategies counteracting the type I IFN pathway. This new knowledge will ultimately improve bTB control, a goal which is so greatly needed at the global level (68).

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 We only used post-mortem sampling at commercial abattoir.

AUTHOR CONTRIBUTIONS

AR designed and did most of the experiments, obtained funding, analyzed the data, prepared all the figures, and wrote the manuscript. FC performed experiments and prepared the inocula for experimental infections under BSL3 conditions. AC cultured AMPs and performed ELISA and q-RT-PCR. ED-D helped in PCLS experiments and revised the figures. MB provided the

Mb3601 strain and revised the manuscript. AA provided the strain Mtb BTB1558. JB improved the RNA extraction protocol. DD and QM performed multiple experiments, and revised the manuscript. FA provided Ab and critically reviewed the imaging data. PG helped with transcriptomic analysis and revised the manuscript. SG obtained funding, designed the experiments, and revised the manuscript. NW obtained funding, supervised all aspects of the work, critically analyzed the data, and wrote the manuscript. All the authors read and approved the manuscript before publication.

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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.2021.696525/full#supplementary-material

Supplementary Table 1 | Sequences of primers used in this study. The primers were designed, using Geneious software, in intron-spanning regions when possible. The annealing temperature was set at 60°C. Housekeeping genes used as the reference to calculate ΔCT are indicated in the gray boxes.

Supplementary Figure 1 | Age and geographical origin of the cows used in the study. The Charolaise and Blonde d'Aquitaine cows used were between 3 and 11 years old and came from eight different French departments. Two Blonde d'Aquitaine cows came from the same farm in Indre et Loire, and three Charolaise cows came from the same farm in Sarthe. All the other animals are from distinct farms. The data represent the age of individual animals and the median and interquartile range.

Supplementary Figure 2 | Transcriptomic signature after infection with different doses of mycobacteria. Bovine precision-cut lung slices were obtained as described in **Figure 1** and infected with 10^5, 5×10^5, 10^6, or 5×10^6 colony-forming units. The RNA was extracted 2 days post-infection, and *SDC4*, *CXCL1*, *HIF1*, and *OAS1* gene expressions were assessed with the Fluidigm Biomark. Individual data and the mean and standard deviation in each group are presented ($n = 3$ Charolaise). The dotted line represents the level of expression in the uninfected group.

Supplementary Figure 3 | Cytokines/chemokines in precision-cut lung slice (PCLS) supernatants. The protein levels were measured in PCLS supernatant at 2 days post-infection with Multiplex. Individual data and the median and interquartile range in each group are presented ($n = 6$). $^*p < 0.05$ (Wilcoxon nonparametric test).

Supplementary Figure 4 | Structure of the bovine precision-cut lung slices (PCLS) under a light microscope. The PCLS were observed under a light microscope (enlargement ×40 to ×200). The PCLS contain numerous alveoli and between one to three bronchioles, with thick and wavy epithelium that can be easily recognized (black asterisk, two views from the same area under two enlargements). Thin blood vessels (red dotted lines) were localized next to the bronchioles and diffused between the alveoli. No blood cells remained inside the endothelium (the cows were bled out at the abattoir). Alveolar macrophages can be seen inside the alveoli (black arrows).

Supplementary Figure 5 | Localization of Mb3601-GFP in bovine precision-cut lung slices (PCLS). The PCLS were fixed at 2 days post-infection with 10^5 colony-forming units of Mb3601-GFP recombinant strain and labeled with anti-pancytokeratine and anti-MHCII antibodies, which, respectively, revealed anti-pancytokeratine and Alexa 555 conjugated secondary Ab. The PCLS were transferred on cover slides and mounted with Fluoromount-G™ mounting medium containing DAPI. **(A)** The 3D images were analyzed with Leica LAS software. Z-stack imaging was performed at ×63 enlargement with a confocal microscope (10–15 μm in thickness, step size of 0.5–1 μm). Dotted white lines are drawn on the alveoli structure. **(B,C)** Crosshead sections illustrating Mb3601 inside **(B)** or near **(C)** an alveolar macrophage. X and Y projections are seen on the bottom and to the right of the picture; the intracellular localization of Mb3601-GFP is indicated by color merging (green + red = yellow). The results from one representative animal are shown (a total of $n = 4$ animals were analyzed).

Supplementary Video 1 | Internalization of Mb3601 in alveolar macrophages after precision-cut lung slice (PCLS) *ex vivo* infection. The PCLS was fixed at 2 dpi with 10^5 colony-forming units of Mb3601-GFP recombinant strain and labeled with anti-pancytokeratine and anti-MHCII antibodies, which, respectively, revealed anti-pancytokeratine and Alexa 555 conjugated secondary Ab. The PCLS was transferred on cover slides and mounted with Fluoromount-G™ mounting medium containing DAPI. Z-stack imaging was performed at ×63 enlargement with a confocal microscope. The 3D images were analyzed with Leica LAS software.

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Human-to-Cattle *Mycobacterium tuberculosis* Complex Transmission in the United States

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The *Mycobacterium tuberculosis* complex (MTBC) species includes both *M. tuberculosis*, the primary cause of human tuberculosis (TB), and *M. bovis*, the primary cause of bovine tuberculosis (bTB), as well as other closely related *Mycobacterium* species. Zoonotic transmission of *M. bovis* from cattle to humans was recognized more than a century ago, but transmission of MTBC species from humans to cattle is less often recognized. Within the last decade, multiple published reports from around the world describe human-to-cattle transmission of MTBC. Three probable cases of human-to-cattle MTBC transmission have occurred in the United States since 2013. In the first case, detection of active TB disease (*M. bovis)* in a dairy employee in North Dakota prompted testing and ultimate detection of bTB infection in the dairy herd. Whole genome sequencing (WGS) demonstrated a match between the bTB strain in the employee and an infected cow. North Dakota animal and public health officials concluded that the employee's infection was the most likely source of disease introduction in the dairy. The second case involved a Wisconsin dairy herd with an employee diagnosed with TB disease in 2015. Subsequently, the herd was tested twice with no disease detected. Three years later, a cow originating from this herd was detected with bTB at slaughter. The strain in the slaughter case matched that of the past employee based on WGS. The third case was a 4-month-old heifer calf born in New Mexico and transported to Texas. The calf was TB tested per Texas entry requirements and found to have *M. tuberculosis*. Humans are the suspected source of *M. tuberculosis* in cattle; however, public health authorities were not able to identify an infected human associated with the cattle operation. These three cases provide strong evidence of human-to-cattle transmission of MTBC organisms and highlight human infection as a

potential source of introduction of MTBC into dairy herds in the United States. To better understand and address the issue, a multisectoral One Health approach is needed, where industry, public health, and animal health work together to better understand the epidemiology and identify preventive measures to protect human and animal health.

Keywords: *Bovine tuberculosis*, zoonotic disease, human-to-cattle transmission, public health, dairy employees

INTRODUCTION

The primary causative agents of human and bovine tuberculosis (bTB) in North America, *Mycobacterium tuberculosis* and *Mycobacterium bovis*, respectively, are included in the *Mycobacterium tuberculosis* complex (MTBC). Other members of the MTBC include *Mycobacterium orygis, Mycobacterium caprae, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium mungi,* and *Mycobacterium suricattae* (1). The MTBC species are so closely related that they are now considered a single species, *M. tuberculosis*, with variants (1, 2).

Worldwide, tuberculosis is the leading cause of human deaths by any single infectious agent and responsible for approximately 1.2 M deaths in HIV-negative people in 2019 (3). Tuberculosis was a leading cause of human morbidity and mortality in the United States at the beginning of the twentieth century. A study conducted in 1912 reported that 66% of New York children diagnosed with tuberculosis (TB) in 1910 were infected with *M. bovis* (4). Based on the transmission of *M. bovis* to children through milk, multiple jurisdictions enacted laws requiring pasteurization. Cincinnati was the first city to establish pasteurization requirements in 1897; New York City followed in 1898 (5). Michigan became the first state to require pasteurization of milk in 1948 and all states have since followed suit. In addition to milk, meat from *M. bovis*-affected cattle is also a potential risk for human infection. Accordingly, the Federal Meat Inspection Act of 1906 (6, 7) gave the U.S. Department of Agriculture (USDA) the authority to inspect cattle before, during, and after slaughter as another tool for preventing zoonotic transmission of *M. bovis*. Lesions detected during this inspection, or entire carcasses if necessary, could be removed from the food chain. Not only did this reduce the risk of human infection, but also cattle with bTB could be identified and trace-back investigations to the herd of origin allowed for identification of bTB-infected herds. Slaughter inspection is the primary means of bTB surveillance in the United States today.

In addition to implementing these two mitigation strategies to reduce human exposure to *M. bovis*, state and federal authorities designed and implemented the U.S. Cooperative State-Federal Bovine TB Eradication Program to reduce the prevalence in cattle populations. The program officially began in 1917; at that time, approximately 1 in 20 cattle were infected with *M. bovis* (8). Reviews of the program and its progress have been published (9–12). Current estimates of animal- and herd-level prevalence of *M. bovis* in the United States are <0.002 and <0.006%, respectively (12). The partnership between State and Federal Animal Health Officials to conduct testing, share data, and conduct disease investigations has been critical to these advancements. In other parts of the world, including parts of Mexico and Central and South America, *M. bovis* infection rates in cattle and other hoof stock are much higher and serve as an important source for human disease (13). Additionally, consumption of unpasteurized dairy products remains a primary cause of human infections with *M. bovis* in North America (14–16).

Despite the early success of the program in the United States, the number of newly identified bTB-affected herds each year has remained relatively steady for the past 30 years (17). The advent of whole genome sequencing (WGS) has markedly advanced our ability to link sources of infection based on genetic similarities. For example, in Michigan WGS routinely supports wildlife as a source of infection. However, for cases outside of Michigan, even after extensive epidemiological investigations, including WGS and trace-back of purchased cattle, the source of disease and method of introduction into a herd is determined only about 40% of the time (17). Without a source of infection, risk mitigation, and disease eradication remain elusive.

Humans are considered the primary host for *M. tuberculosis* and animals are considered accidental hosts (18, 19). Human-to-animal transmission of MTBC organisms, primarily *M. tuberculosis*, is well-documented in many countries around the world. Many of these reports confirm finding *M. tuberculosis* in tissues and fluids from cattle (20–32) and also other animals, including dogs (33), non-human primates (34), elephants (35, 36), and parrots (37, 38). Most reports of cattle with *M. tuberculosis* are from countries where human *M. tuberculosis* prevalence is very high and likely to have been the source of introduction into the cattle populations.

With the technology now available to obtain DNA sequences of *M. bovis* isolates found in U.S. cattle, it is easier to discern the origin of certain *M. bovis* strains. This information may provide possible modes of transmission based on the most common ancestor in the genomic database and similarity of DNA sequences over time. Sharing isolate DNA sequence data between animal and public health will further our understanding of the complex transmission of MTBC organisms between humans and animals.

The objectives of this paper are to present the identified cases of human-to-cattle MTBC transmission in the United States and to highlight the importance of a multisectoral One Health approach in detecting and addressing human-to-cattle transmission of these important human and animal pathogens.

MATERIALS AND METHODS

Case Selection

Records from the 174 bTB affected livestock herds identified in the USA between 1998 and 2020 were reviewed. As part of the State Federal Cooperative Bovine Tuberculosis program, each

herd had been extensively investigated under that cooperative umbrella and if possible, the likely source of the infection was identified and investigated. Also included in this review were 422 records for tuberculous confirmed animals between 2001 and 2020 not associated with a herd, such as feedlot and dairy heifer development facilities.

Inclusion criteria were as follows: All *Mycobacterium tuberculosis* cases detected in livestock; and zoonotic tuberculosis (all were *M. bovis*) cases where humans associated with livestock were identified with active tuberculosis prior to the detection within the herd.

Public Health Investigations

Public health authorities in the U.S. investigate all reported cases of TB disease in humans to ensure patients quarantine until non-infectious and receive and complete appropriate antimicrobial treatment. Contact investigations are routinely performed when patients have pulmonary TB and are considered infectious. Close contacts are screened for exposure risk and tested to determine their TB infection and disease status. The public health investigation may identify animals (especially livestock and captive wildlife) with an epidemiological link to the infectious patient. In North Dakota and Wisconsin, the public health agency alerts their animal health partners and a One Health investigation may be deemed necessary. All information on the human cases contained in this paper was collected during the normal process that occurs in these states when humans with TB are detected.

Phylogenetic Analysis

Whole genome sequences were obtained from both animal health and public health investigations. NVSL's in-house vSNP pipeline was used for the analysis (see https://github.com/USDA-VS/vSNP). vSNP is a reference based, two-step pipeline. Briefly in step one, sequences were aligned to a reference; those identified as *M. bovis* were aligned to the reference genome AF2122/97 (GenBank accession NC_002945.4), and those identified as *M. tuberculosis* were aligned to the reference genome H37Rv (GenBank accession NC_000962.3). The alignment was performed using Burrows-Wheeler Aligner (BWA) (39) and SNPs were called using Freebayes (40). The variant call format (vcf) files created in step one were then added to a database of vcf files and step two was initiated which filters or flags unreliable and low quality variant calls, as well as groups sequences into user defined clades according to relatedness by identifying common SNPs. For each user defined group, step two outputs SNP tables in Excel, an aligned FASTA file and phylogenetic trees constructed with RAxML (41) using the aligned whole-genome SNP sequences under a GTR-CAT model of substitution and a maximum-likelihood algorithm. The annotated and position referenced SNP tables allow for quick error identification and correction. The trees were then manually compared to the SNP table to ensure accuracy of the model.

Tree visualization, annotation, and editing was performed with FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and iTOL (42). **Supplementary File 1** lists the accession numbers for the publicly available sequences from previous studies (17, 43, 44).

RESULTS

Three cases met the criteria for inclusion, two M. *bovis* affected dairy herds, and one *M. tuberculosis* infected 4-month-old calf at a dairy heifer development facility. Those cases are described in detail below.

Case 1: North Dakota Dairy Herd

In October 2013, the North Dakota Department of Agriculture (NDDA), State Board of Animal Health was notified by the North Dakota Department of Health (NDDOH) that an employee at a North Dakota dairy was diagnosed with pulmonary TB with cavitary lung lesions. The case-patient, a man born in Mexico, had been recently diagnosed with another medical condition that likely suppressed his immune system resulting in active TB disease. The case-patient worked for the dairy for at least 3 years prior to his diagnosis. During this time, he worked for 9 consecutive months then returned to Mexico for the remaining 3 months each year. During his employment, the case-patient worked with all ages of dairy cattle. NDDOH tested three household contacts to the case-patient. All were latently infected with TB (LTBI) and were treated prophylactically with a 4-month course of antibiotics per CDC guidelines to prevent future disease.

The North Dakota operation housed 400 dairy cattle and 160 beef cattle. Beef and dairy heifers were often commingled for a few months each year. While the operation had no record of TB skin testing in the dairy or beef herds in the recent past, cull dairy and beef cattle from this operation were slaughtered at abattoirs with high granuloma submission rates for TB surveillance.

After meeting with the NDDOH's TB controller and state animal health officials, the herd owner agreed to herd testing in November 2013. North Dakota Department of Agriculture veterinarians conducted whole-herd testing in consultation with the USDA. All cows and heifers 6 months of age and older were tested using standard protocol of the caudal fold tuberculin (CFT) test in series with the comparative cervical tuberculin (CCT) test.

A 19-month-old pregnant heifer (ND1) was declared a reactor based on CCT testing. Upon necropsy, multiple micro abscesses were identified in a normal appearing mediastinal lymph node that was culture-positive for *M. bovis* at USDA's National Veterinary Services Laboratories (NVSL). Ten additional cows were CCT-negative and sent to slaughter in WI. Samples were collected from all 10 cows and although no gross lesions were identified, samples were submitted for culture at NVSL. Two of the 10 cows (ND2 and ND3) were determined to be infected with *M. bovis*. Microscopic granulomas in a lymph node that contained acid-fast bacteria from ND2 were PCR positive for MTBC but no *mycobacteria* were identified on culture. Representative, normal appearing lymph nodes from the head and thorax were submitted from ND3; these tissues were histologically negative for evidence of mycobacterial infection, but *M. bovis* was isolated from culture. Tissue from the post-mortem exam and the ear tags were DNA tested to confirm they were from the same animal and no errors were made during sampling, labeling, or processing. Over the course of testing,

approximately 40 cattle were removed from the herd and no additional infected cattle identified.

Whole genome sequencing was conducted and the infected pregnant heifer (ND1) had the identical strain to the dairy employee with active TB disease (**Figure 1**; see **Supplementary File 1** for SNP table). The isolate from ND3 had seven single nucleotide polymorphisms (SNPs) compared with the isolates from ND1 and the case-patient. *Mycobacterium bovis* was not detected in the beef cattle. The infected dairy cattle were born and raised on the dairy operation. Two herds with fence line contact were tested and no infected animals were detected. Additionally, surveillance was conducted on barn cats, wild rodents, and hunter harvested deer with no disease detected. North Dakota Game and Fish Department conducted surveillance in the fall of 2014 on hunter-harvested deer and no lesions were identified. After a thorough investigation, no other possible sources of *M. bovis* were found.

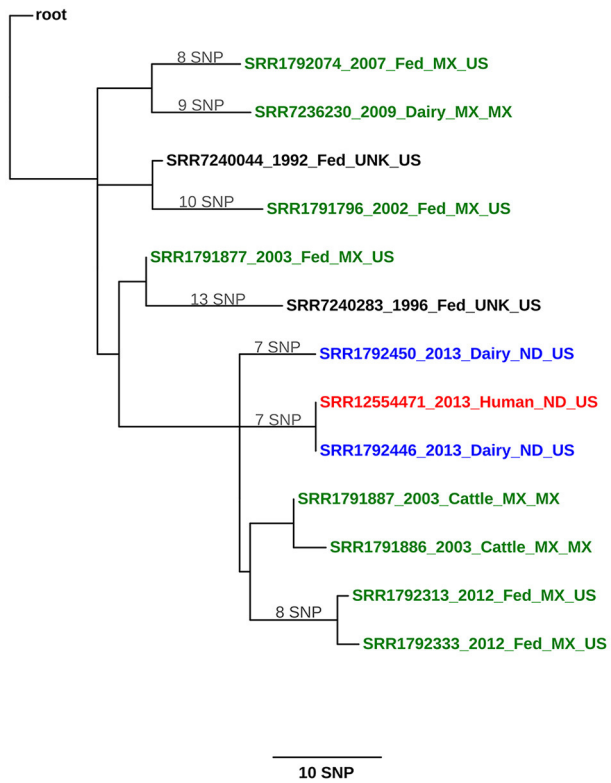

FIGURE 1 | Maximum likelihood phylogenetic tree illustrating the genetic relationship between *M. bovis* isolates from a cattle herd in North Dakota (United States) and a human. The color key indicates the origin of the cattle from which *M. bovis* was isolated: green, Mexico; blue, United States; and black, cattle whose origin could not be traced (unknown). The human isolate is shown in red. Sequences are identified using the following syntax: NCBI SRA accession number_year of isolation_ production type (dairy, fed, cattle [unknown])_geographical origin of the animal (state, country or unknown)_country of detection. The scale bar represents a branch length of 10 SNPs. The tree is rooted to the reference genome *M. bovis* AF2122/97.

Case 2: Wisconsin Dairy Herd

Similar to the identification of the North Dakota herd, the Wisconsin Department of Agriculture, Trade and Consumer Protection (DATCP) was contacted in late April 2015 by the Wisconsin Department of Health Services (WIDHS) about a case-patient with TB disease that worked on a dairy from January to March 2015. The case-patient reportedly became ill in March 2015 before seeking medical care in early April. The case-patient presented in the emergency room with night sweats, cough, and fever. Sputum smears were positive for acid fast bacteria and nucleic acid amplification test (NAAT) was positive for MTBC. Culture revealed the case-patient was infected with *M. bovis*. The case-patient was placed in respiratory isolation and started on a standard four-drug regimen (45). After drug susceptibility testing was complete and pyrazinamide (PZA) resistance detected, PZA was discontinued. Due to severe cavitary disease, the case-patient was treated with TB medications for a full year, with directly observed therapy for the entire course. The case-patient was released from isolation in July 2015 when determined to no longer be contagious. The individual is believed to have become infected while previously living in a Latin-American country where *M. bovis* infections of humans and cattle are prevalent. Public health conducted a routine contact investigation that included TB risk assessment and testing of close contacts to the patient. No additional cases of infectious TB were identified in household or farm employee contacts.

In May 2015, DATCP conducted herd testing, in accordance with USDA guidance, of the 1,500-head herd. Like the ND herd, all cows and heifers 6 months of age and older were testing using the CFT test and CCT-test in series, with no infected animals detected. A single CCT-suspect cow from this first test was euthanized and necropsied with no lesions identified. In September 2015, the herd was tested a second time and was again test negative for bTB with no CCT-positive animals detected. In September 2018, slaughter plant surveillance detected tuberculosis in a carcass from a cow that traced back to this herd, and *M. bovis* was isolated. DATCP conducted another round of whole-herd testing using the CFT and CCT tests in series beginning in October 2018. Seven infected cows were identified during this initial test based on culture of *M. bovis*. Two additional infected cattle were detected during herd testing in March 2019, one infected cow was identified at slaughter in April 2020, and one cow was detected following herd testing in June 2020. Since the detection of the herd as infected, more than 1,500 cows have been examined for bTB by either necropsy or slaughter surveillance. One of the infected cows was test-negative and detected at slaughter. Additionally, surveillance was conducted on wildlife surrounding the premises and included white tailed deer ($n = 232$), raccoons ($n = 10$), and opossums ($n = 6$). One dairy that had heifers housed on the same premises as heifers from the affected dairy including fence line contact will be tested a total of three annual tests (2/3 have been completed and were negative).

At the time of this writing, the herd has had a total of 12 cows, including the slaughter case, detected as infected with *M. bovis*; and the isolates were within a 1–4 SNP difference from the human isolate (**Figure 2**; see **Supplementary File 1** for

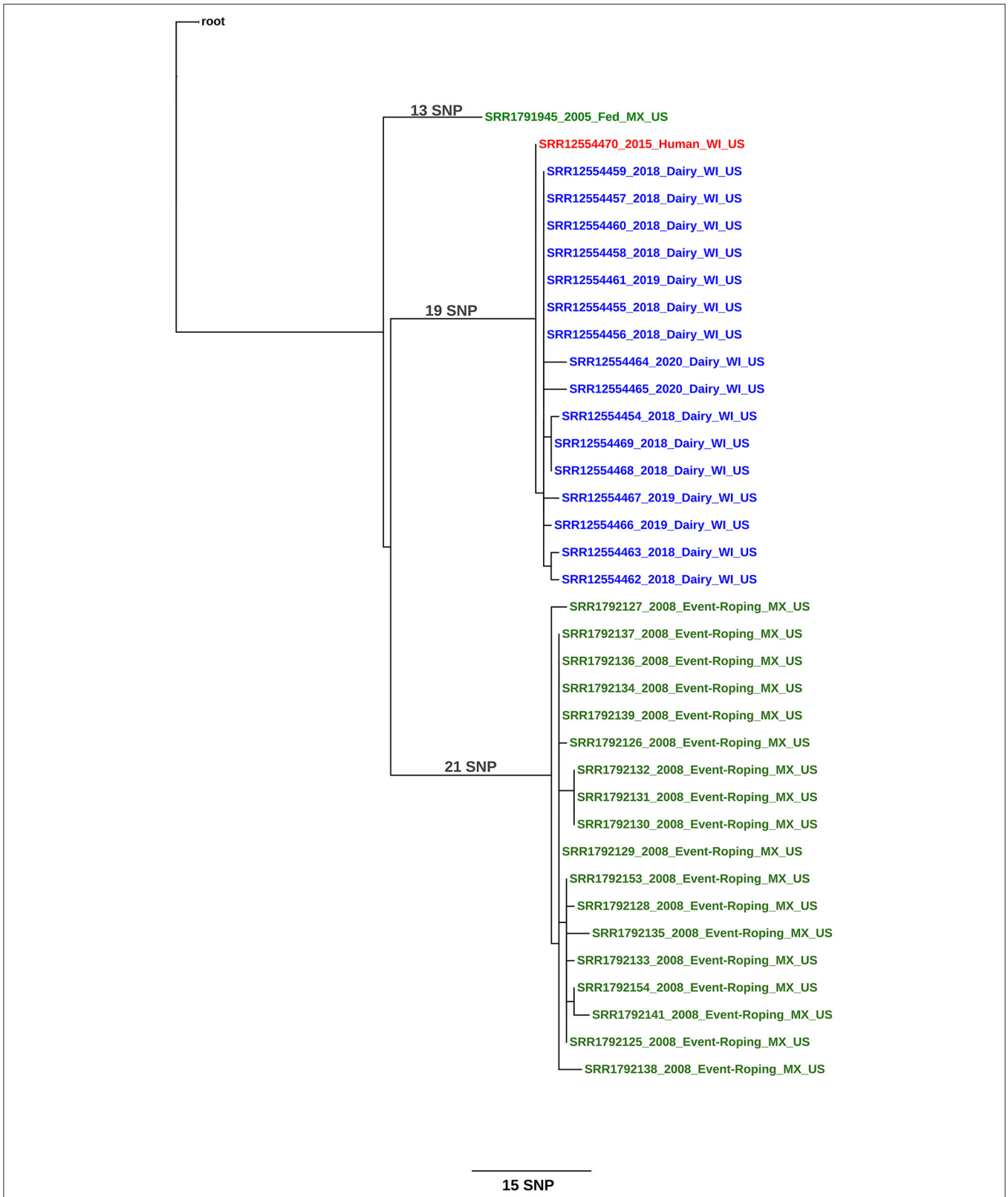

FIGURE 2 | Maximum likelihood phylogenetic tree illustrating the genetic relationship between *M. bovis* isolates a herd in Wisconsin (United States) and a human. The color key indicates the origin of the cattle from which *M. bovis* was isolated: green, Mexico and blue, United States. The human isolate is shown in red. Sequences are identified using the following syntax: NCBI SRA accession number_year of isolation_ production type (dairy, fed, event-roping)_geographical origin of the animal (state, country)_country of detection. The scale bar represents a branch length of 15 SNPs. The tree is rooted to the reference genome *M. bovis* AF2122/97.

SNP table). The public health follow-up with farm employees continues until the farm is released from quarantine. Testing of source herds for purchased cattle added to the Wisconsin herd were not conducted since the epidemiologic investigation and WGS supported the human case-patient as the source of introduction into the herd.

Case 3: Texas Dairy Heifer

In early 2018, a 1-day-old heifer calf from a dairy in New Mexico was transported to Texas where import regulations require post-import TB testing of cattle younger than 2 months of age that move into the state. The heifer was raised at a facility in Texas and was TB test positive in April 2018 at approximately 4 months of age. The heifer was euthanized and necropsied in late April 2018, and tissue samples were sent to NVSL for additional TB testing. There were no gross or microscopic lesions suggestive of MTBC infection. *Mycobacterium tuberculosis* was cultured from the retropharyngeal lymph node collected at necropsy and results were reported in June 2018. The heifer calf isolate grouped with sublineage 4.2.2 (43) and is in NCBI as accession SRR12481506.

The Texas Department of State Health Services (DSHS) conducted a source case investigation to determine if an infectious TB suspect had been identified by public health; however, a source case was not identified. Personnel from DSHS discussed the situation with dairy management, and a dairy employee was identified who exhibited signs and symptoms of TB but was no longer employed at the dairy. Whole genome sequencing of human cases was just being implemented by the Centers of Disease Control and Prevention, and consequently, the previous human cases in this area had not been sequenced. However, both the calf and three adults in the region that were diagnosed in 2017 and 2018 (46) had a matching, rather rare spoligotype for Texas, with an octal code 007000024000000. No employees were tested, but DSHS conducted symptom screening and provided education for the current employees. We were unable to retrospectively obtain WGS from any of the Texas human cases to include in this report.

DISCUSSION

The three cases presented here are the first formal reports of MTBC transmission from humans to cattle in the United States since 1968 (47). In both the North Dakota and Wisconsin cases, the human TB case-patients were exposed to cattle on the operation prior to or at the time bTB was discovered in the cattle. In the North Dakota case, bTB was detected in three dairy cattle within 2 months after the human was diagnosed; in the Wisconsin herd, it was 3 years between the human TB detection and the herd detection.

When bTB-affected herds in the United States are identified, state, and federal animal health agencies conduct epidemiologic investigations to determine the probable source of disease and trace any animals that left the herd to control disease spread. Historically, in the United States the most common sources for bTB infection in cattle have been other infected cattle. The exception to this is in several counties in Michigan where bTB is endemic in the wild white-tailed deer population. Investigations in Michigan have identified deer as the most common source of infection in cattle (48). Animal health authorities conduct interviews with herd owners to determine all potential routes of exposure, including sources of all purchased cattle over a multiyear period and any contact with cattle from other operations, including fence-line contact. These exposures are investigated, and all cattle contacts are tested to determine their bTB status per Bovine tuberculosis eradication uniform methods and rules (49). Additionally, most adult cattle that leave dairies undergo inspection at slaughter for bTB, which is the method by which most infected herds are identified.

Inspection of carcasses at slaughter, or slaughter surveillance has been an effective method of detecting bTB in cattle in the United States. This is highlighted by the WI herd being detected by slaughter surveillance and subsequently, having a test-negative, infected cow that was detected at slaughter. Both the ND and WI herds sent animals to slaughter prior to bTB detection and they continue to do so on a routine basis, providing additional surveillance for bTB. Although infected animals can be missed with slaughter surveillance, 47% of bTB affected herds are detected through slaughter surveillance, excluding herds in the endemic are of Michigan and a localized outbreak in Minnesota (17, 50).

The results of the North Dakota investigation suggest it is highly unlikely that cattle in the herd could have transmitted *M. bovis* to the human. The progression of disease in ND1 appeared to be relatively recent given the finding of *M. bovis* in a single mediastinal lymph node. In the Wisconsin case, the case-patient was diagnosed with TB within 2–3 months of employment. Although *M. bovis* infections in humans have been reported to progress from infection to clinical disease within a few months, more often this progression in humans takes much longer (51, 52). Most of the knowledge of TB in humans is based on infection with *M. tuberculosis* but it might not be the same for *M. bovis* infections.

Additionally, the ND patient's housemates were all tested and considered as LTBI, while the farm family were all negative for bTB. If the cattle were the source of the human infection, one would expect the farm family members to be exposed and potentially infected. The housemates of the patient were exposed to the patient and so it was expected that some or all of them would be infected. None of the patient's housemates were actively shedding *M.* bovis and considered as LTBI.

The North Dakota dairy herd has been tested 9 times since 2013, with only the three infected cows detected at the first herd test, despite this additional testing. Only one of those three infected cows (ND1) had a small gross lesion associated with bTB infection. The WGS of the *M. bovis* strain from the North Dakota case-patient and ND1 were an exact match. Significant epidemiological evidence supports the transmission of *M. bovis* from the employee to the heifer. Evidence includes the degree of illness in the employee with active TB disease (e.g., lung cavitation); the very small lesion in the infected heifer, possibly indicating recent infection; the lack of movement of animals on or off the farm; and the fact that the employee was born in Mexico, a TB-endemic country, and returned annually. Based on the epidemiological and laboratory evidence, the human was

considered the most likely source of disease in the cattle by both animal and public health officials investigating the case.

One of the three bTB-infected cattle, ND2 was PCR positive but culture negative, and no isolate was available for WGS. The last cow, ND3, had normal appearing lymph nodes that were histologically negative but culture positive. The WGS revealed 7 SNP changes from the case-patient and ND1 strain. It is difficult to explain this finding based on the current data but we believe the human patient was likely infected with this strain variation. Humans and cattle have been found to be infected with multiple strains of MTBC, so it is possible the human was infected with more than 1 strain but only 1 was isolated. Unless a more closely related strain is identified in the future, the source of infection for ND3 will remain unknown. Based on the small size of the lesions, however, it is unlikely that ND3 was shedding *M. bovis*. An evaluation of the phylogenetic tree (and **Supplementary File 1**, SNP Table) shows that both ND1 and ND3 share a common ancestor with other Mexican cattle in the region that the dairy worker was known to previously reside and visit on an annual basis. This provides further support that the initial exposure of the case-patient was likely in their home country.

The Wisconsin case report provides the strongest epidemiologic evidence for human-to-cattle transmission, as the case-patient was diagnosed 3 years before the herd was detected. The individual was a very recent addition to the dairy's workforce and was diagnosed with TB disease within 3 months of beginning employment. Collectively, this provides strong evidence that the case-patient's exposure to *M. bovis* occurred prior to employment on the dairy. All cattle having potential contact with the case-patient were tested 2 and 6 months after the employee was no longer working on the dairy, and bTB was not detected. It is highly unlikely that infection was present and circulating in the herd and transmitted from cattle to the case-patient. Further, the WGS from the infected cows had at least 1 SNP change difference from the human and most recent common ancestor, or root sequence, suggesting the cattle isolates were closely related, direct descendants of the human isolate.

Results of testing of other dairy employees and family members continue to be negative since the initial tests in 2015. If the dairy cattle were the source of infection, we would have expected other employees or family members to be infected. The public health investigators were adamant that the employee with TB disease was very sick at the time of diagnosis and could not have progressed to this stage of disease in two months. They were confident the employee was infected prior to entering the dairy's workforce.

Investigators also found no evidence suggesting latent infection in the Wisconsin cattle. Three of the 12 infected cows were purchased additions (WI2, WI3, WI4), while the remaining cows were born and raised on the operation. One of the purchased cows (WI2) was brought on the operation in January 2015, roughly the same time the case-patient began employment. This cow was test negative in May and September 2015 suggesting she was not infected at the time of introduction into the herd. In October 2018, WI2 was found to be infected. The other two purchased cows were brought into the herd in 2016 after the case-patient was no longer present. Their infections are consistent with cattle-to-cattle transmission within the herd. Other than these two cows, the remaining 10 infected cows were all test negative at least once during the 2015 testing.

For both the North Dakota and Wisconsin cases, WGS supports that the TB case-patients were infected in their home country, possibly through contact with infected cattle or consumption of raw dairy products, and subsequently infected cattle on these farms after their disease became active. Others have reported similar scenarios where *M. bovis* infection makes the complete cycle from cow to human and back to cow (20).

While the Texas *M. tuberculosis* case lacks a confirmed case of human TB directly linking a human to the calf, the epidemiologic investigation and evidence of the matching spoligotype circulating in the local community strongly supports human to cattle transmission. Furthermore, a review of the literature found that, with one reported exception, humans are the direct source of *M. tuberculosis* infection for cattle. There is one report of a calf from an experimentally infected cow that was infected via colostrum or milk (53). To our knowledge, that has not been replicated in a naturally infected dairy. This is the first modern reported case *M. tuberculosis* infection in a U.S. bovine.

In addition to the three cases presented here, there have been at least 10 other U.S. bovine cases (i.e., affected herds) since 2009 that investigators were very suspicious of human-to-cattle *M. bovis* transmission as the source of introduction into these herds. These suspicions were based on both epidemiologic data collected, and WGS conducted by animal health officials, but all lacked the active, prospective identification of human cases.

Often, lesions are not present in young cattle diagnosed with *M. tuberculosis* (54–56). Although cattle without gross lesions are not considered a risk for transmission of disease to cattle or humans, *M. tuberculosis* has been found in cows' milk (57–59) and in granulomatous lesions from infected cattle, suggesting infected cattle may be a risk to other cattle and humans (23). Published reports suggest that humans are the primary source of *M. tuberculosis* infections in cattle with transmission occurring via the respiratory route (60). Although evidence for direct human-to-cattle transmission is not always present, *M. tuberculosis* has been found in cattle in multiple countries. Worldwide, human-to-cattle transmission of *M. tuberculosis* has been documented more frequently than *M. bovis,* likely due to the increased prevalence of *M. tuberculosis* in humans. More evaluations are needed to determine the importance of livestock in the transmission of *M. tuberculosis* between cattle and humans. The U.S has been characterizing MTBC isolates in livestock for over 40 years, and the last documented case of *M. tuberculosis* that occurred in U.S. livestock was a llama in 1991, associated with exotic animal trade (unpublished data).

Human-to-cattle transmission of *M. bovis* has infrequently been reported (20, 28, 47, 61–63) and there is only a single report of human-to-cattle *M. orygis* transmission (64). The finding of human-to-cattle transmission of *M. bovis* in the United States may be related to the increased risk of *M. bovis* infection among non-U.S.-born livestock workers compared to U.S.-born workers.

The prevalence of *M. bovis* in humans in the United States has declined from at least 10% of MTBC cases in 1900 to <2% of all

MTBC cases in 2005 (65). Another publication demonstrated that human cases of *M. bovis* in the United States were more likely to be of Hispanic/Latino origin and born outside the United States (66). More recently, Scott et al. (67) reported human *M. bovis* prevalence in the United States with a similar prevalence of 1.3–1.6% of all MTBC cases, and a higher prevalence in children, Hispanics/Latinos, and females.

Although the number of human cases of MTBC infection in the United States has decreased about 90% since 1953 (68), some areas of the United States have reported an increase, especially along the southern U.S. border. A review of pediatric tuberculosis cases in San Diego, CA, from 1980 to 1997 revealed *M. bovis* was responsible for 10.8% of all TB cases and 33.9% of culture-positive cases (14). Hispanics represented 78.9% of the cases. More than half the *M. bovis* culture-positive case-patients (55.2%) had only extra-pulmonary bTB. This study highlights the concern of foodborne exposure via unpasteurized dairy products. Since dairy products appear to be the main source of human *M. bovis* infection in Mexico (17), efforts to eradicate bTB from the Mexican dairy industry must be strengthened to improve human and animal health in both the United States and Mexico.

Although the North Dakota and Wisconsin herds were most likely infected via the respiratory route, given active pulmonary disease in the workers, other publications suggest that extrapulmonary infections are more common with *M. bovis* infection. The authors concluded that the prevalence of extra-pulmonary disease in young, U.S.- or Mexican-born Hispanic/Latino populations suggested recent infection due to foodborne exposure (66). Extra-pulmonary disease was nine times more frequent among those with *M. bovis* than those with *M. tuberculosis*. Since transmission via urine has been reported in the literature in *M. bovis* cases (55), this possible extrapulmonary route of disease spread should be investigated when testing high-risk groups.

The median herd size of U.S. dairies has increased from 80 cows in 1987 to 1,300 cows in 2017 (69). The increase in the average size of dairy operations over the past few decades, in terms of the number of cows per herd, has resulted in dramatic needs for on-farm labor. Non-U.S.-born employees make up a significant portion of the workforce on U.S. dairy farms (70). A 2015 National Milk Producers Federation survey of 1,000 dairies (>50 cows) across the United States reported that 93% of operations hired outside labor and over 51% of employees were immigrants (71). A 2007 Wisconsin survey of dairy farms revealed that 40% of hired labor were immigrants; of these, 88.5% were from Mexico and most of the remainder of employees were from Central and South America (72). Based on the higher risk of MTBC infection in many non-U.S.-born employees compared with U.S. born workers, the Centers for Disease Control and Prevention recommends TB screening testing the high-risk groups (45).

Access to medical care can be challenging for non-U.S.-born employees. Often English language skills are limited, and employees may not have the documentation necessary to reside legally in the United States (71). A pilot project developed at the University of Wisconsin-Eau Claire School of Nursing was developed to immerse nursing students into Hispanic and rural culture. The focus of the program is to provide preventive healthcare and routine health screenings to a population that might not otherwise have access. Tuberculosis screening is included as one of the health screenings offered (73). Although this is a pilot project, it serves as a model that could be used in developing health-care programs that improve dairy employee health and safety.

The case reports presented here provide additional epidemiological support for human-to-cattle MTBC transmission and were largely the result of strong working relationships between animal health and public health in both North Dakota and Wisconsin. The communication and collaboration between animal health and public health officials to investigate cases of zoonotic diseases are crucial for gathering the information necessary to evaluate risk and identify effective preventive measures. While the actions of these two states can serve as a model, there are collaborative opportunities to establish additional best practices for issues important to both animal and human health.

Currently, the U.S. Cooperative State-Federal Bovine TB Eradication Program does not include mitigation strategies to address the risk of human introduction of MTBC into U.S. cattle herds, and these findings could change the paradigm of the program. A collaborative One Health approach is needed to address the health of the dairy workers and the animals. The U.S. government has defined One Health as "a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment." (74). In response, the U.S. dairy industry convened a multisectoral working group of state and federal animal and public health officials to address this challenge.

CONCLUSION

This is this first published report using epidemiological and genotype evidence to establish human-to-cattle *M. bovis* transmission in the United States. This is also the first report of *M. tuberculosis* infection of cattle in the United States. In order to advance eradication of bTB in the U.S. cattle herd, the program must incorporate and address humans as another potential source of *M. bovis* or other MTBC species for cattle. This effort can only be achieved with a collaborative One Health approach that includes federal and state animal, public health, and wildlife agencies, livestock industries, producers, and healthcare workers and is focused on safeguarding both human and animal health.

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 study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Ethical review and approval was not required for the animal study because this is a case series involving client owned cattle and is not a research project. Written informed consent for participation was not obtained from the owners because Regulatory procedures were performed that didn't necessarily require written consent. Written consent was obtained for animals removed based on results of regulatory testing.

AUTHOR CONTRIBUTIONS

JL, TD, CY, and SR-A contributed to the conception of the paper. EP, SG-B, RK, JT-K, BC, SK, DP, SR, WH, TT, and SR-A performed the investigations. JL, EP, TD, and SR-A wrote the first draft. CP constructed the phylogenetic trees. All authors contributed to manuscript revisions, read, and 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.2021.691192/full#supplementary-material

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Enhanced Detection of *Mycobacterium bovis*-Specific T Cells in Experimentally-Infected Cattle

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Bovine tuberculosis (bTB), caused by infection with *Mycobacterium bovis*, continues to be a major economic burden associated with production losses and a public health concern due to its zoonotic nature. As with other intracellular pathogens, cell-mediated immunity plays an important role in the control of infection. Characterization of such responses is important for understanding the immune status of the host, and to identify mechanisms of protective immunity or immunopathology. This type of information can be important in the development of vaccination strategies, diagnostic assays, and in predicting protection or disease progression. However, the frequency of circulating *M. bovis*-specific T cells are often low, making the analysis of such responses difficult. As previously demonstrated in a different cattle infection model, antigenic expansion allows us to increase the frequency of antigen-specific T cells. Moreover, the concurrent assessment of cytokine production and proliferation provides a deeper understanding of the functional nature of these cells. The work presented here, analyzes the T cell response following experimental *M. bovis* infection in cattle via *in vitro* antigenic expansion and re-stimulation to characterize antigen-specific CD4, CD8, and γδ T cells and their functional phenotype, shedding light on the variable functional ability of these cells. Data gathered from these studies can help us better understand the cellular response to *M. bovis* infection and develop improved vaccines and diagnostic tools.

Keywords: Bovine tuberculosis, *Mycobacterium bovis*, T cell responses, proliferation, IFN-g, functional potential

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INTRODUCTION

Bovine tuberculosis (bTB), is a chronic bacterial infection caused primarily by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (1). This group of genetically-related mycobacteria also includes *M. tuberculosis*, *M. cannetii*, *M. africanum*, *M. pinnipeii*, *M. microti*, *M. caprae*, and *M. mungi*, which are known to infect and result in similar disease pathology in multiple hosts (2). Worldwide, bTB is a major cause of economic hardship. In 1995, it was estimated that bTB causes >50 million cattle infections resulting in $3 billion of losses annually (3, 4). The number of infected cattle and associated economic loss are likely higher today and its zoonotic nature poses a legitimate public health risk.

As with other intracellular pathogens, protective immune responses against tuberculosis (TB)

are associated with interferon gamma (IFN-γ) production derived from T helper 1 (T_H1) CD4 T cells (5–9). Delayed-type hypersensitivity reactions and IFN-γ release assays (IGRA) are commonly used to assess Mycobacterial reactivity or infection in various species [reviewed in Schiller et al. (10), Walzl et al. (11)]. However, these responses may not necessarily correlate with protection. In cattle, vaccination against bTB results in the induction of IFN-γ responses that can be measured *ex vivo* following overnight antigen stimulation, yet neither the presence nor the levels of IFN-γ induced translate into levels of protection afforded by vaccination (4, 11, 12).

Immune protection from future infections is mediated by the induction and maintenance of memory responses. Memory T cells are a heterogenous population of cells including T effector memory (T_{EM}), T central memory (T_{CM}), and T resident memory (T_{RM}) cells, which display distinct functional, effector, and migratory phenotypes (13). T_{EM} tend to have a fast response to antigen, retain their effector function (i.e., cytokine production), and are relatively short-lived. In contrast, T_{CM} respond slower to antigen, show increased proliferative capabilities, can generate T effector and T_{EM} cells, and are long-lived. Previously, our laboratory demonstrated that following *M. bovis* infection in cattle, both T_{EM} and T_{CM} CD4 T cells are generated (14). In addition, we demonstrated that following *M. bovis* infection, bovine T_{CM} are highly proliferative to antigen stimulation, and that T_{CM} cells can revert in phenotype to generate T_{EM} and T effector phenotypes (14).

While IFN-γ may not serve as a correlate of protection, it nevertheless plays a central role in the response to TB. Long-term culture systems, that measure IFN-γ from T_{CM} cells, appear to be better predictors of vaccine efficacy as compared to *ex vivo* IFN-γ production (15–17). These data would suggest that perhaps the T cell source of IFN-γ is a better predictor of protection rather than the overall levels of IFN-γ. Since proliferation is another characteristic feature of memory responses, typically associated with T_{CM}, we wondered if the concurrent assessment of proliferation and IFN-γ production would allow us to better understand the source of IFN-γ and the overall functional phenotype of memory responses following *M. bovis* infection.

In the work presented here, we utilize an *in vitro* recall response assay, whereby antigen-specific cells are expanded, and proliferation and IFN-γ production are assessed concurrently. Additionally, we assess the potential of these antigen-specific cells to produce IFN-γ via restimulation, thereby enhancing their detection.

MATERIALS AND METHODS

Animals and *Mycobacterium bovis* Aerosol Challenge

Holstein steers (∼6 months of age) were obtained from a tuberculosis-free source and housed at the National Animal Disease Center, agricultural biosafety level 3 (AgBSL3) animal facility. Animals were allowed to acclimate for 2 weeks prior to challenge. All animal studies were conducted with approval from the Institutional Animal Care and Use Committee (AICUC) at the National Animal Disease Center in Ames, Iowa.

Mycobacterium bovis strain 10-7428, a field strain of low passage (<3), which has been shown to be virulent in a calf aerosol model (18). The inoculum was prepared using standard techniques (19) in Middlebrook's 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO). Mid log-phase growth bacilli were pelleted by centrifugation at 750x g, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2) and stored at −80° C until used. Frozen stock was warmed to room temperature and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson). A single dose was determined to be 1.12×10^4 CFU per steer.

M. bovis aerosol infection in cattle has been previously described (18, 20, 21). Briefly, eight (8) steers were infected with a single dose of virulent *M. bovis* strain 10-7428 by nebulization of inoculum into a mask (Equine AeroMask®, Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Six (6) age-matched steers were used as non-infected controls. All experimental animal procedures were conducted in accordance with recommendations in the Care and Use of Laboratory Animals of the National Institutes of Health and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (22, 23). All animal-related procedures were also approved by the USDA-National Animal Disease Center Animal Care and Use Committee.

Isolation of Peripheral Blood Mononuclear Cells

Whole blood was collected via venipuncture of the jugular vein into EDTA tubes. Blood was processed for isolation of PBMC as described earlier (24), with some modifications. Briefly, 10 ml of blood were diluted 1:2 in sterile, culture grade, Dubelcco's phosphate-buffered saline (DPBS) (Gibco, Thermo Fisher, Waltham, MA) and centrifuged at 1,200 × g for 30 min at room temperature (RT). The buffy coats were harvested and overlayed onto 5 ml of 1.077 Ficoll (Sigma-Aldrich, St. Louis, MO), and centrifuged again at 1,200 × g for 30 min at RT. PBMC were then harvested and washed once in sterile PBS at 300 × g for 10 min. Cells were then counted on a hemocytometer using trypan blue staining to determine number and viability. Cells were then resuspended to the desired concentration using complete RPMI 1640 (Gibco Life Tech, Thermo Fisher) media, as described previously (24).

PBMC Labeling, *in vitro* Antigen Stimulation, and Restimulation

In order to assess antigen-specific responses, PBMC were first labeled using the CellTrace® violet proliferation kit (Invitrogen, Thermo Fisher), according to manufacturer's recommendations. Following labeling, 1×10^6 cells were plated onto 96-well, flat bottom plates and left unstimulated, or stimulated with

PPDb (5 μg/well), or Concanavalin A (ConA, 0.5μg/well, Sigma-Aldrich), in a total volume of 200 μl. Cells were then incubated at 37°C with 5% CO_2 for 7 days. In order to assess intracellular cytokine production, cultured PBMC were treated with either a 1× solution of eBioscience™ Protein transport inhibitor (500× Brefeldin A, Thermo Fisher) or with a 1× solution of eBioscience™ Cell Stimulation cocktail plus Protein transport inhibitor [500× phorbol 12-myristate 13-acetate (PMA), ionomycin, Brefeldin A] (Thermo Fisher) and incubated overnight for ∼16 h prior to harvest on day 7 (24).

Surface and Intracellular Staining

For surface and intracellular cytokine staining, PBMC were harvested on day 7 of culture and washed with DPBS via centrifugation at 300 × g for 5 min at RT. Staining was performed as described previously (24). Briefly, cells were incubated with a fixable viability dye (eBioscience™, Thermo Fisher) and then stained for surface markers using FITC-labeled anti-bovine CD4 (CC8, Bio-Rad, Hercules, CA), APC-labeled anti-bovine CD8 (CC63, Bio-Rad), and anti-bovine γδ (IgG2b, TCR1-N24, Washington State University, Pullman, WA; BUV-labeled anti-IgG2b, BD Bioscience, San Jose, CA) antibodies. Cells were then fixed and permeabilized using BD Cytofix/Cytoperm™ kit (BD Bioscience) according to manufacturer's recommendation and stained with an anti-bovine, PE-labeled IFN-γ antibody (CC302, Bio-Rad). Cells were resuspended in FACS buffer and then analyzed using a BD FACSymphony™ A5 flow cytometer (BD Bioscience). Data was analyzed using FlowJo® software (Tree Star, Inc., San Diego, CA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad software, San Diego, CA). Pair-wise comparisons of means were performed using *t*-tests, and multiple comparisons mixed-effects analysis were performed using Sidak's multiple comparisons test. $P \leq 0.05$ were considered statistically-significant.

RESULTS

T Cell Population Subsets

Analysis of T cell population subsets from cultured PMBC from control and *M. bovis*-infected animals at various time points post-challenge was performed; gating scheme for the analysis is shown in **Supplementary Figure 1**. Frequencies of CD4, CD8, and γδ T cells did not differ significantly between control and infected animals at all time points analyzed (**Figures 1A–C**). Overall, frequencies of all three subsets were relatively stable through the course of infection, with γδ T cells comprising the majority of the circulating pool of T cells (**Figure 1C**).

T Cell Proliferative Responses Following *M. bovis* Infection

In order to assess antigen-specific responses, PBMC from control and *M. bovis*-infected animals were stimulated *in vitro* with PPDb and the frequency of proliferating CD4, CD8, and γδ T cells were determined. Representative histograms for assessment of proliferation can be seen in **Supplementary Figure 2**. Following antigen stimulation, we observed a significant increase in the frequency of proliferating CD4 T cells from *M. bovis*-infected animals as compared to controls at 4, 8-, and 48-weeks post-infection (**Figure 2A**). CD8 T cells from infected animals at 4- and 8-weeks post-infection showed an increase in the frequency of proliferating cells, as compared to control animals, however, this change was not statistically significant (**Figure 2B**). Similarly, proliferation was observed in γδ T cells from *M. bovis*-infected animals, however, the frequency of proliferating cells was only statistically different from control animals at 4 weeks post-infection (**Figure 2C**). Altogether, these data indicate that the majority of the proliferative response to PPDb antigens occurred within the CD4 T cell compartment of PBMCs from *M. bovis*-infected animals.

T Cell IFN-γ Responses Following *M. bovis* Infection

The frequency of IFN-γ-producing cells following *in vitro* stimulation of PBMC from control and infected animals was also assessed. Similar to the proliferation data, IFN-γ production was predominantly observed within the CD4 T cell compartment of infected animals, at all time points analyzed (**Figure 3**). However, despite observing an increased frequency of IFN-γ-producing CD4 T cells from *M. bovis*-infected animals as compared to controls, these differences were not statistically significant ($p = 0.09$, 0.08, and 0.30, respectively, for each time point) (**Figure 3A**). At 4- and 8-weeks post-infection, we observed an increase in the frequency of IFN-γ-producing CD8 T cells as compared to controls, however, this increase was not statistically significant (**Figure 3B**). The IFN-γ contribution from γδ T cells was relatively minimal, and no differences were seen between infected and control animals (**Figure 3C**). Congruent with the proliferation findings from above, the majority of IFN-γ produced in response to PPDb antigens is derived from CD4 T cells following *M. bovis* infection.

Concurrent Assessment of Proliferation and IFN-γ Responses to Mycobacterial Antigens

Assessing proliferation and cytokine production concurrently, allows for further characterization of the functional potential of antigen-specific cells. By analyzing cells in this fashion, four distinct functional subsets were identified: cells that proliferate and produce IFN-γ (double function), cells that only produce IFN-γ, cells that only proliferate, and cells that neither proliferate nor produce IFN-γ (double negative) in response to antigen (**Supplementary Figure 3**). These functional subsets were identifiable for CD4 (**Figure 4**), CD8 (**Supplementary Figure 4**), and γδ (**Supplementary Figure 5**) T cells. However, not unexpectedly, these subsets were more discernable within the CD4 T cell compartment, as this is the T cell population with the greatest frequency of cells responding to antigen stimulation. Functionally, antigen-specific CD4 T cells are primarily capable of either proliferating or both proliferating and producing IFN-γ. At 4, 8-, and 48-weeks post-infection, CD4 T cells that

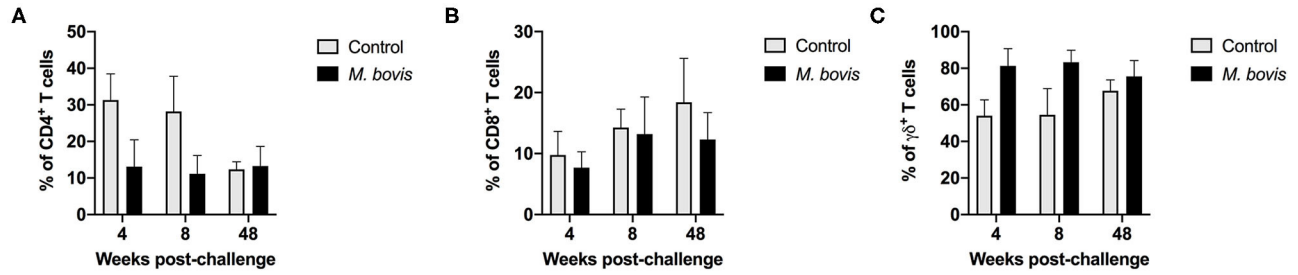

FIGURE 1 | Frequency of T cell subsets in control and *M. bovis* infected animals following challenge and *in vitro* antigen stimulation. PBMC from control (gray bars) and *M. bovis*-infected (black bars) animals were isolated at different time points after infection and stimulated *in vitro* with PPDb. Shown are the frequency of CD4 **(A)**, CD8 **(B)**, and γδ **(C)** T cells following *in vitro* stimulation. Presented are mean frequency values ± S.D.

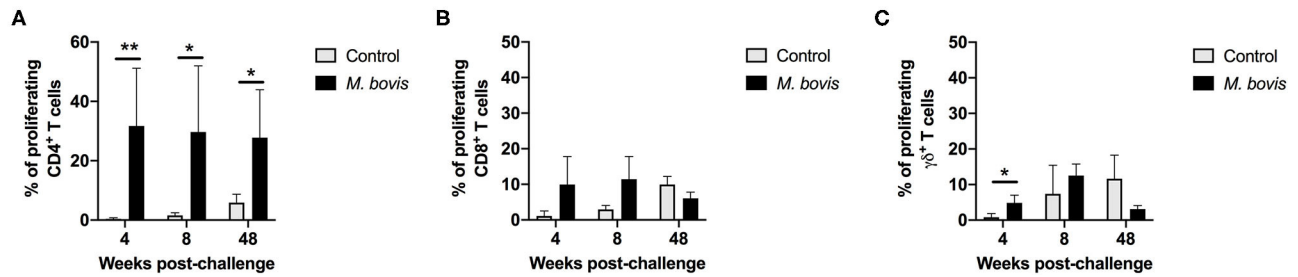

FIGURE 2 | Proliferation responses of T cell subsets from control and *M. bovis*-infected animals, following challenge and *in vitro* antigen stimulation. Shown are the frequencies of proliferating CD4 **(A)**, CD8 **(B)**, and γδ **(C)** T cells for control (gray bars) and *M. bovis* infected (black bars) animals at 4, 8, and 48 weeks post-infection in response to PPDb *in vitro* stimulation. Presented are mean frequency values ± S.D. Statistically significant differences are indicated; $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

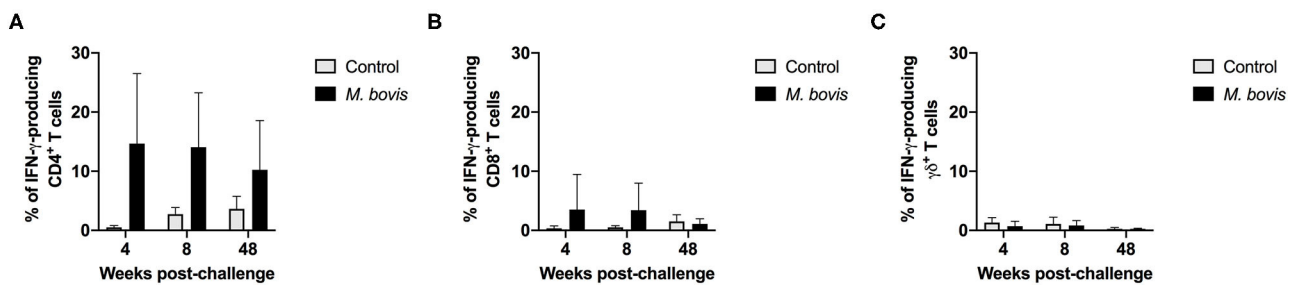

FIGURE 3 | Frequency of IFN-γ responses of T cell subsets from control and *M. bovis*-infected animals following *in vitro* antigen stimulation. Shown are the frequencies of IFN-γ-producing CD4 **(A)**, CD8 **(B)**, and γδ **(C)** T cells from control (gray bars) and *M. bovis*-infected (black bars) animals at 4-, 8-, and 48-weeks post-infection, following *in vitro* PPDb stimulation. Presented are mean frequency values ± S.D.

proliferate and produce IFN-γ to antigen stimulation comprise approximately 8.19, 11.91, and 9.74% of the total CD4 response, respectively (**Figure 4**, pie charts, green color). In comparison, cells that only proliferate in response to antigen stimulation make up 21.65, 17.78, and 18.03% at 4, 8-, and 48-weeks post-infection, respectively (**Figure 4**, pie charts, purple color). These data indicate that a smaller frequency of CD4 T cells display dual function at all times points analyzed. In fact, further analysis of the CD4 antigen-specific response, which excludes the double negative population (i.e., cells not responding to antigen stimulation), revealed that the proliferation-only population comprised over 50% of the response at all time points analyzed (**Supplementary Figure 6**, top panel). CD4 T cells that showed dual function (i.e., proliferation and IFN-γ) comprised 25–37% of the antigen specific cells (**Supplementary Figure 6**, top panel, green), while single IFN-γ producers only made up a very small percentage of the population (1–6%) (**Supplementary Figure 6**, top panel, blue).

Despite the reduced frequency of responding CD8 T cells, a similar pattern of functional phenotype was observed for this subset (**Supplementary Figure 4**). Proliferative responses made up the majority of the functional potential of CD8 T

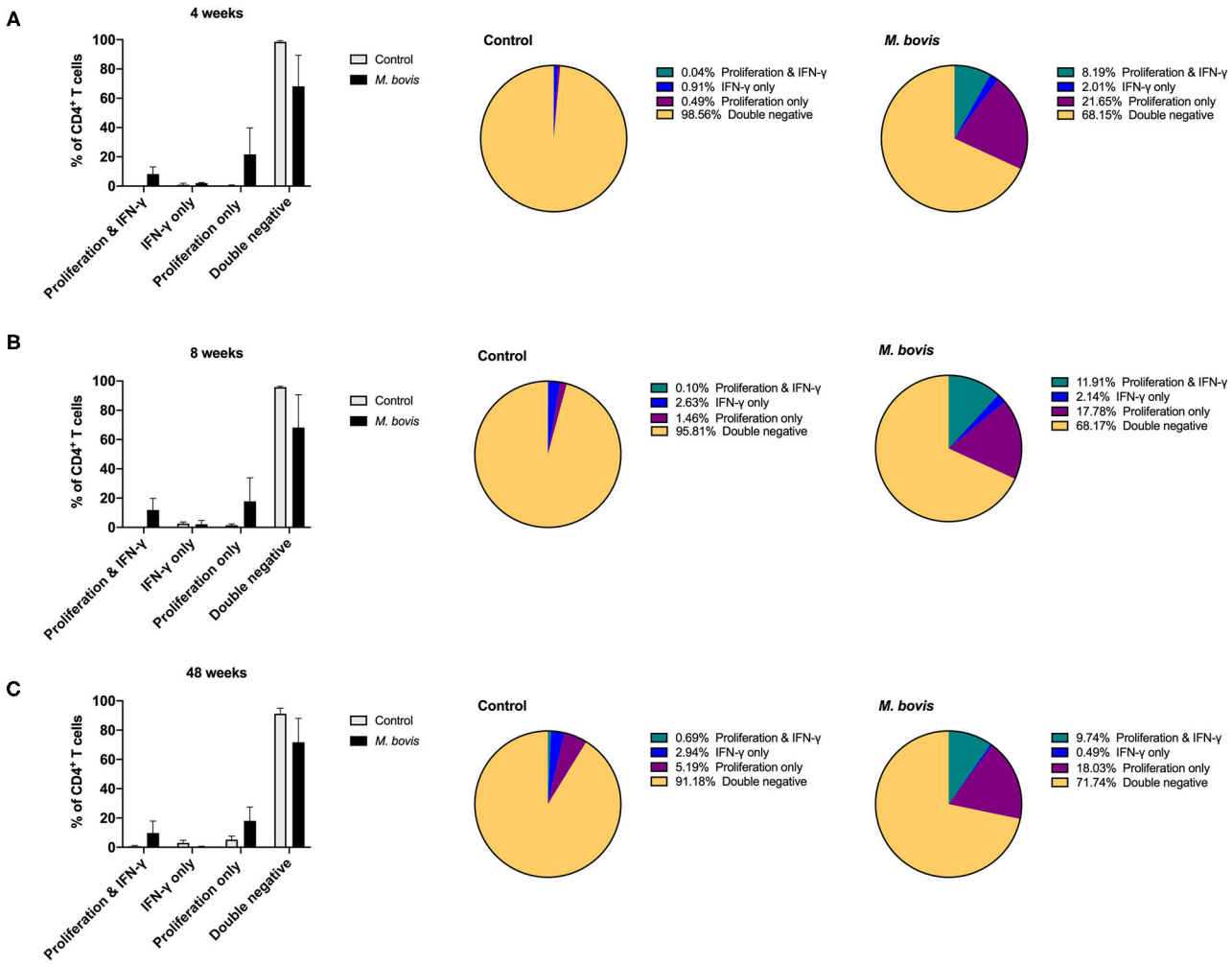

FIGURE 4 | Distinct functional subsets of *M. bovis*-specific CD4 T cells following concurrent assessment of proliferation and IFN-γ production. Bar graphs (left) and pie charts (right) showing the frequency of CD4 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected animals (black bars) at 4-**(A)**, 8-**(B),** and 48-**(C)** weeks post-infection. Functional phenotypes are denoted as CD4 T cells that in response to *in vitro* PPDb stimulation show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

cells, followed by double producers, and a smaller percentage of IFN-γ-only producing cells (**Supplementary Figures 4, 6**, middle panel). Interestingly, γδ T cells showed primarily a proliferative response (**Supplementary Figure 5**), making up over 90% of the antigen-specific response at all time points analyzed (**Supplementary Figure 6**, bottom panel). Altogether, these data showed that when functional phenotypes are assessed concurrently, proliferation appears to predominate for CD4, CD8, and γδ T cells. Additionally, CD4-, CD8-, and γδ-derived IFN-γ arises from cells that have also proliferated in response to antigen, with a much smaller contribution arising from non-proliferating cells.

Enhancing Cytokine Production Following Mycobacterial Antigen Stimulation

Above findings indicated that proliferation comprises the majority of the antigen-specific response, and that only a subset of proliferating cells produced IFN-γ in response to PPDb stimulation. We wondered if the remainder of proliferating cells had the potential to make cytokines, when re-stimulation was provided *in vitro*. In order to determine if these cells had the potential to produce IFN-γ, 16 h prior to harvest on day 7, cells were re-stimulated with PMA/Ionomycin, a pan-T cell stimulator, in the presence of brefeldin A, as described previously (24). Indeed, following restimulation with PMA/Ionomycin, we observed a lower percentage of cells that only proliferated (**Supplementary Figure 3**, last column). This is particularly clear for CD4 T cells from *M. bovis*-infected animals, where cells displaying dual function (proliferation and IFN-γ) now constitute ∼44, 40, and 22% of the response at 4-, 8-, and 48-weeks post-infection, respectively (**Figure 5**). The frequency of proliferating-only CD4 T cells constitutes a mere 5, 0.15, and 2.13% of responding cells (**Figure 5**).

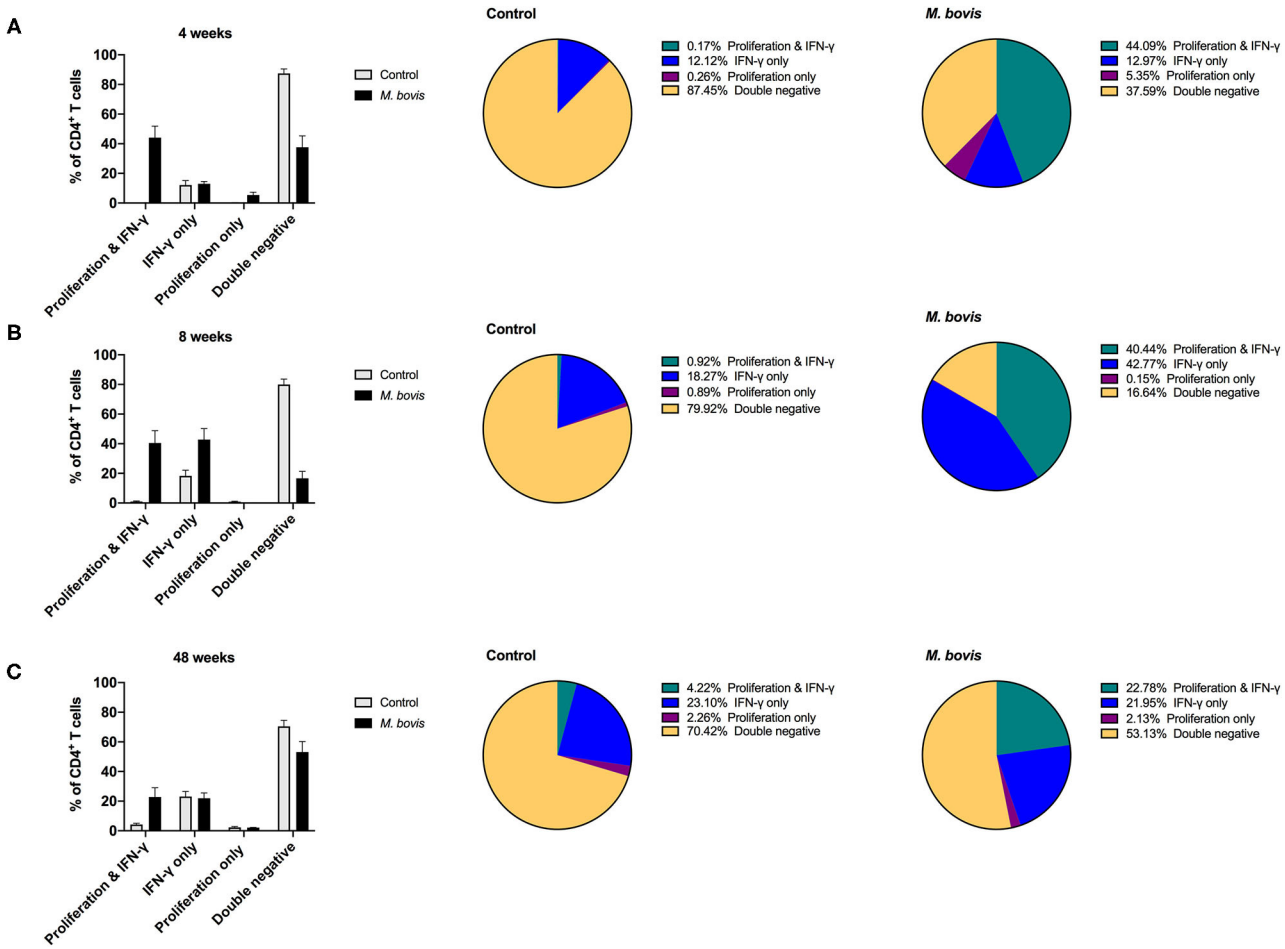

FIGURE 5 | Distinct functional subsets of *M. bovis*-specific CD4 T cells following concurrent assessment of proliferation and IFN-γ production following antigen stimulation and restimulation *in vitro*. Bar graphs (left) and pie charts (right) showing the frequency of CD4 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected animals (black bars) at 4-**(A)**, 8-**(B)**, and 48-**(C)** weeks post-infection. PBMC were stimulated with PPDb for 7 days and restimulated with PMA/ionomycin overnight for the last 16 h of culture. Proliferation and IFN-γ production were then assessed concurrently via flow cytometry. Functional phenotypes are denoted as CD4 T cells that show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

This switch in the functional profile of antigen-specific T cells following restimulation is also observed in antigen-specific CD8 (**Supplementary Figure 7**) and γδ (**Supplementary Figure 8**) T cells, albeit not to the extent that was observed for CD4 T cells. When analyzing this response solely within the subset of cells responding to antigen, CD4 T cells that proliferate and produce IFN-γ constitute 70% of the response at 4 weeks post-infection and 48% at 8- and 48-weeks post infection (**Supplementary Figure 9**, top row). In comparison, antigen-responsive cells CD8 T cells that proliferate and produce IFN-γ consistently constitute ∼30% of the response at 4-, 8-, and 48-weeks post infection (**Supplementary Figure 9**, middle row, green). At 4- and 48-weeks post-infection, there remains a substantial population of CD8 T cells (33 and 23%, respectively), that only demonstrate proliferative potential despite the addition of restimulation (**Supplementary Figure 9**, middle row, purple). A similar pattern of response is observed for γδ T cells responding to antigen stimulation; there is an increase in the frequency of proliferating and IFN-γ-producing cells at week 8 post-infection, yet at 4- and 48 weeks post infection proliferation predominates as the antigen-specific response (**Supplementary Figure 9**, bottom row, green and purple). Altogether, these data demonstrate that *M. bovis*-specific T cells that proliferate to antigen stimulation are all capable of producing IFN-γ, and that this response can be enhanced by providing restimulation.

CONCLUSIONS

Understanding the functional role of T cells following *M. bovis* infection in cattle will provide insights for the development of vaccine and diagnostic interventions. Proliferation and cytokine production are two major functional characteristics of activated,

antigen-specific T cells. Production of IFN-γ from T cells is intimately tied with T_H1 responses, which are necessary for the clearance of intracellular pathogens, including mycobacteria. However, IFN-γ levels do not always correlate with protection (4, 11). In the work presented here, we sought to further dissect the functional potential of *M. bovis*-specific T cells by concurrently assessing two commonly-measured functional phenotypes: proliferation and IFN-γ production. In doing so, we were able to characterize three distinct functional subsets for all three T cell populations: cells with the potential to proliferate and produce IFN-γ (dual function), cells that only produce IFN-γ, and cells that only proliferate in response to antigen stimulation. Furthermore, we demonstrate that T cells with dual function and cells that proliferate-only make up the majority of the *M. bovis*-specific T cell response. We also demonstrate that when restimulated, antigen-specific cells that proliferate-only, are capable of producing IFN-γ.

The frequency of CD4, CD8, and γδ T cells remains relatively stable throughout the course of infection, or at least through the time points analyzed in this study (4, 8, and 48 weeks post-infection). In addition, we found that *M. bovis*-specific proliferative responses can be seen in all three T cell populations, yet significant increases in the frequency of proliferating cells were only observed for CD4 T cells at all three time points. Similarly, IFN-γ responses were primarily seen within CD4 T cells, and to a lesser extent with CD8 T cells. Interestingly, despite this increase in the frequency of IFN-γ-producing CD4 T cells in *M. bovis*-infected animals, these changes were not statistically significant when compared to CD4 T cells from control animals. We attribute this to the variability in responses observed for IFN-γ in outbred populations, such as cattle. It should be noted that moderate variability was also observed for proliferative responses. Cattle represent an outbred population, and while experimental infections provide some level of control, variability in animal responses are expected. Overall, however, we show that proliferation and IFN-γ responses following *M. bovis* infection are primarily found within the CD4 T cell compartment, with CD8 and γδ T cells providing minor contribution to these responses, consistent with previous observations from our laboratory (14).

Concurrent measurement of proliferation and IFN-γ provides another level of insight into the functional potential of antigen-specific T cells. As a result, we were able to identify three distinct antigen-specific T cell populations with functional potential. We observed that following *M. bovis* challenge, there are two proliferating populations, one that produces IFN-γ and one that only proliferates. These two populations could represent distinct effector or memory subsets. We have previously demonstrated that following *M. bovis* infection, cattle do develop T central memory (T_{CM}) and T effector memory (T_{EM}) responses (14). Furthermore, we have demonstrated that antigen stimulation not only results in proliferation of T_{CM}, but also in a switch from T_{CM} to T_{EM}, which are capable of producing IFN-γ (14). By measuring the functional phenotypes concurrently, we are likely seeing those distinct subsets. The work presented here did not include surface markers to corroborate memory phenotypes and only characterized proliferation and IFN-γ production. Despite this, it should be noted that by measuring proliferation and cytokine production concurrently, we were able to identify two functionally-distinct populations of cells responding to antigen stimulation. This approach may allow further characterization into other memory and/or effector subsets, which cannot be identified using surface markers as they have either not yet been identified or are not available for cattle.

Concomitant immunity, is a mechanism of immunity whereby a persistent, low-grade infection results in protection from subsequent re-infection with the same pathogen. This type of protection has been shown in other infectious models such as *Leishmania* (25). Unlike the classical idea of "T cell memory," concomitant immunity is primarily driven by a unique subset of CD4 effector cells that are derived from T_{CM} cells, are non-proliferative, are high IFN-γ producers, and are long-lived but only under conditions of persistent antigen availability [reviewed in Reyed and Rafati (26)]. In the mouse, this subset of T effector cells can be characterized by expression of the surface marker Ly6C (26), but this marker has not been characterized in other species. Therefore, tracking of this specific cell type becomes difficult in other species. This phenomenon, and its potential role in mediating protection in tuberculosis, have been recently described in a non-human primate model (27). Further support for concomitant immunity is found in human cohort studies as well as epidemiological data suggesting that prior infection with *M. tuberculosis* provides protection against subsequent infections (28, 29). The role of concomitant immunity has not yet been explored for bTB. While we may be unable to characterize Ly6C-expressing T effector memory subset based on surface expression markers, it may be possible to characterize these cells based on their non-proliferative and high-IFN-γ expression profile, which this assay would facilitate. Additional surface and intracellular markers could be added in order to expand the profiling of memory subsets using this assay. We propose that by refining the concurrent analysis of functional phenotypes, we may be able to explore some of these questions in cattle, thus providing further insights into the cellular immune response to *M. bovis* in its natural host.

The presence of antigen-specific cells that proliferate but do not produce IFN-γ in response to antigen stimulation led us to ask the question about the functional potential of these cells. To address this, we stimulated these cells with PMA/Ionomycin and measured cytokine production. Interestingly, as seen with T cells from cattle vaccinated against brucellosis (24), this subset of proliferating T cells is capable of producing IFN-γ. These data beg the question as to why the distinction in functional phenotype. As mentioned earlier this could be related to memory subtypes (T_{CM} vs. T_{EM}), or perhaps this distinction is related to the nature of antigen stimulation. We assume that these antigen-specific T cells constitute a heterogenous populations of cells with a wide T cell repertoire and antigenic specificity. One hypothesis would be that the quantity and quality of the antigen may be responsible for driving function. Indeed, it has been previously shown that thresholds of T cell receptor signaling and duration of signaling determine T cell fate including functions such as cytokine production and proliferation [reviewed in Zikherman and Au-Yeung (30)]. We cannot discard the possibility that

the antigen in this assay reaches varying degrees of stimulation for different T cell receptors. Further analysis utilizing defined antigens (i.e., single peptides or peptide cocktails) for stimulation using this assay, would allow us to determine if antigen-specificity and/or availability drives the functional distinction observed here.

Restimulation with PMA/ionomycin also allowed for the enhanced detection of antigen-specific, IFN-γ-producing CD4 cells (i.e., cells proliferating and producing IFN-γ). The restimulation step demonstrated that these proliferating cells are capable of producing IFN-γ when added stimulation is provided. However, we did not assess whether these cells could produce any other cytokines. Polyfunctional T cells (i.e., cells with the ability to produce multiple cytokines) have been shown to correlate with protection in various infectious models (31–33). However, the role of polyfunctional T cells in TB remains poorly understood, with conflicting data for their role in protection vs. active disease (34, 35). Work from our laboratory has shown that in cattle polyfunctional (IFN-γ/TNF-α/IL-2) CD4 T_{CM} cells are associated with protective responses following BCG vaccination, while IFN-γ/TNF-α T_{CM} cells are associated with higher bacterial burdens (36). The data presented here suggests that a large proportion of *M. bovis*-specific CD4 T cells only proliferate upon antigen stimulation, but are capable of producing cytokines in response to restimulation. It may be possible that a significant portion of antigen-specific cells are missed when cytokine analysis is performed in isolation. Assessment of cytokine polyfunctionality using restimulation, as described here, may provide a way to enhance our ability to detect these T cell subsets with polyfunctional phenotypes.

Altogether, the work presented here utilized an *in vitro* assay relying on enrichment of *M. bovis*-specific T cells via antigen stimulation to characterize functional phenotypes of proliferation and/or IFN-γ-production. The data demonstrate that the majority of antigen-responsive CD4 T cells proliferate in response to antigen, followed by cells capable of proliferating and producing cytokine. This type of approach, concurrent assessment of two major functions of activated T cells, along with further phenotypic analysis, are likely to increase our fundamental understanding of T cell responses involved in bTB.

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

The animal study was reviewed and approved by National Animal Disease Center Institutional Animal Care and Use Committee (NADC IACUC).

AUTHOR CONTRIBUTIONS

PB, CK, and MP: experiment design, sample collection, experiments, and manuscript editing. PB: data analysis and manuscript preparation. All authors contributed to the article and 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.2021.676710/full#supplementary-material

Supplementary Figure 1 | Flow cytometry gating scheme for T cell subsets. Shown are representative dot plots demonstrating gating strategies for lymphocytes and singlet discrimination based on FSC and SSC. Also shown are live/dead discrimination based on uptake of a fixable viability dye, and CD4, CD8, and γδ gating based on SSC and fluorescent signal.

Supplementary Figure 2 | Flow cytometry analysis of proliferating cells based on CellTrace™ violet dilution. Shown are representative histograms for determination of the frequency of proliferating CD4, CD8, and γδ T cells without stimulation (first column), PPD-B antigen stimulation (middle column), and ConA stimulation (last column).

Supplementary Figure 3 | Flow cytometry gating for the concurrent assessment of proliferation and IFN-γ responses for T cell subsets. Shown are representative dot plots for cells gated on CD4 (top row), CD8 (middle row), and γδ (bottom row) following *in vitro* culture without stimulation (first column), with PPDb stimulation (middle column) and PPDb + PMA/Ionomycin (last column), for the assessment of proliferation (CellTrace dilution, y-axis), and IFN-γ production (x-axis).

Supplementary Figure 4 | Distinct functional subsets of *M. bovis*-specific CD8 T cells following concurrent assessment of proliferation and IFN-γ production. Bar graphs (left) and pie charts (right) showing the frequency of CD4 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected animals (black bars) at 4-**(A)**, 8-**(B)**, and 48-**(C)** weeks post-infection. Functional phenotypes are denoted as CD8 T cells that in response to *in vitro* PPDb stimulation show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

Supplementary Figure 5 | Distinct functional subsets of *M. bovis*-specific γδ T cells following concurrent assessment of proliferation and IFN-γ production. Bar graphs (left) and pie charts (right) showing the frequency of CD8 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected

animals (black bars) at 4**-(A)**, 8**-(B)**, and 48**-(C)** weeks post-infection. Functional phenotypes are denoted as γδ T cells that in response to *in vitro* PPDb stimulation show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

Supplementary Figure 6 | Breakdown of *M. bovis*-specific T cell subsets by functional phenotype. Shown are pie charts showing the distribution of CD4 (top row), CD8 (middle row), and γδ (bottom row) T cells responding to antigen stimulation from *M. bovis*-infected animals via proliferation and IFN-γ production (green), IFN-γ production only (blue), and proliferation only (purple).

Supplementary Figure 7 | Distinct functional subsets of *M. bovis*-specific CD8 T cells following concurrent assessment of proliferation and IFN-γ production following antigen stimulation and restimulation *in vitro*. Bar graphs (left) and pie charts (right) showing the frequency of CD4 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected animals (black bars) at 4**-(A)**, 8**-(B)**, and 48**-(C)** weeks post-infection. PBMC were stimulated *in vitro* with PPDb for 7 days and restimulated with PMA/ionomycin overnight for the last 16 h of culture. Proliferation and IFN-γ production were then assessed concurrently via flow cytometry. Functional phenotypes are denoted as CD8 T cells that show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

Supplementary Figure 8 | Distinct functional subsets of *M. bovis*-specific γδ T cells following concurrent assessment of proliferation and IFN-γ production following antigen stimulation and restimulation *in vitro*. Bar graphs (left) and pie charts (right) showing the frequency of CD4 T cells with distinct functional phenotypes from control (gray bars) and *M. bovis*-infected animals (black bars) at 4**-(A)**, 8**-(B)**, and 48**-(C)** weeks post-infection. PBMC were stimulated *in vitro* with PPDb for 7 days and restimulated with PMA/ionomycin overnight for the last 16 h of culture. Proliferation and IFN-γ production were then assessed concurrently via flow cytometry. Functional phenotypes are denoted as γδ T cells that show proliferation and IFN-γ production (green), IFN-γ production only (blue), proliferation only (purple), or do not respond [double negative (gold)]. Shown are mean frequencies ± S.D.

Supplementary Figure 9 | Breakdown of *M. bovis*-specific T cell subsets by functional phenotype following restimulation. Shown are pie charts showing the distribution of CD4 (top row), CD8 (middle row), and γδ (bottom row) T cells responding to *in vitro* PPDb stimulation from *M. bovis*-infected animals via proliferation and IFN-γ production (green), IFN-γ production only (blue), and proliferation only (purple).

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Whole Genome Sequencing Links *Mycobacterium bovis* From Cattle, Cheese and Humans in Baja California, Mexico

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Mycobacterium bovis causes tuberculosis (TB) in cattle, which in turn can transmit the pathogen to humans. Tuberculosis in dairy cattle is of particular concern where the consumption of raw milk and dairy products is customary. Baja California (BCA), Mexico, presents high prevalence of TB in both cattle and humans, making it important to investigate the molecular epidemiology of the disease in the region. A long-term study was undertaken to fully characterize the diversity of *M. bovis* genotypes circulating in dairy cattle, cheese and humans in BCA by whole-genome sequencing (WGS). During a 2-year period, 412 granulomatous tissue samples were collected from local abattoirs and 314 cheese samples were purchased from local stores and vendors in BCA and sent to the laboratory for mycobacterial culture, histology, direct PCR and WGS. For tissue samples *M. bovis* was recovered from 86.8%, direct PCR detected 90% and histology confirmed 85.9% as mycobacteriosis-compatible. For cheese, *M. bovis* was recovered from 2.5% and direct PCR detected 6% of the samples. There was good agreement between diagnostic tests. Subsequently, a total of 345 whole-genome SNP sequences were obtained. Phylogenetic analysis grouped these isolates into 10 major clades. SNP analysis revealed putative transmission clusters where the pairwise SNP distance between isolates from different dairies was $\leq$3 SNP. Also, human and/or cheese isolates were within 8.45 (range 0–17) and 5.8 SNP (range 0–15), respectively, from cattle isolates. Finally, a comparison between the genotypes obtained in this study and those reported previously suggests that the genetic diversity of *M. bovis* in BCA is well-characterized, and can be used to determine if BCA is the likely source of *M. bovis* in humans and cattle in routine epidemiologic investigations and future studies. In conclusion, WGS provided evidence of ongoing local transmission of *M. bovis* among the dairies in this high-TB

burden region of BCA, as well as show close relationships between isolates recovered from humans, cheese, and cattle. This confirms the need for a coordinated One Health approach in addressing the elimination of TB in animals and humans. Overall, the study contributes to the knowledge of the molecular epidemiology of *M. bovis* in BCA, providing insight into the pathogen's dynamics in a high prevalence setting.

Keywords: whole genome sequencing, Baja California, bovine tuberculosis, single nucleotide polymorphism, *M. bovis*, cheese

INTRODUCTION

Bovine tuberculosis (bTB), most commonly caused by *Mycobacterium bovis*, is characterized by the formation of granulomas in the lymph nodes and lungs of infected individuals, though other organs may also be affected (1, 2). It is an OIE (World Organization for Animal Health) reportable disease that infects a broad variety of mammals including humans. Infection in cattle can occur through direct contact by the inhalation of infected aerosols from sick animals and through oral ingestion of contaminated milk, fodder and pastures (3). Humans can acquire the infection also by direct contact with infected animals and through the consumption of contaminated unpasteurized milk and dairy products (4). Due to the significant impact this disease can have on public health and international trade of cattle and their byproducts, programs for the control and eradication of bTB have been implemented in many countries. In developed countries, significant success has been achieved, but wildlife reservoirs have challenged total eradication (5, 6); in least-developed or developing countries, however, the lack of economic compensation for culled animals due to test and slaughter strategies, or the absence of such strategies, complicates control (7, 8).

In Mexico, the bTB National Program classifies geographic territories into two zones (eradication or control) based on the regional bTB herd prevalence over a 12-month period. The program's strategies are to reduce or eliminate the prevalence of the disease in "eradication" zones, which have a bTB prevalence of <0.5%, as well as to prevent reinfection by applying mitigation measures in the movement of cattle from control zones to eradication zones. Eradication zones are primarily populated by beef cattle and currently 86.02% of the country is recognized as being an eradication zone. The bTB prevalence in the remaining control zones (13.98%) is >0.5% (range of 0.1–14.2%) or is unknown and contain primarily dairy cattle (9). The state of Baja California (BCA) is divided into two zones: an eradication zone with <0.5% of bTB herd prevalence and a control zone to the north, which borders California in the US, that is mainly populated by dairy cattle and has reported prevalence rates as high as 40% (10). As previously reported in the literature (11), there is very high prevalence of tuberculosis in cattle and humans in BCA and although *M. tuberculosis* is the main causative agent of TB in humans, *M. bovis* may play an important role in areas where bTB is endemic and even more so where bTB prevalence rates in cattle are high (12). In Mexico there is limited information available with respect to human TB caused by *M. bovis* and some studies have reported a median percentage of 7.6% (range 0–31.6%) (13, 14). However, due to a lack of species identification in the diagnosis, cases of bTB in humans are likely underestimated (15). Additionally, in the US, 90% of human bTB cases are usually traced to people of Hispanic communities, most of which have origins in Mexico (16). Interestingly, San Diego County in California is the only county in the US which borders a control zone and reports the highest levels of *M. bovis* infection in people (17, 18). Consequently, this high TB burden dairy region of BCA may be contributing to that high level of *M. bovis* detection. Furthermore, isolation of *M. bovis* from cheese has been reported (19) and the association of human bTB cases in Hispanic people from Mexico has been attributed to the consumption of contaminated cheese produced with unpasteurized milk (20). The production of artisanal cheese is customary in Mexico and is often carried out by traditional "cheese-makers" in the rural areas of the country, to be later sold at the open markets and small stores (21). Comparably, cases of human bTB in the US have also been associated to fresh cheese brought into the US from Mexico (22).

Recovery of *M. bovis* from raw milk and cheese is challenging and relies on decontamination methods that can maintain the delicate balance between inactivation of undesirable microorganisms and the viability of mycobacteria, thus appropriate processing procedures such as homogenization, decontamination, concentration and culture media, must be selected to facilitate optimum recovery of mycobacteria (23). Previous studies at the National Veterinary Services Laboratories (NVSL) have evaluated the isolation of *M. bovis* and *M. avium* subsp. *paratuberculosis* from milk and cheese using various combinations of decontamination processes and have eventually settled on a chemical combination of N-acetyl-l-cysteine–sodium hydroxide (NALC-NaOH) for decontamination (19, 24, 25). Furthermore, comparisons of different types of media for the optimal recovery of *M. bovis* have yielded the best results for 7H11P (Middlebrook 7H11 agar supplemented with sodium pyruvate, calf serum, lysed sheep blood and malachite green) and BACTEC MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD) supplemented with an antibiotic mixture (BBL MGIT PANTA, Becton Dickinson) (26–28).

In contrast to the bTB prevalence in BCA dairy cattle, the US cattle herd has a very low prevalence of bTB (<0.001%) and several studies have suggested most new cattle herd detections are the result of new introductions and not continued spread within local cattle (29, 30). While many sources of bTB introduction remain unknown, some of the genotypes have closely matched

isolates recovered from previous studies in BCA (11). Since dairy cattle movements are strictly controlled in Mexico, humans and fomites may be a likely source as this region serves as a major port of transit for people and goods, including fresh cheese. To address this issue, a binational collaboration was initiated with three overall objectives: (1) fully characterize the diversity of *M. bovis* genotypes circulating in BCA by whole-genome sequencing, (2) to determine the role of fresh cheese from the region as a potential source of infection to humans and (3) compare the genotypes identified in BCA to those previously reported for the region, the rest of Mexico and the US.

MATERIALS AND METHODS

Study Area

The study was performed in the control zone of Baja California, Mexico, which is the dairy region located in the far northwest of the state (**Figure 1**). The location of the dairy herds from which the sampled cattle originated was mapped and clusters were formed based on a maximum distance (radius) of 5 km between the dairies. Thirteen clusters were assigned as follows: Ensenada 1 (ENS-1), Ensenada 2 (ENS-2), Ensenada 3 (ENS-3), Ensenada 4 (ENS-4), Mexicali 1 (MEX-1), Rosarito 1 (ROS-1), Rosarito 2 (ROS-2), Tecate 1 (TEC-1), Tecate 2 (TEC-2), Tijuana 1 (TIJ-1), Tijuana 2 (TIJ-2), Tijuana 3 (TIJ-3) and Tijuana 4 (TIJ-4). A full list of the dairies that corresponded to each cluster is in **Supplementary File 1**.

Tissue Samples

From October 2016 to November 2018, tissue samples with bTB-suspicious lesions (granulomas) were collected by an accredited veterinarian during post-mortem inspection of dairy cull cattle, under the supervision of the abattoir's official veterinarian. Three abattoirs, each in the municipalities of Ensenada, Tecate and Tijuana, were targeted. Based on estimations by the Mexican Secretariat of Agriculture and Rural Development (Secretaría de Agricultura y Desarrollo Rural, SADER), these three abattoirs receive over 90% of the cull dairy cows in the region. The abattoirs were visited 2–3 times per week on days in which the volume of animals was highest. Each sample was divided in two: one half was stored in formalin for histopathological analysis and the second half was frozen for bacteriological analysis. Epidemiological data associated to each animal's official identification tag (SINIIGA) was collected, such as owner, farm of origin (location, production unit), dealer, transit document number (movement authorization), etc. (https://www.siniiga.org.mx/identifica.html). Finally, samples were submitted to the USDA's National Veterinary Services Laboratories (NVSL) in Ames, Iowa for analysis.

Cheese Samples

Fresh cheese samples were collected throughout the region of Ensenada, Rosarito, Tecate and Tijuana in BCA from October 2016 to December 2019. Approximately 250 g pieces were purchased from informal sellers, small stores and markets, 2–4 times per week. Samples were stored in sterile, airtight containers and shipped in styrofoam coolers with icepacks to NVSL for analysis.

Mycobacterial Isolation and Identification

Tissues

Prior to culture, a pea-sized sample was obtained for direct PCR. Then, granuloma samples were trimmed of excess fat and connective tissue and soaked for 20−30 min in a 1:100 solution of bleach and R/O water, then tissues were homogenized. Seven mL of macerated tissue were placed in 5 mL of 1 N NaOH and decontaminated for 7–10 min and neutralized to effect with the MycoDDR Neutralization Buffer B (Immuno-Mycologics, Inc., US) to a final volume of 35 ± 5 mL. Specimens were centrifuged at 4,700x*g* for 25 ± 2 min at 10°C and the supernatant decanted off. Pellet was resuspended in 2–3 mL of PBS and was inoculated into BACTEC MGIT 960 (Becton Dickinson, Sparks, Md.) for up to 42 days and two tubes of Middlebrook 7H11 media with sodium pyruvate and incubated at 37°C for up to 8 weeks.

Cheese

Mycobacterial isolation from cheese samples was performed following a previously described methodology (19). Briefly, 5 g portions of cheese were weighed and aseptically transferred into a blender jar containing 45 mL of 2% sodium citrate. The cheese was homogenized and the jars were placed in a 37°C water bath for 1 h to help liquefy the specimen. The cheese suspension was decontaminated using the N-acetyl-L-cysteine (NALC)-NaOH method (31). 10 mL of the liquefied and homogenized sample was mixed with 10 mL of digestant containing NaOH-Sodium citrate and NALC. The mixture was allowed to stand at room temperature for 15–20 min. 30 mL of phosphate buffer was then added. The mixture was then centrifuged at 4,700x*g* for 25 min at 10°C and the supernatant decanted off. Pellet was resuspended in 2–3 mL of PBS and was inoculated into BACTEC MGIT 960 (Becton Dickinson, Sparks, Md.) and incubated for up to 42 days and two tubes of Middlebrook 7H11 media with sodium pyruvate (7H11P) and incubated at 37°C for up to 8 weeks.

Isolate Identification by PCR and Sanger Sequencing

Real-time PCR against *IS*1081was performed on DNA extracted from acid fast colonies either from solid media or MGIT media. If the Ct value was below 14, the DNA was sent for whole genome sequencing. If the PCR was above 14, the isolate was subcultured on to fresh 7H11P solid media and allowed to grow. If the PCR was negative, the DNA was sent for Sanger sequencing using both universal primers against 16S rDNA and mycobacterial specific primers for rpoB and the sequences were blasted against GenBank.

Direct PCR

Direct real-time PCR was performed directly from tissue as previously described (32), with modifications. Direct PCR was performed on a pea-sized sample obtained from the tissue used for culture. Briefly, tissues were examined for granulomatous lesions and dissected to obtain a pea-sized subsample. Subsamples were transferred into 2 mL screw-cap microcentrifuge tubes with a glass bead mixture of approximately

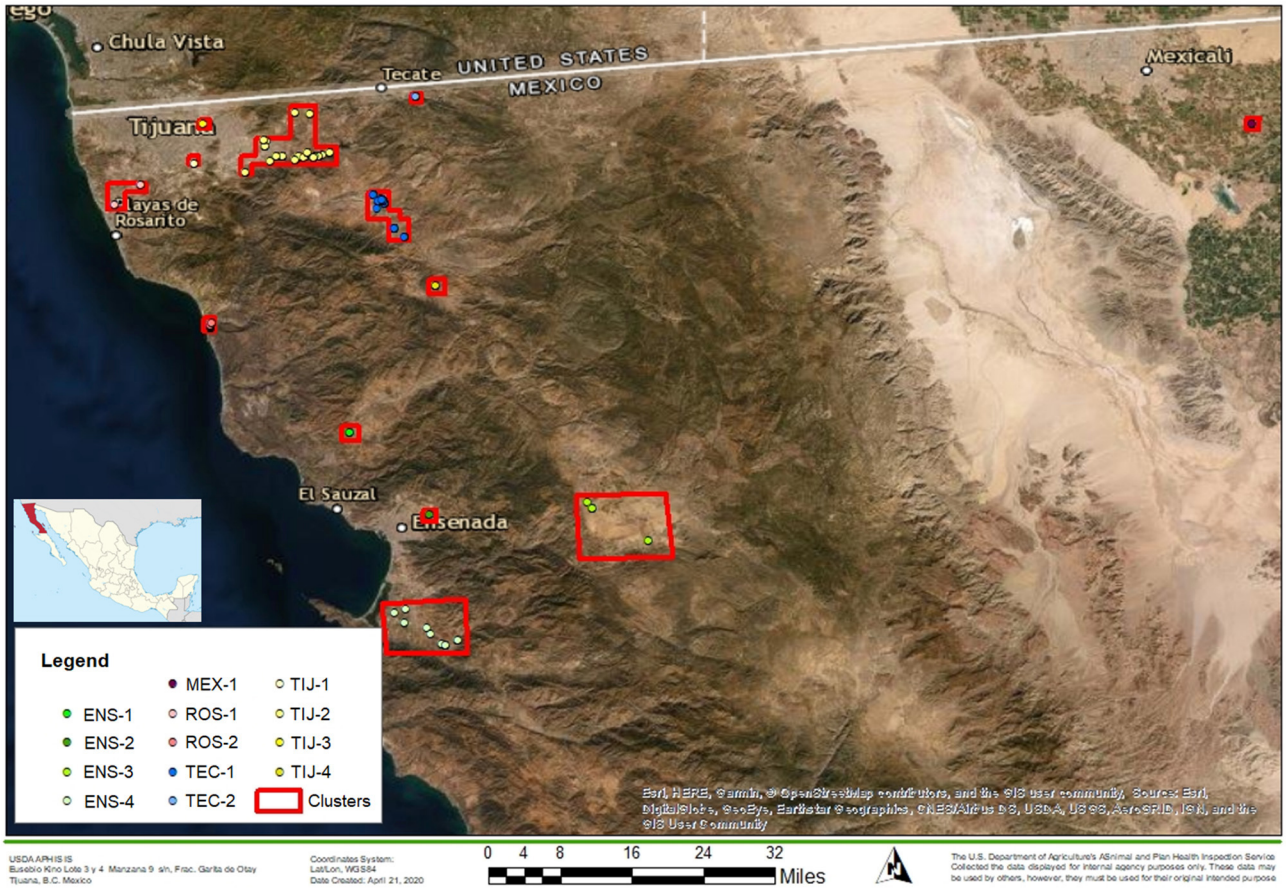

FIGURE 1 | Map of the northern region of Baja California. The location of BCA with respect to Mexico is shown on the left. Red squares represent dairy clusters (n = 13) formed based on a maximum distance of 5 km (radius) between dairies. Clusters' names are indicated in the legend. The map was built using ArcGIS software (basemap WGS1984).

125 μL of 1.0 mm and 125 μL of 0.1 mm beads. 400 μL of a buffer solution containing approximately 400 μL 1X TE buffer and 2.5 μL of DNA Extraction Control 670 were added per sample. Samples were heat-inactivated in a heat-block at 100°C for 30 min and posteriorly bead-disrupted at full speed for 2 min using a mini-bead beater. Samples were then centrifuged at 16,000x*g* for 5 min. The top aqueous layer was used to extract mycobacterial DNA using the MagMax CORE nucleic acid purification kit (Applied Biosystems, ThermoFisher Scientific, US) and a KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA, USA). Real time PCR was performed on the QuantStudio or Viia7 instruments (Applied Biosystems, California, USA).

For cheese, 400 μL of the homogenate were transferred into 2 mL screw-cap microcentrifuge tubes with a glass bead mixture of approximately 125 μL of 1.0 mm and 125 μL of 0.1 mm beads. PCR tubes contained a total volume of 500 μL: 98.5 μL of 1X TE buffer, 2.5 μL of DNA Extraction Control 670 and 400 μL of phenol/chloroform. After inactivation with the phenol/chloroform, samples were bead-disrupted and processed the same as for tissue as mentioned above.

Whole Genome Sequencing and Data Analysis

DNA from colonies was extracted using the MagMax CORE nucleic acid purification kit (Applied Biosystems, ThermoFisher Scientific, US) and a KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA. USA). Real time PCR was performed on the QuantStudio or Viia7 instruments (Applied Biosystems, California, USA). A minimum of 20 μL of DNA sample with a minimum concentration of 5 ng/μL was required for sequencing. Sequencing was performed in an Illumina MiSeq device (Illumina, San Diego, CA, USA), according to manufacturer's instructions, using 250 bp paired-end read chemistry and libraries were prepared using the Nextera XT Library Preparation Kit (Illumina, San Diego, CA, USA) also according to manufacturer's instructions. Raw FASTQ files were analyzed with the vSNP pipeline (https://github.com/USDA-VS/vSNP). Quality check is done as part of the vSNP package (see **Supplementary File 5** for sequencing metrics). Briefly, FASTQ files were used to align reads against the reference genome *M. bovis* AF2122/97 (NCBI RefSeq Accession NC_002945.4) using BWA-mem (33). 80X depth of coverage was targeted. SNPs were

called using FreeBayes (34) and visually validated with IGV (35). Phylogenetic trees were constructed based on whole genome concatenated SNP sequences using RAxML (36) under a GTR-CAT model of substitution. Tree visualization, annotation and editing was performed with FigTree (37). As output from the vSNP pipeline, SNP tables for each major clade were generated; these are formatted Excel tables that group and sort isolates and SNP according to relatedness and reflect exactly what is shown by the phylogenetic tree, which provides transparency of the results. In the SNP tables the columns identify the genome location of the SNP calls and the isolates are listed in the rows. The reference (*M. bovis* AF2122/97, NC_002945.4) is listed across the top and is identified as the "reference call." All SNP are highlighted. Map-quality for each SNP is indicated and is an average of the map quality scores of each isolate at that position. A score of 60 is the highest possible. Finally, the annotation of the position is listed at the bottom of the SNP table.

The complete analysis involved the sequences generated in this study and sequences from the NVSL database generated by previous studies (11, 30, 38), which are publicly available in the NCBI Sequence Read Archive under Bioprojects PRJNA384996, PRJNA251692 and PRJNA449507, respectively (**Supplementary File 4**).

Cluster Analysis

A cut-off value for pairwise SNP distances between isolates of 12 SNP has been widely used for *M. tuberculosis* transmission studies (39–41). Here, a cut-off value of 10 SNP was used based on patterns observed in the data by the careful visual inspection of the SNP matrices (Excel tables) obtained from the vSNP pipeline. Each cluster was identified and labeled based on the defining SNP for that cluster according to its genome position with respect to the reference genome (*M. bovis* AF2122/97, RefSeq accession number NC002945.4). Additionally, a cut-off value of 3 SNP was also used for identifying putative transmission between the dairies.

Statistical Analysis

To evaluate concordance between laboratory tests for the diagnosis of *M. bovis* (culture, histology and PCR), Cohen's kappa coefficient was determined for histology and culture and for PCR and culture, as culture remains the gold standard for *M. bovis* confirmatory diagnosis. The following formula was applied:

$$K = (Po - Pe)\,/(1 - Pe) \tag{1}$$

where Po refers to the observed agreement between tests and Pe refers to the expected agreement. For this, Pe was determined with the following formula:

$$Pe = [(n1/n) * (m1/n)] \; + \; [(n0/n) * (m0/n)] \tag{2}$$

where the values n1, n, m1, n0 and m0 are based on the following:

Histology/PCR	Culture Positive	Negative	Total
Positive	a	b	m_1
Negative	C	d	m_0
Total	n_1	n_0	n

Cohen suggested the Kappa result be interpreted as follows: values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial and 0.81–1.00 as almost perfect agreement (42).

RESULTS

Source Herd Information

Based on cattle census data by Mexico (43), over 95% of the dairies that were larger than 100 head were sampled during the study. Animals were housed in open-air dry-lot dairies where modern management practices are in place, similar to what can be seen in developed countries such as the US. The main breeds in these dairies were Holstein and Swedish Red, with an average milk production of 25 liters per day. This region of the country is an ideal environment for dairy production and upon observation, the animals appear healthy and with adequate body condition and clinical tuberculosis is not typically recognized.

Tissue Samples

A total of 445 tissue samples with TB-suspicious lesions were obtained from dairy cattle, which represented a total of 90 dairies. Unfortunately, 33 samples failed to make it to the laboratory in acceptable condition for testing due to shipping logistics. Consequently, 412 samples were included in the analysis, representing a final total of 61 dairies. Ten samples could not be traced back to a dairy, so they were labeled as "Unknown." Of the total, 363 (88.1%) samples corresponded to lymph nodes (superficial cervical, mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal and hepatic), 10 were from lung (2.4%) and 39 from liver (9.5%). The number of granulomas collected per dairy varied widely between 0 and 35. The overall detection rate for *M. bovis* from tissue samples was 86.9%, with lymph nodes achieving the highest rate at 91.7% (**Table 1**).

Cheese Samples

A total of 314 cheese samples were included in the analysis (**Supplementary File 6**). Overall, 22 (7%) were reported out as contaminated (overgrowth of non-acid fast bacteria); 262 (83.4%) were reported as no isolation made, and 30 (9.6%) contained acid fast bacteria, of which 8 (2.5%) were *M. bovis*. Other acid fast bacteria recovered from the cheese included 17 *M. porcinum* isolates, and one each of *M. bolletii, M. fortuitum and M. neoaurum*; and two atypical, likely unnamed mycobacteria with sequences that did not match close enough for species determination.

A total of 85 stores were visited throughout the four municipalities. *M. bovis* was cultured from all four municipalities, one store in Tecate, two different stores in Rosarito, one store

TABLE 1 | Total tissue samples obtained from dairy cattle in Baja California, Mexico for the detection of *M. bovis*.

Type of tissue	Total number of samples	Samples detected with *M. bovis*	Proportion detected (%)
Lymph nodes	363	333	91.7
Superficial cervical	1	1	100.0
Hepatic	12	12	100.0
Mandibular	8	6	75.0
Mediastinal	24	21	87.5
Parotid	12	12	100.0
Retropharyngeal	217	196	90.3
Tracheobronchial	89	85	95.5
Lung	10	6	60.0
Liver	39	19	48.7
Total	412	358	86.9

TABLE 2 | Distribution of culture, histology and direct PCR results for the detection of *M. bovis* from tissue from dairy cattle from Baja California, Mexico.

	Culture		
Direct PCR	**Positive**	**Negative**	**Total**
Detected	351	20	371
Not Detected	6	34	40
Inconclusive	1	0	1
Total	358	54	412
Histology			
Mycobacteriosis compatible	345	9	354
Other diagnosis	13	45	58
Total	358	54	412

TABLE 3 | Comparison between histology and culture results for the detection of *M. bovis* from granulomas obtained from dairy cattle in Baja California, Mexico.

	Culture results		
	***M. bovis* isolated**	**No isolation**	**Total**
Histology diagnosis			
Mycobacteriosis-compatible	345	9	354
Abscess	0	4	4
Actinobacillosis or mycosis	0	9	9
Chronic pneumonia	0	2	2
Coccidioidomycosis	0	2	2
Eosinophilic granuloma	1	0	1
Granuloma, unknown etiology	2	1	3
Hepatitis	0	1	1
Lymphoid hyperplasia	1	1	2
Lymphoplasmitic hepatitis	1	0	1
Lymphosarcoma	0	2	2
Microgranuloma	0	1	1
Mycotic granuloma	0	1	1
No significant findings	2	5	7
Pyogranuloma	6	16	22
Total	358	54	412

in Tijuana (two samples collected at different times), and one store in Ensenada (three samples collected at different times). Even though the number of samples for "fresh" cheese (188) was greater than "panela" cheese (126), the isolation rate of *M. bovis* by culture was the same for both (2.7 and 2.4%, respectively). Similarly, the detection rate of *M. bovis* from fresh and panela cheese by direct PCR was not significantly different between the two types, having obtained detection rates of 5.8% (11/188) and 6.3% (8/126), respectively. Overall, the detection rate was higher for direct PCR (5.7%) compared to culture (2.5%). All the *M. bovis* isolates recovered from these cheese samples had different WGS sequences, suggesting different cow sources despite several obtained from the same store.

Culture, Histology, and Direct PCR

For *M. bovis* detection, cattle tissues were processed by direct PCR, culture and histology. A comparison of culture, histology and direct PCR results is shown in **Table 2**. Of the 412 tissue samples, 358 were positive for culture, 354 were positive for histology (mycobacteriosis-compatible) and 345 were detected by both. Direct PCR alone detected 371 samples and 351 were detected by both PCR and culture. Six culture-positive samples were not detected by direct PCR and 20 culture-negative were detected by direct PCR. For culture and PCR, the kappa obtained was 0.6774 (CI 95% 0.6398–0.7149), indicating substantial agreement between these tests. For histology and culture, 345 samples were detected by both tests, nine were "mycobacteriosis compatible" but culture-negative and 13 identified as "other diagnosis" by histology were positive for culture. Thereby, a kappa statistic of 0.7727 (CI 95% 0.7449–0.8004) was obtained for culture and histology, also indicating a substantial level of concordance between the two tests (**Table 2**).

Additionally, **Table 3** details the various diagnosis classified as "other diagnosis," representing the samples identified as negative by histology. Thirteen samples fell under this classification and included eosinophilic granuloma ($n = 1$), granuloma of unknown etiology ($n = 2$), lymphoid hyperplasia ($n = 1$), lymphoplasmitic hepatitis ($n = 1$), pyogranuloma ($n = 6$) and no significant findings ($n = 2$).

WGS and SNP Clusters

Of the 61 final total dairies, 58 (93.5%) were confirmed to contain animals infected with *M. bovis*, having obtained at least one isolate from an animal in the dairy (**Figure 2**). The highest number of isolates obtained from a single dairy was 31 (Dairy001) and a single isolate was recovered for 17 dairies. Also, at least one and up to 12 SNP clusters were identified for a single dairy (Dairy039), only Dairy053 and Dairy059 were not associated to a SNP cluster. Additionally, 20 isolates that corresponded to 13 dairies were indicative of mixed infection due to their

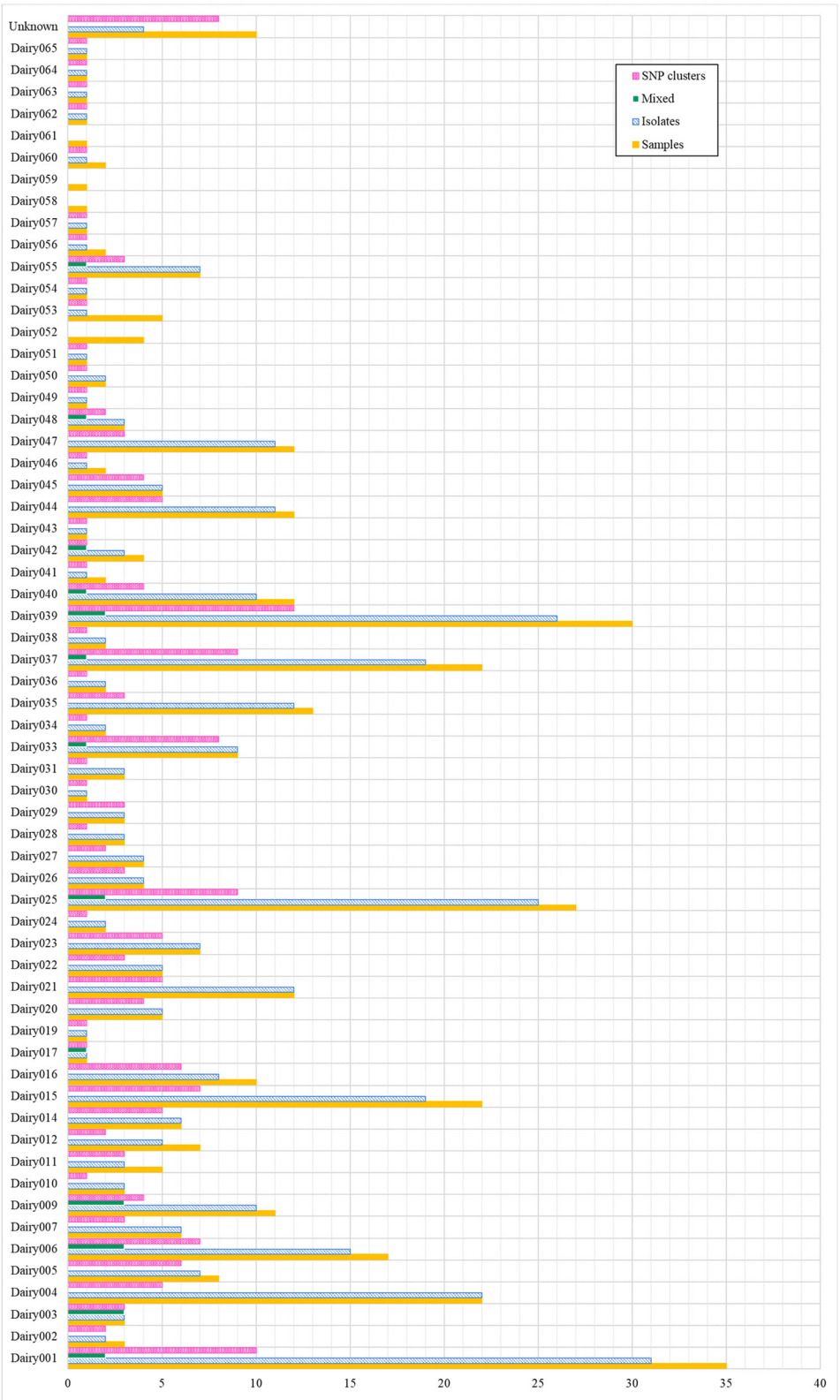

FIGURE 2 | Baja California dairies with positive detection of *M. bovis*. For each dairy, the figure indicates the total number of samples, isolates and SNP clusters. "Mixed" refers to isolates that had a high proportion (≥50%) of ambiguous SNP calls, indicating the presence of more than one genotype. "Unknown" refers to dairies that were not possible to trace back.

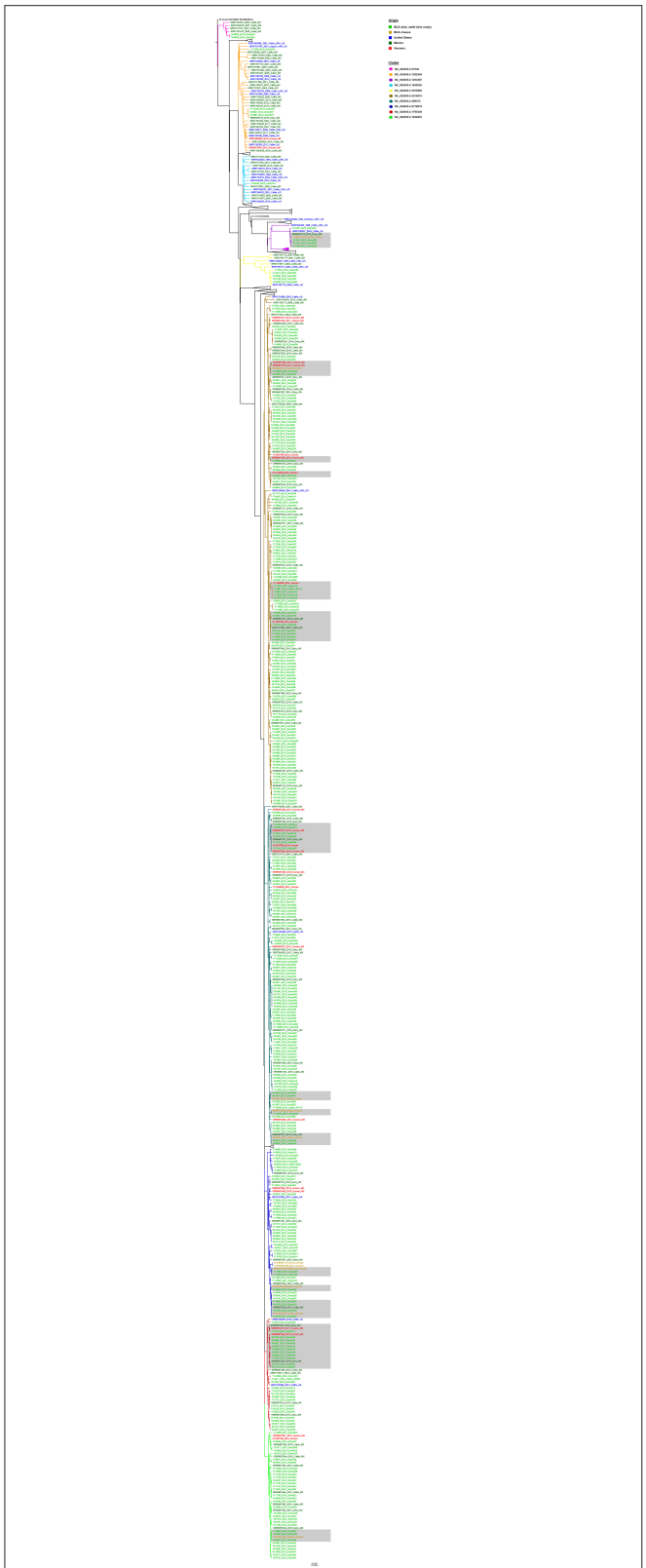

FIGURE 3 | Whole-genome SNP-based phylogeny of 642 *M. bovis* isolates obtained from cattle, cheese and humans from Baja California, Mexico (BCA) *(Continued)*

FIGURE 3 | and the US. The BCA isolates from dairy cattle and cheese obtained from this study correspond to 10 main clades, as indicated in the legend. Each clade is labeled according to the genome position of the defining SNP for that clade, with respect to the reference genome *M. bovis* AF2122/97 (NC002945.4). Gray-shaded areas indicate clusters of human and/or cheese isolates that are within 10 SNPs of a recent common shared ancestor. Some clades (of unrelated isolates to BCA) were collapsed for to improve visibility. The scale bar represents a distance of 40 SNPs (branch length).

high proportion of ambiguous calls (≥50%), which suggests the presence of more than one genotype.

A total of 642 whole genome SNP sequences were included for analysis: 346 from this study (eight from cheese and 338 from dairy cattle) and 297 obtained from GenBank from previous studies (26 human—Mexico, four cheese—Mexico and 267 cattle—from USA and Mexico) (11, 30, 44). Overall, 26 main groups/clades were identified, of which 10 corresponded to the isolates from this study and were labeled according to the defining SNP for each clade (with respect to its genome position in the reference) (**Figure 3**).

Final alignments (SNP matrices) for each clade are shown in **Supplementary File 2**. Most of the isolates from this study fell in clades NC_002945.4:4219410 (38%, 132/345) and NC_002945.4:389472 (26%, 90/345). Clades NC_002945.4:57046 and NC_002945.4:1945505 included the least number isolates with 2 and 1, respectively. All the clades included cattle isolates, five clades included cheese isolates (NC_002945.4:389472 = 3; NC_002945.4:2778919 = 5; NC_002945.4:4219410 = 1; NC_002945.4:1254487 = 1; and NC_002945.4:1622803 = 1) and seven clades included human isolates (NC_002945.4:389472 = 8; NC_002945.4:2778919 = 2; NC_002945.4:4219410 = 9; NC_002945.4:1295549 = 2; NC_002945.4:1790349 = 2; NC_002945.4:2232592; and NC_002945.4:1622803 = 2), one of which does not include any isolates from this study.

In general, all the cheese and human isolates had genotypes that were also found in cattle. On average, human and/or cheese isolates were within 8.45 (range 0–17) and 5.8 SNPs (range 0–15), respectively, from cattle isolates (**Figure 3**, gray shaded clusters). Consequently, nine out of 11 of the cheese isolates were ≤10 SNPs from a cattle isolate from a BCA dairy and this was also true for 11 out of 26 of the human isolates. Within the global context of the *M. bovis* phylogeny, most of the isolates belonged to the European 1 Clonal Complex (Eu1) and only isolates from clade NC_002945.4:1254487 belonged to the European 2 Clonal Complex (Eu2).

To determine how much of the diversity of *M. bovis* in BCA has been captured, the genotypes identified in this study were compared to those from previous reports (**Figure 4A**). From a total of 97 SNP clusters, only two were exclusive to this study (BCA-P), while none were exclusive to what was previously reported in other studies (BCA-O). In total, 19 SNP clusters were identified as specific to the BCA region, while 29 were common to other regions in Mexico (MEX). A total of 49 SNP clusters were found for other regions of Mexico only and not in BCA. Additionally, a comparison between the genotypes isolated from

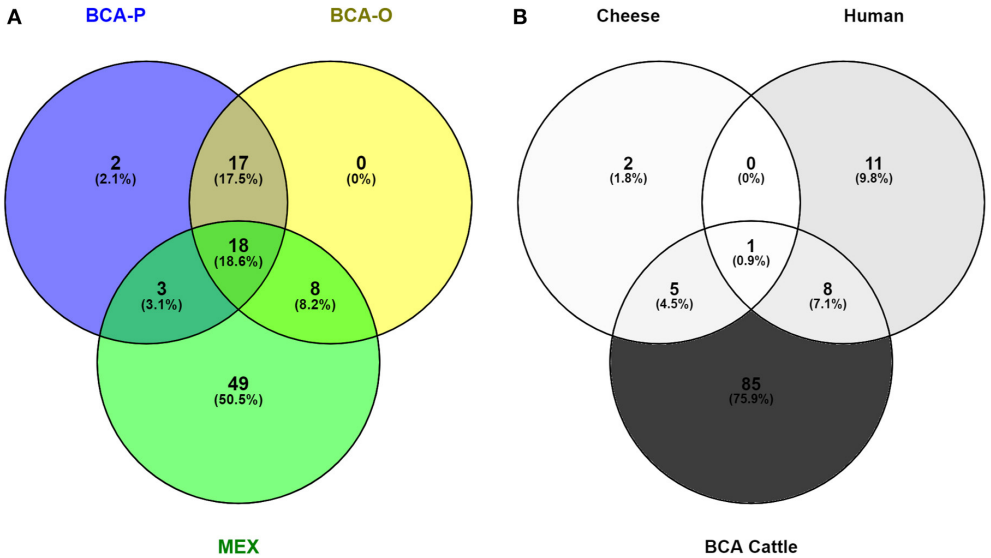

FIGURE 4 | Venn diagrams representing comparisons of *M. bovis* genotypes **(A)** identified in Baja California in this study (BCA-P) and previously (BCA-O, MEX) **(B)** from cattle, humans and cheese.

cheese, humans and cattle revealed that one was common to all three sources, five were common to both cattle and cheese and eight were common to cattle and humans (**Figure 4B**). The larger proportion of SNP clusters (75.9%) belonged only to cattle. A list of the SNP clusters identified in this study can be consulted in **Supplementary File 3**.

Putative Transmission Among Dairies

A total of 64 isolates were found that had a pairwise SNP distance of $\leq$3 SNP to at least one other isolate and the dairies of origin were identified (**Table 4**). Thirty-three out of the total 61 dairies, which represents 54% of the dairies included in this study, were found to have very closely related isolates, thereby suggesting epidemiological associations between dairies. Most of these associations involved only two dairies and in two instances there were three dairies involved. Moreover, nine pairs of these closely related isolates had identical SNP profiles. Dairy001 was found to have the most associations ($n = 8$), four of which involved isolates with identical SNP profiles (**Supplementary File 2**). Dairy039 followed closely with a total of six associations, while Dairies 004, 025 and 037 had four each. The rest had only one or two associations.

These dairies belonged to nine of the 13 previously defined dairy clusters: ROS-1, ROS-2, TIJ-2, TIJ-4, TEC-1, TEC-2, ENS-2, ENS-3 and ENS-4. The frequency at which genotypes were common to two or more dairy clusters is represented in **Figure 5**. At least one genotype was common to all clusters and each cluster had at least one genotype in common with another cluster. Cluster TIJ-2 had SNP clusters in common with at least six other clusters; of these, ROS-2 had the most shared SNP clusters. Cluster TEC-1 had genotypes in common with at least four other clusters (TIJ-2, TIJ-4, TEC-2 and ROS-2). The rest of the clusters shared genotypes with at least two others and three clusters (ENS-2, ENS-3 and ROS-1) only shared genotype(s) with one other cluster.

DISCUSSION

The state of Baja California borders the United States, more specifically the state of California, and contains San Ysidro, the busiest border port in the world. Consequently, a high level of cooperation between the governments of Mexico and the US in both human and animal disease surveillance is necessary to address the transmission of the disease between animals, dairy products or people. Tuberculosis is particularly problematic as BCA is one of the states with the highest incidence of TB in cattle and humans in Mexico (10, 45). This study was carried out as a binational effort between the US and Mexico to use WGS to characterize the bTB genotypes circulating in BCA, determine the role of fresh cheese from the region as a potential source of infection to humans and compare the genotypes identified here to those previously reported in the region, Mexico and the US.

Overall, the detection of *M. bovis* from granulomatous tissue at abattoirs in BCA was high, with 84% from histology/culture and 85% from direct PCR/culture. In contrast, the bordering Mexican State of Sonora, which has strict controls to prevent cattle movement from BCA, has an *M. bovis* detection rate of ∼1.5% from granulomatous lesions sampled in abattoirs (46). In this study, histology and direct PCR performed nearly the same as culture, which is regarded as the gold standard. Only six and 13 culture positive samples were negative by direct PCR and histology, respectively. Several factors can affect the sensitivity of histopathologic diagnosis, including the multifocal distribution of small granulomas, which may prevent histopathologic identification. On the other hand, 20 and nine samples were negative to culture, but detected by the direct

TABLE 4 | Possible epidemiological associations between BCA dairies based on isolates with a difference of ≤3 SNP.

	Dairies with closely related isolates (≤3 SNP)	Total matches within 3 SNPs	Total genomes per dairy
Dairy001*	Dairy003*, Dairy004, Dairy005*, Dairy007*, Dairy011, Dairy047, Dairy050*, Dairy057	8	29
Dairy002	Dairy062	1	2
Dairy003	Dairy001, Dairy004, Dairy025	3	3
Dairy004	Dairy001, Dairy003, Dairy015, Dairy037	4	22
Dairy005*	Dairy001*, Dairy014*	2	7
Diary006*	Dairy064*	1	12
Dairy007*	Dairy001*	1	6
Dairy009	Dairy039, Dairy053	2	7
Dairy010	Dairy037	1	3
Dairy011	Dairy001, Dairy039	2	3
Dairy012	Dairy028, Dairy044	2	5
Dairy014	Dairy005	1	6
Dairy015	Dairy004, Dairy026	2	19
Dairy016	Dairy039	1	8
Dairy023	Dairy025	1	7
Dairy025*	Dairy003, Dairy023, Dairy039*, Dairy040	4	23
Dairy026	Dairy015, Dairy037	2	4
Dairy028*	Dairy012*	1	3
Dairy029*	Dairy037*	1	3
Dairy033	Dairy041	1	8
Dairy037*	Dairy004, Dairy010, Dairy026, Dairy029*	4	18
Dairy039	Diary006, Dairy009, Dairy011, Dairy016, Dairy025, Dairy053	6	24
Dairy040	Dairy025	1	9
Dairy041	Dairy033	1	1
Dairy044	Dairy012	1	11
Dairy047	Dairy001	1	11
Dairy050	Dairy001	1	2
Dairy053	Dairy009, Dairy039	2	1
Dairy055	Unknown	1	6
Dairy057	Dairy001	1	1
Dairy062	Dairy002	1	1
Dairy064*	Diary006*	1	4
Unknown	Dairy055	1	4

Total associations found for each dairy and total number of high-quality genomes for the specific dairy are indicated. An asterisk indicates associations based on a 0 SNP difference (identical SNP profiles).

PCR and histology. For PCR, this could reflect amplification of mycobacterial DNA from non-viable organisms; and the absence of acid-fast bacteria upon histological examination may be because of degradation of bacteria or scarcity of bacteria in lesions. Nonetheless, correlation between diagnostic tests (culture vs. PCR and culture vs. histology) was substantial based on kappa coefficients of 0.6774 and 0.7727, respectively.

Genotype characterization of pathogens is essential for disease surveillance, epidemiology and the development of proper control strategies. Previous studies on the diversity of *M. bovis* in cattle in BCA used spoligotyping and VNTR (47, 48) and one study also used WGS (11) to characterize the strains in the region. In comparison to (11), which evaluated 155 *M. bovis* WGS from BCA cattle, our study, which contained 338 WGS, only identified two additional groups, for a total of 10 main *M. bovis* genetic groups circulating in the region (**Figure 4A**). Overall, 726 WGS sequences, including cattle and cheese have been collected from this region, and based on the significant overlap, we suggest that the genetic diversity of *M. bovis* in BCA is now well-represented. In comparison to other Mexican states, about 30% of the genotypes were common to both BCA and the rest of the country, 20% were exclusive to BCA and 50% were exclusive to the rest of Mexico (**Figure 4A**). Due to its proximity to the US, it is possible that heifers that were historically imported into BCA may have introduced the disease, as the genotypes found here coincided with those once dominant in the US cattle population (29). Current normativity in Mexico restricts the movement of animals from high-prevalence to low-to-zero prevalence regions, which may explain why the genotypes exclusive to BCA have not been spread. In this regard, the state of Sonora, which separates BCA from the rest of Mexico, is classified as "Advanced Modified Accredited" due to the extremely low prevalence, which may act as a deterrent (or geographical barrier) against the movement of dairy cattle to-and-from BCA and the rest of Mexico through this region (49).

The recovery of *M. bovis* from 2.5% of cheese samples collected from local markets is startling and has far reaching consequences. This suggests that routine consumption of fresh cheese by the local population on both sides of the boarder will likely result in the exposure of infectious tuberculous bacteria by most regular consumers of this product over time. Such a claim does require a high level of evidence and merits further discussion. **Supplementary File 6** contains in-depth details of collection date, type of cheese, municipality and store code. While some stores had multiple culture positive samples, all isolates recovered had different WGS profiles, and were collected at different times. Also of note, the isolates recovered from cheese samples most closely matched the diaries located within the same municipality.

Isolation of *M. bovis* from raw milk and cheese is known to present complications due to their complex matrix (high protein and lipid components), as well-susceptibility to contamination by background microflora (50). NVSL has extensive experience developing methods to improve the rate of recovery from these difficult sample types. A contamination rate between 5 and 10%, and a 9.6% recovery rate of acid-fast bacteria suggests decontamination was optimal. Despite this, it is likely that recovery of *M. bovis* does not fully capture the overall prevalence of cheese containing infectious *M. bovis*. This is indicated by the total PCR positivity rate of 6% (19/314). However, because PCR only detects the presence of DNA, the conservative approach is to focus on the samples proven to have live bacteria.

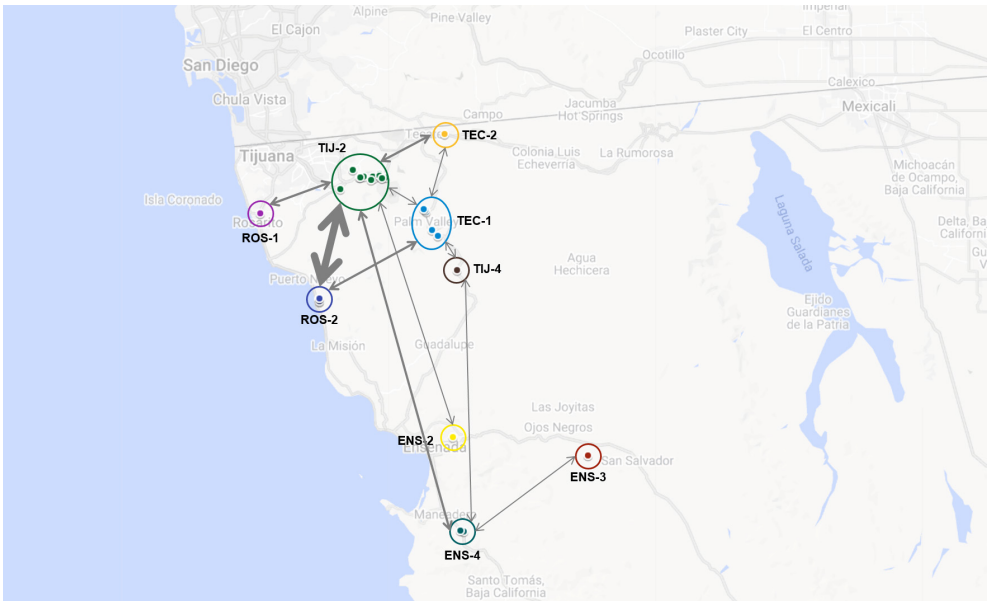

FIGURE 5 | Putative interaction among dairies based on the identification of one or more shared genotypes. Dairies' clusters are indicated and labeled accordingly (ROS-1, ROS-2, TIJ-2, TIJ-4, TEC-1, TEC-2, ENS-2, ENS-3 and ENS-4). Bidirectional arrows are indicative of shared genotypes between dairy clusters and thickness is directly related to the number of shared genotypes (the thicker the arrow, the more shared genotypes). Map was built using GoogleMaps.

Previous studies have established a relationship between *M. bovis* genotypes found in cattle and humans (51) and positive correlations between the consumption of Mexican fresh cheese and cases of zoonotic tuberculosis (20, 52, 53). Through WGS, this study was able to find a direct relationship (transmission clusters) between *M. bovis* infected dairy cattle in BCA, fresh cheese that originated from the same region, *M. bovis* isolates from humans also from BCA and sporadic dairy cattle in California (**Figure 3**). Only one previous study, also performed in BCA, used WGS to determine relationships between cases of zoonotic tuberculosis and cattle from the region, also finding positive results (11). The fact that the cheese contains *M. bovis* from the local dairy cattle suggests it is in fact being made with unpasteurized milk from these infected herds. The official norm NOM-243-SSA1-2010 states that milk destined for human consumption is required to undergo "a thermal treatment of a determined time and temperature that guarantees its innocuity," such as boiling, pasteurization, ultra-pasteurization, sterilization or dehydration, but it exempts the milk that is used for making cheese "whose typical characteristics may not allow it to be made from milk that has undergone thermal treatment" (54). Additionally, NOM-031-ZOO-1995, which is the official norm for the control of bTB in Mexico, states that only 50% of the total national milk production is pasteurized and the rest is consumed [as raw milk] or transformed into dairy products. Consequently, the threat of raw [unpasteurized] milk and its derivatives, possibly from bTB infected cattle, is apparent. Fresh cheeses are a staple food in Mexican households, making them an important source of infection to humans. If pasteurization is not a viable option due to the intrinsic organoleptic characteristics of these types of cheese, cheese-makers must make sure that the raw milk they use comes from healthy animals as to guarantee the innocuity of the product, as stipulated in the official norm for the sanitary requirements of milk and its derivatives (54). In addition to this, education to the public regarding the risks of consuming unpasteurized dairy products could have an impact on their decision to continue to consume these products (55, 56).

For bTB control programs to be successful, strict quarantine measures and animal movement restrictions of infected animals are key. In this study, WGS of *M. bovis* isolates obtained from different dairies throughout the area of study revealed that at least 50% of these dairies were identified with the same SNP clusters or very closely related SNP clusters. This is indicative of herd-to-herd transmission either through the exchange of infected animals or by the acquisition of animals from a same infected source, among other possible causes (57–59). For this study, many dairy owners reported no testing was routinely performed (such as tuberculin skin test for bTB) when introducing new cattle into their herd. In Mexico, the bTB National Program implemented in 1995 has had great success in the beef cattle sector, with most of the focus being on cattle for export to the US. Unfortunately, due to the characteristics of the dairy production systems (longer life/production cycle, higher density of animals, cost of replacement animals, etc.) and relaxed herd management practices (such as lack of testing for newly introduced animals), as well as no compensation of culled animals for farmers, it has been more difficult to reduce the prevalence in dairy regions, where it has been reported at up to 16% (60). Based on experience of the authors, there is a strong and widespread misconception amongst dairy farmers regarding the risk of bTB to human health: because they believe that the milk will ultimately be pasteurized, they don't consider the status of bTB in their herd to be critical.

Another important aspect observed in this study regarding the burden of infection in these BCA dairies is the presence of mixed infections. A mixed infection refers to the simultaneous presence of multiple strains (i.e., genotypes, variants, etc.) of the same pathogen in an individual host. In this study, based on the identification of 20 isolates that presented a high proportion of ambiguous (heterozygous) SNP calls (>50%), nearly 6% of the infected animals had a mixed infection. The high number of heterogenous SNPs point to clearly distinct DNA fingerprints, which support co-infection with different genotypes acquired either at a single point in time or as separate events. This could be indicative of multiple introductions of bTB into the diaries and highlights the lack of control in the movement of animals in the region. Previous studies in *M. tuberculosis* have also found mixed infections in humans more frequently in high-TB burden regions (61). Though a well-studied occurrence in humans, information on identification of mixed *M. bovis* infections in cattle through WGS is scarce; thus, the results shown here may be useful for future comparisons.

A limitation of the study was that sampling only focused on granulomatous lesions observed at carcass inspection. This may have led to the exclusion of animals with an early stage of infection and thus an underestimation of further micro diversity within the herds. However, the large number of animals sampled and the long-term sampling period, as well as the comparison to previously published genomes, support the overall diversity of *M. bovis* seen in the region. Another limitation of the study was the lack of *M. bovis* isolates recovered from humans. In Mexico, mycobacterial culture is not commonly performed in humans, as diagnosis and treatments are initiated using acid fast staining of sputum and PCR. Typically, only cases refractory to treatment are cultured and further characterized. A better understanding of the TB strains infecting humans in the region is needed.

CONCLUSIONS

Despite the well-managed dairy production of this region, a high proportion (93.5%) of the dairies sampled was found infected with bovine tuberculosis. WGS provided evidence of ongoing local transmission of *M. bovis* among these dairies as several of them shared at least one genotype with at least one other dairy in the region. This study was successful at characterizing the diversity of *M. bovis* circulating in the region. This will allow future studies to evaluate the regional and global spread of these genotypes in humans and animals, allowing for a coordinated One Health approach to be used in animal and human TB elimination programs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all samples were collected as part of authorized regulatory surveillance (NOM-031-ZOO-1995) on animals after harvesting. Written informed consent for participation was not obtained from the owners because all samples were collected as part of authorized regulatory surveillance.

AUTHOR CONTRIBUTIONS

SR-A, AP, RM, and EF conceived and designed the study. ED, ER, and KS collected the samples. All authors analyzed the data, wrote, and reviewed the 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.2021.674307/full#supplementary-material

Supplementary File 1 | Total dairies and dairy-clusters included in this study.

Supplementary File 2 | SNP tables for the 10 clades associated to *M. bovis* isolates obtained from dairy cattle, humans and cheese in Baja California.

Supplementary File 3 | List of *M. bovis* SNP clusters (genotypes) identified in BCA dairies.

Supplementary File 4 | List of accessions retrieved from NCBI's Sequence Read Archive.

Supplementary File 5 | Sequencing metrics for the genomes obtained in this study.

Supplementary File 6 | Metadata and results associated to the cheese samples obtained from Baja California Mexico.

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BRIEF RESEARCH REPORT
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Evaluation of P22 ELISA for the Detection of *Mycobacterium bovis*-Specific Antibody in the Oral Fluid of Goats

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The ante-mortem diagnosis of tuberculosis (TB) in ruminants is based mainly on the intradermal tuberculin test and the IFN-γ assay. Antibody (Ab)-based tests have emerged as potential tools for the detection of TB infected animals using serum, plasma, or even milk samples. Oral fluids have also been evaluated as alternative samples with which to detect specific Abs against *Mycobacterium bovis* in pigs or wild boars, but not in ruminants. The objective of this study was, therefore, to evaluate the performance of an in house-ELISA for TB diagnosis (P22 ELISA) in goats as an experimental model for the diagnosis of TB using oral fluid samples. Oral fluid samples from 64 goats from a TB-infected herd ($n = 197$) and all the animals from a TB-free herd ($n = 113$) were analyzed using the P22 ELISA. The estimated sensitivity (Se) and specificity (Sp) were 34.4% (95% CI: 22.4–45.6) and 100% (95% CI: 97.4–100), respectively. The optimal cut-off point was set at 100% according to the ROC analysis. Those animals with a higher level of Abs in their oral fluid attained a higher lesion score ($p = 0.018$). In fact, when taking into account only the setting of the animals with severe lesions ($n = 16$), the ELISA showed a Se of 75% (95% CI: 53.7–96.2). Results of the present study suggest that the P22 ELISA is highly specific but has a limited value detecting infected animals in oral fluid samples. Nevertheless, its performance is significantly higher in the presence of severe lesions.

Keywords: diagnosis, goat, tuberculosis, oral fluid, P22 ELISA

INTRODUCTION

Animal tuberculosis (TB) is a zoonotic infection that is caused mainly by *Mycobacterium bovis* and, more rarely, by other members of the *Mycobacterium tuberculosis* complex (MTBC) (1). The control programmes carried out for ruminants such as cattle and goats are based principally on a test and cull strategy using the single and comparative intradermal tuberculin (SIT and CIT) tests, both of which are based on cell-mediated immune response (2). The interferon-gamma release

assay (IGRA), which is an official ancillary diagnostic test for bovine TB, is used to maximize the detection of infected animals and is also based on the cellular immune response (3).

Serological tests have, in recent years, emerged as a potential ancillary test for livestock, and may even be a first option for wildlife owing to their advantages when compared to cell-based tests (4, 5). Moreover, serological tests could be a valuable diagnostic tool in the form of screening tests with which to detect TB at the herd level and have been shown to maximize the detection of TB infected ruminants when they are used in combination with cellular-based tests (6–8). An ELISA based on the recently developed P22 protein complex (P22 ELISA) has shown high performance in terms of sensitivity (Se) and specificity (Sp) in ruminants (4, 6, 8, 9).

Non-invasive and easy-to-collect samples other than serum and plasma have also been evaluated for the detection of specific Abs against several diseases including TB, one of which is oral fluids (10–13). Previous studies have evaluated the performance of the ELISA by comparing milk samples with those of serum for TB diagnosis, and have obtained a similar Se and Sp (14, 15). The use of milk samples is, however, restricted to dairy animals. Oral fluid, which is a biological fluid, can, meanwhile, allow the routine monitoring of animals' health status owing to the minimally invasive and non-stressful method employed to collect it, which can be performed by personnel with minimal training. Moreover, unlike the case of milk samples, it is not necessary for the animal to be lactating and allows males and those animals that do not produce milk (kids, females not lactating or meat herds) to be sampled (11) Oral fluid samples have additionally been proposed as an alternative biological specimen by which to detect specific Abs against *M. bovis* in wild boar, attaining a Se and a Sp of 67.3 and 100%, respectively (13). In fact, oral fluid samples have been used in the fight against other swine diseases, such as classical swine fever, influenza, or porcine reproductive and respiratory syndrome (PRRS) (16–20), thus suggesting that oral fluids are valuable samples for the surveillance and control of TB and other diseases in suids through the use of Ab-based diagnostic platforms (21).

Despite their use in swine populations, few studies have evaluated serological tests by employing oral fluid samples in ruminants in order to detect antibodies against foot and mouth disease virus (FMDV) or Schmallenberg virus (SBV) in bovines (22, 23). With regard to TB, no studies using oral fluid samples for the antibody-based diagnosis of TB in domestic ruminants have, to the best of the authors' knowledge, been published previously.

Given the usefulness of oral fluid samples in other species, we have adapted a P22 ELISA in order to analyze oral fluid samples in goats. The main objective of the present study was to evaluate, for the first time, the usefulness of oral fluid samples as regards detecting specific antibodies against *M. bovis* in ruminants.

MATERIALS AND METHODS

Study Design and Herds of Study

The study was performed with two herds of Guadarrama-breed goats located in central Spain, one of which was *M. bovis*-infected (range: 1–7 year old animals) and one of which was TB-free (range: 1–6 year old animals), which were used for Se and Sp estimations, respectively. The Se was evaluated in a herd ($n = 197$) in which *M. bovis* SB0121 was isolated. This herd was subjected to a SIT test, a CIT test and an IGRA, which showed an apparent prevalence of 87.5, 67.7, and 53.8%, respectively. Owing to the high proportion of reactors, all the animals were slaughtered and subjected to *post-mortem* analysis. The presence of TB-compatible lesions was evaluated during slaughtering, and tissue samples were collected for the bacteriological culture and isolation of bacteria in the laboratory. Culture positive (*M. bovis* SB0121 was isolated) animals and those with TB compatible lesions ($n = 64$) were included in the study in order to evaluate the Se of the P22 ELISA in oral fluid samples. The Sp was evaluated in a TB-free herd ($n = 113$), based on its history of TB-free status and the negative results obtained from the animals' SIT, CIT, and serological tests (P22 ELISA) in the last three testing events in the last 2 years. The Gudair vaccine (CZ Vaccines, Porriño, Spain) against *M. avium* subsp. *paratuberculosis* (MAP) had been administered to the animals in both herds at the age of 6 months as part of their vaccination programmes.

The animals included in the study were subjected to SIT and CIT tests and IGRA. Serum and oral fluid samples were collected before the intradermal test and analyzed using a P22 ELISA. The results obtained were compared with the *post-mortem* analysis.

The animals in the present study were not considered as experimental animals. All handling and sampling procedures were performed in compliance with Spanish legislation (Royal Decree 720.7/2011).

Serum and Oral Fluid Sample Collection

Blood samples were collected from the jugular vein by means of venipuncture, using plastic serum tubes (BD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA). The samples were stored at room temperature for 24 h and then centrifuged for 15 min at 650 g, after which sera were stored at −20°C until assaying. Oral fluid samples were obtained from the same goats by dry swabbing the animals' mouths, which were cleaned beforehand. In the laboratory, the dry swabs were introduced into 2 ml tubes with a medium composed of 4 µl of azidiol (Panreac, Spain) and 1 ml of phosphate buffered saline (PBS). These dry swabs were conserved at 4°C for 8 h, after which the samples were centrifuged at 13,000 g for 5 min, and an aliquot of 500 µl of the supernatant was obtained and stored at −20°C until the assay test.

P22 ELISA

Serum samples were analyzed by employing a P22 ELISA, as previously described by Infantes-Lorenzo et al. (4). The protocol was adapted to oral fluid samples as follows: the optimal dilution of oral fluid was determined by evaluating the reactivity of samples diluted from 1:2 to 1:128, and a dilution of 1:2 was eventually chosen. One hundred microliters of detection antibody at 1:2,000 were added, and the plates were incubated at room temperature for 30 min. As before, the secondary antibody [Rabbit anti sheep IgG(H/L)-HRP] (Southern Biotech, USA) was titrated from 1:500 to 1:8,000 in order to choose the optimal dilution.

The results were expressed as a P22 ELISA percentage (E%), which was calculated using the following formula:

$$E\% = [\text{mean sample OD}/(2\times\text{mean of negative control OD})] \times 100$$

In the case of the serum samples, E% values of 100 or higher were considered positive, as described elsewhere (4), while in that of oral fluid samples, a cut-off was calculated using a receiver operating characteristic (ROC) analysis, and E% values of 100 or higher were considered positive.

Intradermal Tuberculin Tests

The SIT and CIT tests were carried out by means of the intradermal inoculation of 0.1 ml of bovine and avian PPDs (CZ Vaccines, Porriño, Spain) in the right and left site of the cervical region, respectively, using a Dermojet syringe (Akra Dermojet, Pau, France). All tests were performed according to Council Directive 64/432/EEC and Royal Decree RD2611/1996, and the reactions were interpreted as previously described (24).

Interferon-Gamma Release Assay

Blood samples were collected from the jugular vein using evacuated tubes (BD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA) with heparin in order to detect IFN-γ production. The blood samples were then processed as previously described (25). The blood was incubated at 37°C in a humidified atmosphere in the presence of antigens (PPD-B and PPD-A) for 18–20 h. The samples were then centrifuged at 2,500 rpm for 15 min, and the supernatant was collected. Interferon-gamma release was measured using a commercial IGRA designed for goats (Bovigam TB kit, Thermo Fisher Scientific, Waltham, USA), according to manufacturer's instructions, and the results were interpreted as described elsewhere (26).

Post-mortem Analysis

TB-compatible gross lesions (TBL) include nodular off-white lesions containing caseous material, which may be mineralized in the center and encapsulated by fibrous tissue (27, 28). The inspection and semi-quantitative scoring of the TBL present in the lung lobes and lymph nodes (LNs) of culled animals were carried out on the basis of a previous lesion valuation model proposed by Vordermeier et al. (29), with some modifications. This scoring system relies on the size and number of lesions, in addition to the percentage of the organ affected, as follows: 0, no visible lesions; 1, one small lesion apparent on slicing; 2, <5 lesions of <10 mm in diameter; 3, more than five lesions of <10 mm in diameter or one lesion >10–30 mm in diameter and/or <50% of the organ affected; 4, more than one lesion >30 mm in diameter and/or >50% of the organ affected; and 5, coalescing lesions and >70% of the organ affected. In the lungs, one extra point was awarded to animals that had pleural adhesions. All the lung lobes (left apical, left diaphragmatic, right apical, right cardiac, right accessory, and right diaphragmatic) were examined individually and the scores of these lobes were added up in order to calculate the total lung score. The head LN (retropharyngeal) and pulmonary LNs (tracheobronchial and mediastinal) were likewise examined individually, and the scores of the different LNs were added up in order to calculate the total score.

Tissue samples from the lungs and the retropharyngeal, tracheobronchial, and mediastinal LNs from 64 animals with TB lesions were used for bacteriological culture in Löwenstein-Jensen with sodium pyruvate medium (Difco, Spain), as described previously (15). Animals with TB-compatible lesions in the lungs or in the different LNs analyzed in the present study were considered as TB positive. A comparison between the P22 ELISA results and the lesion score obtained for the lungs and/or head and pulmonary LNs was then carried out.

Statistical Analysis

Wilson's 95% confidence intervals (95% CI) were calculated for the percentage of positive reactors to the different tests. ROC analysis was performed to define the optimal cut-off value (**Supplementary Material**). Quantitative values, such as E% in oral fluid samples with regard to the TBL score, were attained for the two different herds and were compared using the Mann–Whitney *U*-test. Moreover, the variation of quantitative values of the diagnostic techniques regarding to the TBL score was calculated using an *R*-squared (R^2) and interpretated as follows: 0.00–0.25 poor, 0.26–0.50 fair, 0.51–0.75 moderate, and 0.76–1.00 substantial. All the statistical tests were carried out using SPSS Statistics 25 (IBM, New York, NY, USA), and interpreted by considering a *p*-value of 0.05 in order to determine statistical significance.

RESULTS

With regard to the P22 ELISA carried out using oral fluid samples, a ROC curve was calculated using samples from TB-infected ($n = 64$) and TB-free ($n = 113$) animals. The optimal cut-off point was set at 100%, at which the highest Se and Sp were observed. A higher or lower cut-off point caused a loss of Se with a constant Sp, or vice versa, and it was for this reason that an E% of 100 was chosen as the cut-off point.

In the present study, 22 out of the 64 TB-infected animals attained positive results to the P22 ELISA when using oral fluids, yielding an estimated Se of 34.4% (95% CI: 22.4–45.6). All the animals from the TB-free herd tested negative to the P22 ELISA when using oral fluid samples, attaining an Sp of 100% (95% CI: 96.7–100; **Table 1**). The use of the selected cut-off point made it possible to obtain the positive predictive value [100% (95% CI: 97.4–100)], negative predictive value [77.2% (95% CI: 71.1–83.2)], and area under the curve (0.827). Nineteen out of the 22 P22 ELISA-positive goats similarly had TB-compatible lesions in their lungs, and 20 out of 22 had them in different LNs (17 out of 22 in both locations). Taking into account only the setting of 16 animals with severe lesions (lesion score over 10), the ELISA showed an Se of 75% (95% CI: 53.7–96.2). Moreover, those animals that were positive to the P22 ELISA as regards oral fluid samples had a significantly ($p = 0.018$) higher lung TBL score (**Figure 1**).

When using the serum samples, 56 out of the 64 *M. bovis*-infected goats were positive to the P22 ELISA (E% > 100),

with an Se of 87.5% (77.2–93.5, 95% CI) (**Table 2**). The *M. bovis*-infected animals had a higher E% when employing the serum (median = 653.9 E%; $p < 0.0001$) than when employing the oral fluid samples (median = 66.6 E%) (**Figure 2**). Similar E% for oral fluid (median = 46.2 E%) and serum (median = 55.2 E%) samples were obtained from animals in the TB-free herd, and no significant differences were observed ($p = 0.852$). Finally, there was a very small correlation between the P22 results obtained for the oral fluid and serum samples ($R^2 = 0.032$) and between the E% results obtained for the oral fluid and the TB compatible lesions observed in the lungs of the slaughtered goats ($R^2 = 0.142$) and the head and pulmonary LNs: retropharyngeal ($R^2 = 0.206$), tracheobronchial ($R^2 = 0.081$), and mediastinal ($R^2 = 0.116$) (**Supplementary Material**). The correlation between the oral fluid E% and the total lesion score was slightly higher ($R^2 = 0.225$).

Finally, with regard to the techniques based on cellular immune response, of the 64 goats in the TB-infected herd, 55 and 37 were considered positive reactors to the SIT test [(85.9% (95% CI: 75.3–92.4)] and the CIT test [(57.8% (95% CI: 45.6–69.1)], respectively. In this herd, 38/64 animals were positive to IGRA, with an apparent prevalence of 59.4% (95% CI: 47.1–70.5) (**Table 2**). In this respect, there was no correlation between IGRA and the total lesion score results ($R^2 = 0.004$), which was lower than the correlation observed with E% when using oral fluid samples. A poor correlation was observed between SIT and CIT tests and the total lesion score results ($R^2 = 0.042$ and 0.069, respectively) (**Supplementary Material**).

TABLE 1 | Results used to determine the sensitivity and specificity of the oral fluid P22 ELISA for diagnosis of tuberculosis in goats.

Sample type	Presence of TB-lesions	Absence of TB-lesions	Total test results
Oral fluid ELISA test positive	22* (TP)	0 (FP)	22 (total test positives)
Oral fluid ELISA test negative	42 (FN)	113** (TN)	184 (total test negatives)
Total samples analyzed	64	113	206 (total population)

**Sensitivity of the P22 ELISA was 34.4% (22/64); **Specificity of the P22 ELISA was 100% (113/113).*

DISCUSSION

In the present study, an in-house ELISA with which to detect specific antibodies against *M. bovis* in oral fluid samples was developed and evaluated for the first time in goats. The results showed a high Sp but a limited Se of the P22 ELISA for TB diagnosis using oral fluid samples from goats.

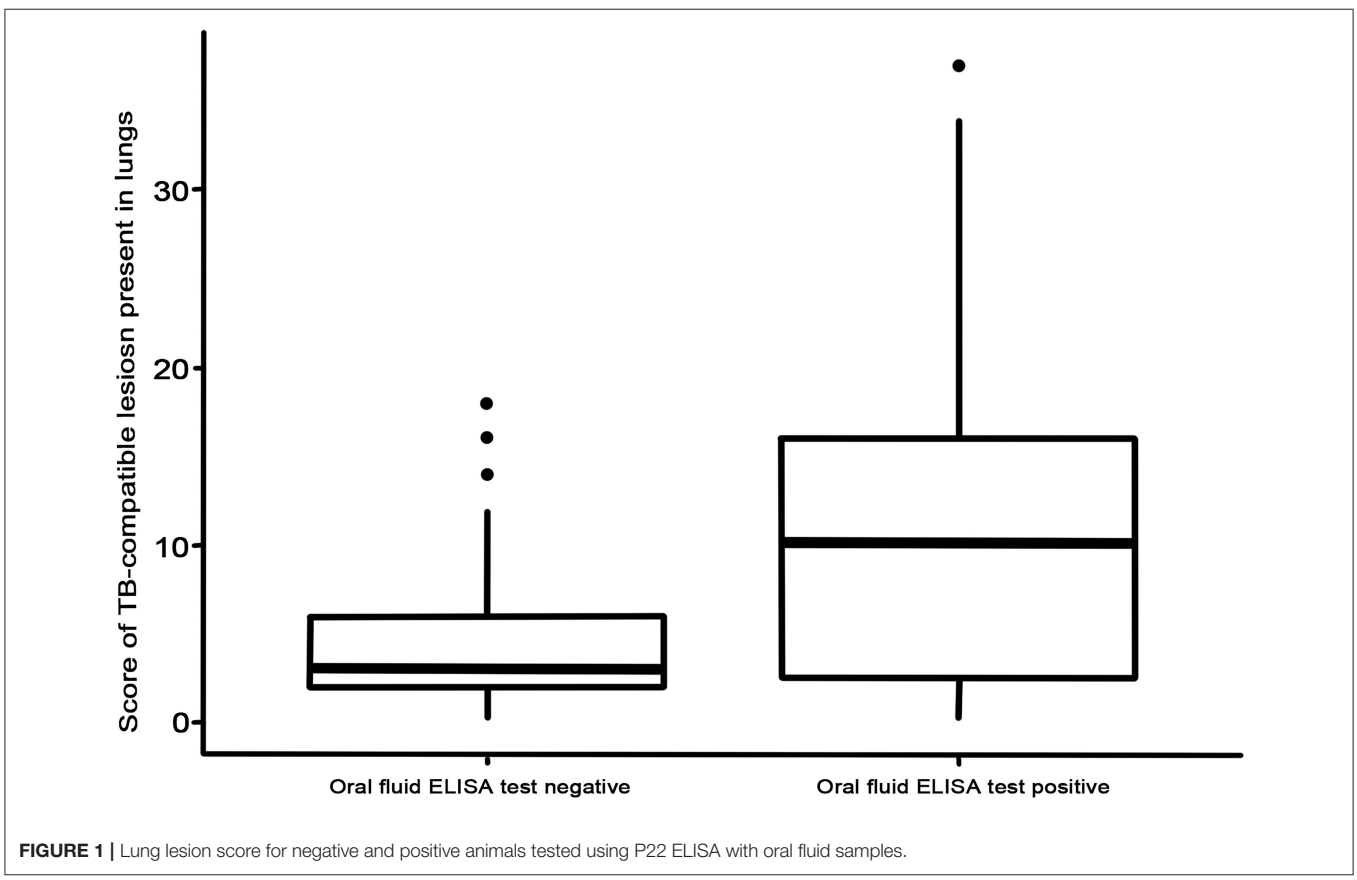

FIGURE 1 | Lung lesion score for negative and positive animals tested using P22 ELISA with oral fluid samples.

TABLE 2 | Summary of the ante-mortem tuberculosis (TB) diagnostic tests and post-mortem analysis in the goats under study.

Herd	Animals	SIT test[a]	CIT test[b]	IGRA[c]	P22 ELISA (serum)[d]	P22 ELISA (oral fluid)[d]	TB lesions in lungs	TB lesions in head and pulmonary lymph nodes
***M. bovis*-infected**	64	55 (85.9%)	37 (57.8%)	38 (59.4%)	56 (87.5%)	22 (34.4%)	54 (84.4%)	60 (93.7%)
TB-free	113	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[a] *SIT, Single intradermal tuberculin test: protocol and interpretation was performed according to Council Directive 64/432/EEC and Royal Decree RD2611/1996.*
[b] *CIT, Comparative intradermal tuberculin test: protocol and interpretation was performed according to Council Directive 64/432/EEC and Royal Decree RD2611/1996.*
[c] *IGRA, interferon-gamma release assay: an animal was considered positive to IGRA if the optical density (OD) of a sample stimulated with bovine PPD minus the OD of the aliquot stimulated with PBS (nil) was >0.05 and greater than the OD of the sample stimulated with avian PPD.*
[d] *P22 ELISA, an animal was considered positive to ELISA p22 when the E% value was >150 (serum) or 100 (oral fluid).*

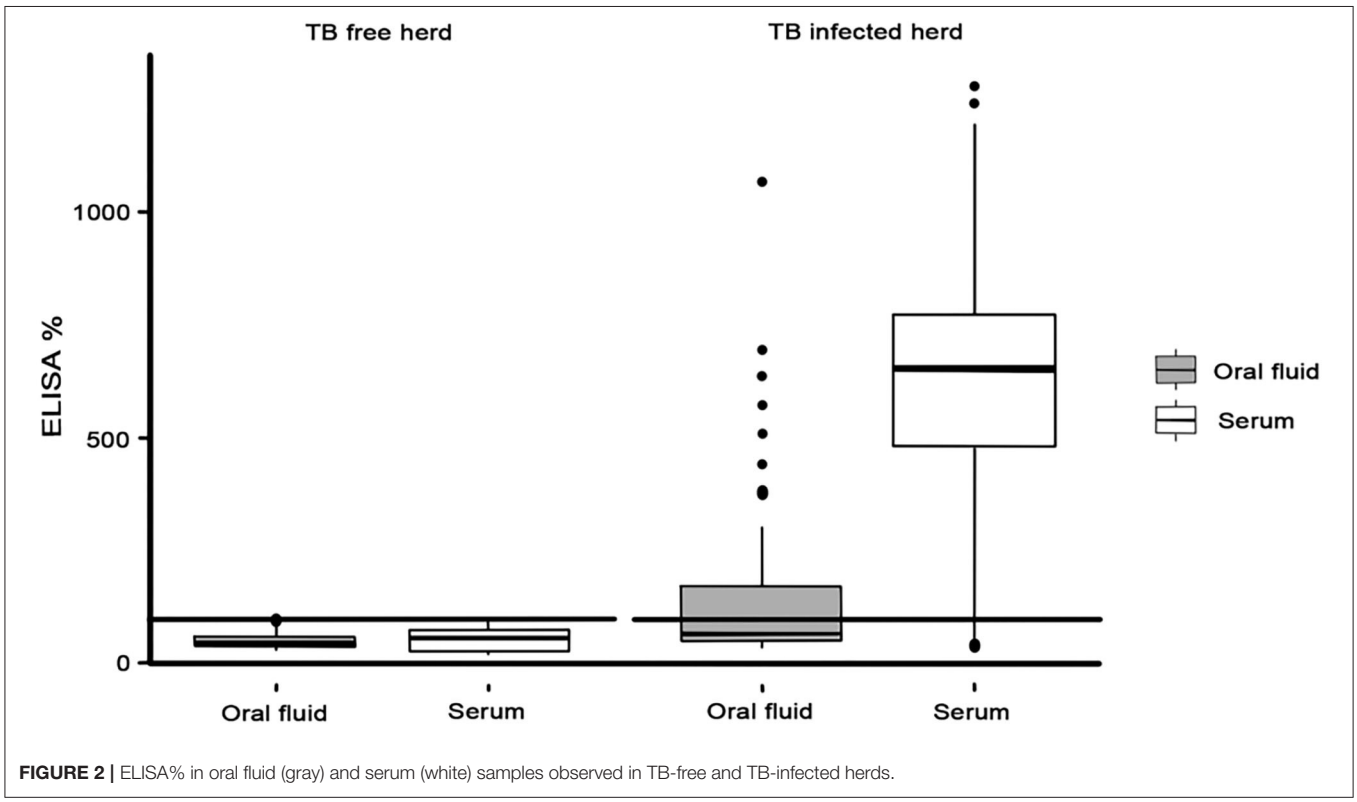

FIGURE 2 | ELISA% in oral fluid (gray) and serum (white) samples observed in TB-free and TB-infected herds.

The P22 ELISA had previously been evaluated for TB diagnosis in goats using sera and milk samples, showing promising results as regards Se and Sp (4, 8, 15). With regard to oral fluid samples for TB diagnosis, a previous study was carried out using a PPD-B-based ELISA in wild boar (13). However, to the best of the authors' knowledge, no previous studies using an ELISA for the detection of specific antibodies against TB in oral fluid samples from ruminants are available. With regard to Sp, the P22 ELISA achieved an excellent Sp of 100%, which was higher than that obtained by the serological tests described to date for the diagnosis of TB in goats using serum or milk samples (4, 7). Interference of vaccination against MAP on the diagnosis of TB in goats has been previously described in TB-free herds in other countries (30). Moreover, different studies have reported a lower Sp [58.3 (95% CI: 42.2–72.9)−96 (95% CI: 90.1–98.4)] of the P22 ELISA in MAP vaccinated goats than in non-vaccinated goats (4, 31). The higher Sp obtained in our study [100% (95% CI: 96.7–100)] using oral fluids was related to the low Se achieved. Therefore, although both the herds studied herein were vaccinated against *M. avium* subsp. *paratuberculosis* (MAP), a cross-reaction in the TB oral fluid test owing to MAP vaccination was not demonstrated in our study using the recommended cut-off point. However, despite the optimal performance of the P22 ELISA when using serum and milk samples, the present study showed that the usefulness of oral fluid samples as regards detecting *M. bovis* infection in goats was limited, unlike that which occurred in a previous study with wild boar that reported an Se of 67.3% (13). The performance of a diagnostic test is usually evaluated by taking into account the results of a bacteriological culture that is considered the gold standard. None of the *ante-mortem* tests used to define TB infection in goats are perfect in terms of Se and Sp, and they are

not, therefore, accurate indicators of the real TB status of the animals. In this respect, the results of serology were used as a reference to define the infection status in the study of wild boar, and this could have affected the accuracy of the test (13). In fact, when the Se of the P22 ELISA carried out using oral fluid samples was estimated using serum results as a gold standard, the Se was slightly higher in our study [39.3% (95% CI: 27.6–52.4) data not shown], but still lower than that reported for wild boar.

It is necessary to state that in the present study, the P22 ELISA attained a higher Se when using serum samples than when using oral fluid samples. Moreover, the Se of TB diagnostic techniques based on specific-antibody detection can be improved by using samples collected after the PPD inoculation. This phenomenon, which is denominated as the booster effect, has been reported in different species (8, 32, 33). It has been suggested that the booster effect on the antibody response is a valuable methodology by which to increase the Se of serological assays in ruminants (6, 8). Further studies with which to evaluate this booster effect in oral fluid samples are, however, required.

The Se of the antibody detection tools is generally lower than that reported when using diagnostic tools based on cellular immune response (34). However, in this work, a higher Se was obtained when using serum samples (87.5%) than when using IGRA (59.4%), SIT (85.9%), and CIT (57.8%) tests, even in the absence of the booster effect. These results support the value of serological tests as a tool for TB diagnosis in ruminants, as observed in recent studies (4, 6, 8, 9), and the importance of developing new TB diagnostic tools in order to maximize the detection of infected animals.

It is widely known that the production and concentration in the samples of the different immunoglobulin (Ig) may differ between species. In this context, the IgG concentration in the oral fluid from goats is significantly lower than that in swine (35), which may explain the differences in the Se previously stated. The IgA-antibody levels in ruminants are, on the contrary, higher than in other species, which could explain the differences observed in the diagnosis when employing IgA or IgG based-ELISAs for other infections such as Schmallenberg disease (23). Moreover, recent studies support the theory that the detection of IgA in oral fluid samples appears to be more robust and stable over time in pigs (20). Ab production in oral fluid is related to mucosal immunity, and local antigen stimulation is, therefore, of paramount importance if high levels of IgA are to be attained in that sample. This could also explain the differences observed between serum and oral fluid samples. In this respect, in the present study, a better correlation between E% in oral fluid samples and the severity of lesions was observed in the retropharyngeal LNs. These LNs are closer to the salivary glands when compared to the pulmonary LNs (mediastinal and tracheobronchial), but additional studies are required in order to confirm the potential correlation between a more severe local immune response and a higher antibody production in ruminants.

Finally, it is necessary to state that, in this study, a goat model was used to evaluate the performance of the P22 ELISA when using oral fluid samples. Previous studies have shown promising results of antibody-based platforms for the diagnosis of other diseases (e.g., FMDV or SVB) when using oral fluid samples in cattle (22, 23). The results from the present study suggest a similar or limited performance of the P22 ELISA in oral fluid samples from cattle owing to the high diagnostic pressure as a consequence of the official eradication programmes, since it is difficult to find animals in an advance stage of infection. The realization of similar studies with which to confirm this hypothesis would, nevertheless, be interesting.

In conclusion, the use of oral fluid sample biomarkers for TB diagnosis in ruminants is still far from being routinely applied and requires further validation and research. The overall results obtained from the present study suggest that employing the P22 ELISA for the detection of specific antibodies in oral fluid samples is highly specific but has a limited value as regards detecting infected animals. Nevertheless, its performance is significantly higher in the presence of severe lesions, detecting a high proportion of those animals in the herd that have these lesions.

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.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the animals in the present study were not considered as experimental animals. All handling and sampling procedures were performed in compliance with Spanish legislation (Royal Decree 720.7/2011). Written informed consent for participation was not obtained from the owners because the animals has an owner but he collaborates with our research center and he is aware of all the studies and publications (he prefers to be anonymous).

AUTHOR CONTRIBUTIONS

JO, JI-L, and JB wrote the manuscript and designed the figures. JO and JI-L performed the literature search. JO, JI-L, AG-B, and IA-R performed the experiments. JO, JI-L, JB, LJ, IM, IA-R, ÁR, LJ, BR, and MD interpreted the data. All the authors reviewed and approved the 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.2021.674636/full#supplementary-material

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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A Defined Antigen Skin Test for Diagnosis of Bovine Tuberculosis in Domestic Water Buffaloes (*Bubalus bubalis*)

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Bovine tuberculosis (bTB) remains endemic in domestic water buffaloes (*Bubalus bubalis*) in India and elsewhere, with limited options for control other than testing and slaughter. The prescribed tuberculin skin tests with purified protein derivative (PPD) for diagnosis of bTB preclude the use of Bacille Calmette-Guérin (BCG)-based vaccination because of the antigenic cross-reactivity of vaccine strains with *Mycobacterium bovis* and related pathogenic members of the *M. tuberculosis* complex (MTBC). For the diagnosis of bTB in domestic water buffaloes, we here assessed a recently described defined-antigen skin test (DST) that comprises overlapping peptides representing the ESAT-6, CFP-10 and Rv3615c antigens, present in disease-causing members of the MTBC but missing in BCG strains. The performance characteristics of three doses (5, 10 or 20 μg/peptide) of the DST were assessed in natural tuberculin skin test reactor ($n = 11$) and non-reactor ($n = 35$) water buffaloes at an organized dairy farm in Hisar, India, and results were compared with the single intradermal skin test (SIT) using standard bovine tuberculin (PPD-B). The results showed a dose-dependent response of DST in natural reactor water buffaloes, although the SIT induced a significantly greater ($P < 0.001$) skin test response than the highest dose of DST used. However, using a cut-off of 2 mm or greater, the 5, 10, and 20 μg DST cocktail correctly classified eight, 10 and all 11 of the SIT-positive reactors, respectively, suggesting that the 20 μg DST cocktail has a diagnostic sensitivity (Se) of 1.0 (95% CI: 0.72–1.0) identical to that of the SIT. Importantly, none of the tested DST doses induced any measurable skin induration responses in the 35 SIT-negative animals, suggesting a specificity point estimate of 1.0 (95% CI: 0.9–1.0), also identical to that of the SIT and compares favorably with that of the comparative cervical test (Se = 0.85; 95%

CI: 0.55–0.98). Overall, the results suggest that similar to tuberculin, the DST enables sensitive and specific diagnosis of bTB in water buffaloes. Future field trials to explore the utility of DST as a defined antigen replacement for tuberculin in routine surveillance programs and to enable BCG vaccination of water buffaloes are warranted.

Keywords: water buffaloes (*Bubalus bubalis*), defined antigen skin test, sensitivity, specificity, bovine tuberculosis

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic inflammatory disease of cattle caused by members of the *Mycobacterium tuberculosis* complex, and in addition to being an important animal health problem, bTB also poses a significant threat to public health (1). It has been estimated that annual worldwide economic losses associated with bTB are ∼USD 3 billion (2). The disease is well-controlled in high-income countries, however, bTB remains endemic in most low- and middle-income countries (LMICs), including India where bTB has significant impacts in terms of decreased productivity, increased mortality and zoonotic threat. While national control programs involving test-and-cull strategies have proven to be hugely successful in high-income countries, such approaches are often not feasible in LMICs for both social and economic reasons.

India has the largest livestock population in the world, including nearly 191 million cows and 109 million buffaloes (3). Livestock rearing is one of the most important activities in the rural areas of the country, and for many individuals, it is the only source of livelihood. Haryana, a state in northern India, has a large number (∼4.3 million) of the Murrah breed of domestic water buffaloes (*Bubalus bubalis*). Indigenous buffaloes are important economically, contributing nearly 35% of the country's total milk (3). Although bTB has been well-studied in cattle generally, studies on this disease in buffalo are scarce, especially for high-producing breeds like Murrah.

A recent meta-analysis on bTB in India reported a pooled prevalence of bTB of 4.3% (95% CI: 2.7, 6.7) in buffaloes, calculated from a total of 29,037 animals tested between 1942 and 2016 (4). Without the implementation of any disease control program, this current level of endemicity is predicted to increase in the coming years, especially given the predicted intensification of dairy farming in India. Given that test-and-cull-based control programs are not implementable in India, a vaccine-based intervention strategy may be a promising solution. The Bacille Calmette-Guérin (BCG) vaccine was initially developed for control of human tuberculosis by Albert Calmette and Camille Guérin and was first used in humans in 1921 (5). While there is evidence supporting BCG-induced protection against bTB, the vaccine has not yet been licensed for use in cattle due to the presence of cross-reactive antigens that interfere with the specificity of the tuberculin skin tests recommended by the World Organisation for Animal Health (6, 7).

Recent developments in the field of bTB diagnostics have focused on fit-for-purpose tools that can reliably differentiate infected and (BCG) vaccinated animals (DIVA) in order to make implementation of BCG-based intervention strategies a possibility in LMICs. Several antigens with DIVA capability have been evaluated, of which ESAT-6, CFP-10, and Rv3615c appear to be the most promising (8, 9). These antigens have been extensively evaluated both in experimental and field conditions, and a peptide-based formulation of these antigens, henceforth referred to as the defined antigen skin test (DST), was previously evaluated for its utility in field studies in cross-bred cattle both in natural reactors and in BCG vaccinates (9, 10). Here, we assess the utility and performance of DST in buffaloes in India.

MATERIALS AND METHODS

Antigens and Peptides

The DST used in the study is comprised of *M. bovis* antigens ESAT-6, CFP-10, and Rv3615c. Peptides ($n = 13$) representing these antigens were chemically synthesized at >98% purity by GenScript USA, Inc. and USV Private Limited, India (see **Supplementary Table 1** for peptide sequences). The safety of DST has been demonstrated in *Bos taurus* subsp. *indicus* under Good Laboratory Practice (GLP) conditions in India with repeat and overdosing experiments (unpublished data). The bovine tuberculin (PPD-B) and avian tuberculin (PPD-A) were sourced from Prionics, Thermo Fisher, Schlieren, Switzerland.

Animals

To determine the performance characteristics of DST in buffaloes, skin tests were conducted in adult female Murrah buffaloes (3–5 years old) recruited from an animal farm at the Lala Lajpat Rai University of Veterinary and Animal Science (LUVAS), Hisar, Haryana, India. Recruited animals that were known bTB reactors based on prior single intradermal testing with PPD-B ($n = 11$), were housed separately from the healthy herd. Skin tests were also conducted in control naïve animals ($n = 35$) from the organized dairy farm. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of the institute (letter number VCC/IAEC/2590-2619 dated 27-12-2018).

Interferon-Gamma Enzyme-Linked Immunosorbent Assay

For *in vitro* stimulation of whole blood, PPD-B and PPD-A were used at a final concentration of 300 and 250 IU/ml, respectively, as per the BOVIGAM™ kit (Thermo Fisher Scientific) instructions. The DST peptide cocktail was used at 10 μg/ml in *in vitro* assays. Whole blood was collected and stimulated overnight at 37°C, 5% CO_2, with the antigens *in vitro*. BOVIGAM™ kits were used to determine IFN-γ concentrations in whole-blood culture supernatants. Results

for antigen-stimulated cultures were expressed as background-corrected optical density at 450 nm (i.e., ΔOD450).

Intradermal Skin Test Procedures

Skin tests using PPD-tuberculins (PPD-A at 25,000 IU/ml and PPD-B at 30,000 IU/ml) were performed as recommended by the manufacturer (Thermo Fisher Scientific, USA), and the results were interpreted as per OIE guidelines (11). Skin thickness was measured by the same operator before administration of PPD and at 72 h post-injection. Skin test readings were measured in millimeters as per OIE-prescribed guidelines. The DST was administered in three different doses, i.e., 5, 10, and 20 μg of each peptide constituent (final injection volume = 0.1 ml), and the sites of injection were randomized. For the single intradermal test (SIT) involving only PPD-B, an increase in skin thickness of 4 mm or more was considered a positive reaction (11), whereas for DST, an increase in skin thickness of 2 mm or more was considered a positive reaction based on the studies conducted in cattle (9). For the comparative cervical test (CCT) involving injection of both PPD-A and PPD-B, a difference in increase in skin thickness between the two infection sites (B-A) is calculated. Per OIE guidelines, a measurement of >4 mm was considered positive for CCT.

Statistical Analyses

All statistical analyses were performed using Prism 8 (GraphPad Software, La Jolla, CA). Confidence intervals (CI) for the sensitivity estimates in natural reactors for DST and PPDs were calculated using the Clopper-Pearson method. For PPD-B, we calculated a one-sided CI (lower 95% CI: 76). Standard two-sided CIs were calculated for CCT, i.e., PPD (B-A) and DST using the same method with point estimates of 82% (95% CI: 48, 98).

RESULTS

Performance of the Defined Antigen Cocktail in the *in vitro* IFN-γ Release Assay

IGRAs were conducted to compare the performance of the DST peptide cocktail, composed of ESAT-6, CFP-10, and Rv3615c, with that of the PPDs (**Figure 1**). The data demonstrated that PPD-B induced a significantly greater IFN-γ response in reactor animals when compared to the DST cocktail ($P < 0.05$; mean of difference = 0.053), while there was no statistical significance in the response induced by DST vs. PPD-B minus PPD-A ($P = 0.98$). Out of 11 animals tested, nine showed IGRA positivity (IGRA cut-off > 0.1) when PPD-B alone was considered, eight were IGRA-positive by PPD-B minus PPD-A, and seven were IGRA-positive by DST; however, the differences were not statistically significant. The data suggest that the DST peptide cocktail may be used as a stimulating antigen in blood tests for diagnosis of bTB in buffaloes.

Defined Antigens Induce a Sensitive and Specific Skin Test Response

The performance of the DST was assessed in natural reactor ($n = 11$) and non-reactor ($n = 35$) buffaloes. The results showed that, when using a cut-off of 2 mm or more, the DST cocktail at 5, 10, and 20 μg correctly classified eight, 10 and 11 of the 11 reactors as positive, respectively (**Figure 2** and **Table 1**). The standard bovine tuberculin antigen (PPD-B), used in the SIT, induced a significantly stronger skin test response than that induced by the highest dose of DST used in this study ($P < 0.001$) and identified all 11 reactors as positive, whereas the CCT identified nine of the 11 buffaloes as reactors. Notably, none of the tested DST doses induced any measurable skin induration responses in the control group ($n = 35$).

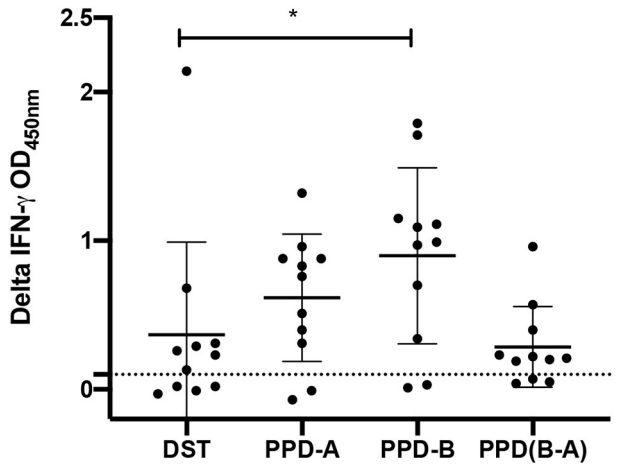

FIGURE 1 | Capacity of DST antigens to induce an *in vitro* IFN-γ response in whole blood collected from naturally infected buffaloes ($n = 11$). The background-corrected optical density (OD) values for all antigens tested are shown. The horizontal line indicates the mean (±SD), and the statistical difference between the responses was determined by using the paired *t*-test (*$P < 0.05$). The means of background-subtracted OD values for DST, PPD-A, PPD-B, and PPD(B-A) were found to be 0.37, 0.62, 0.90, and 0.29, respectively. The standard deviations for DST, PPD-A, PPD-B, and PPD(B-A) were 0.62, 0.43, 0.60, and 0.27, respectively.

DISCUSSION

Recent developments in the field of bTB diagnostics have centered around identifying candidate antigens for DIVA in order to enable vaccine-based interventions in LMICs. Previous studies on the use of defined skin test antigens in cattle, using a combination of ESAT-6, CFP-10, MPB70, and MPB83, concluded that these antigens are promising candidates for performing the IGRA in cattle, with the ability to differentiate between vaccinated animals and those infected with *M. bovis* (8, 12). The current study was performed to assess the performance of a recently developed peptide-based DST cocktail in Murrah buffaloes, an economically important breed of buffaloes in India. In this study, we first performed IGRAs with DST peptides at 10 μg/ml. Although PPD-B was the most sensitive in eliciting IFN-γ responses *in vitro*, there was no significant difference in the responses elicited by the DST peptides and PPD(B-A). To our knowledge, this is the first study where potential of defined skin test antigens has been demonstrated in naturally tuberculin skin test reactor domestic buffaloes. Our data demonstrated

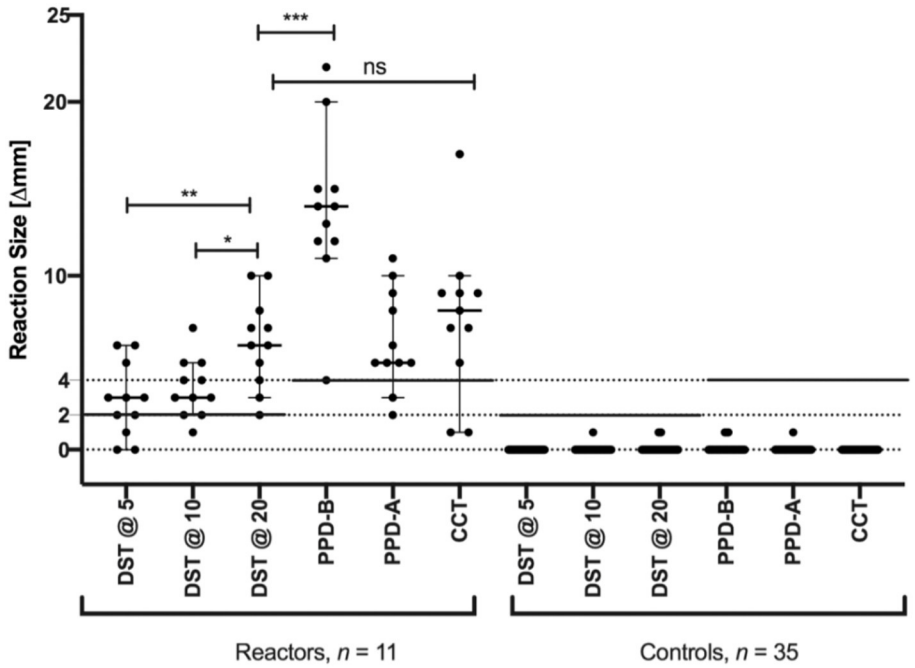

FIGURE 2 | Skin test responses for PPD-A, PPD-B and DST at three doses (5, 10, and 20 ug/ml). Responses were measured at 72 h after injection in naturally infected ($n = 11$) and naive control ($n = 35$) buffaloes. Results are expressed as the difference in skin thickness (in millimeters) between the pre- and post-skin-test readings, with the horizontal line providing the median (±95% CI). The statistical difference between the responses was determined using analysis of variance (ANOVA) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The solid horizontal lines at 2 and 4 mm are the cut-offs used for DST, and CCT and PPD-B, respectively. The mean skin thickness recorded for DST@5, DST@10, DST@20, PPD-B, PPD-A, and CCT in reactor buffaloes are 2.8, 3.5, 6.2, 13.8, 6.3, and 7.5 mm respectively.

TABLE 1 | Relative sensitivities of SIT, CCT, and DST at different doses.

Test[a]	Positive	Negative	Sensitivity
SIT[b]	11	0	100% (lower 95% CI: 71.5)
CCT	9	2	84.6% (95% CI: 54.5, 98.1)
DST, 5 μg	8	3	78.6% (95% CI: 49.2, 95.3)
DST, 10 μg	10	1	91.7% (95% CI: 61.5, 99.8)
DST, 20 μg[b]	11	0	100% (lower 95% CI: 71.5)

[a] *The diagnostic specificity for all tests listed was found to be 100% (lower 95% CI: 71.5).*
[b] *The SIT and the DST with 20 μg identified all 11 natural reactors as test-positive.*

that, these peptide-based antigens have promising potential for use in IGRAs as an ancillary test in concert with the skin test for distinguishing between reactor and uninfected animals. However, as this proof-of-concept study has only been performed on a small number of animals, it will be important to validate the performance of DST in a larger cohort of known infected and naïve buffaloes.

We also assessed the utility of the DST peptide cocktail as a skin test reagent in buffaloes. Of the three different doses (5, 10, and 20 μg per peptide) of DST that were compared, 20 μg/ml appeared to have the highest sensitivity, although higher concentrations may need to be tested to achieve an optimal balance between sensitivity and specificity. Most importantly, the peptide cocktail also proved to be highly specific, as no measurable skin induration response was observed following injection into naïve uninfected buffaloes. Both SIT and the highest dose of DST were found to be 100% sensitive, identifying all 11 naturally-infected buffaloes as reactors. In this context, it is important to note that, given the high burden of environmental mycobacteria in India, PPD-B can induce non-specific responses, often leading to high rates of false-positivity with SIT. The CCT with the simultaneous injection of PPD-A helps overcome this loss of specificity associated with SIT, but only at the expense of sensitivity. The results of this study showed that the peptide-based DST provides sensitivity and specificity equivalent to that of the OIE-recommended CCT, without the need for a second injection. Moreover, DST at its highest tested dose (20 μg/ml) elicits a lower amplitude of skin test response in infected animals (6.18 ± 2.60 mm) as compared with PPD-B (13.81 ± 4.68 mm) without hindering the specificity estimate. Such exuberant non-specific PPD-B responses tend to raise concerns and limit acceptability of the SIT among farmers. In reactor buffaloes, we found that 20 μg DST produced a skin response greater than the cut-off of 2 mm in all of the animals tested. In this context, it is crucial to highlight the difference in antigen dosage that may be necessary for accurate bTB diagnosis in cattle and buffaloes. In a previous study with cross-bred cattle, pilot dose titration experiments showed that DST@10 may be the optimal dose in cross-bred cattle (9). However, here the data show that DST@20 may be better suited for bTB diagnosis in buffaloes as it identified all reactors as test-positive without compromising

test Specificity. Future studies that are adequately statistically powered are required to accurately determine the antigen dosage required for diagnosis in buffaloes.

In 2019, the World Health Organization reported that over nine million people developed TB, of which ∼1.5 million died (13). Only eight countries account for about two-thirds of the total reported cases of TB, with India leading the count. Moreover, some recent estimates suggest that ∼9% of all human TB cases in India may be of zoonotic origin (14). Hence, it is increasingly recognized that controlling TB in the livestock population in these settings could be a major step toward attaining the ambitious End TB goal of 2035 (15). As part of these control efforts, effective methods of identifying infected animals are needed. However, there are several major limitations to the OIE-recommended tuberculin-based skin tests, including the fact that the active components in the reagent are undefined and their interference with BCG-based vaccination. In contrast, the peptide-based DST offers superior quality control and potential for DIVA has previously been demonstrated in cattle (10), and the current study indicates that the DST antigens are promising candidates for differentiating between reactor and uninfected buffaloes. Limitations of the study include the relatively small number of animals and their origin from a single dairy farm. Hence, these results will need to be verified in larger cohorts of naturally infected and naïve animals to establish more refined estimates of sensitivity and specificity of the DST in buffaloes prior to consideration for replacement of the traditional tuberculin standard. Moreover, while the DIVA capability of DST enables implementation of BCG vaccination as an intervention, the efficacy of BCG vaccination in buffaloes remains unknown. Field trials are in process to evaluate the performance of DST in larger cohorts of infected, BCG-vaccinated and naïve buffaloes, and the efficacy of BCG in buffaloes in natural transmission settings in India is being planned.

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

The animal study was reviewed and approved by Institutional Animal Ethics Committee, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India.

AUTHOR CONTRIBUTIONS

SS, VK, MV, DBa, and NJ conceptualized the study. TK, MS, BJ, DA, DBi, and DY conducted the animal experiments. MS, SS, and MV conducted the lab experiments. TK, MS, and SS analyzed the data. TK prepared the first draft. All authors contributed to the article and 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.2021.669898/full#supplementary-material

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Conflict of Interest: MV was employed by company Cisgen Biotech Discoveries Pvt. Ltd. SS and VK have filed intellectual property protection for the DST.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genotypic Characterization of *Mycobacterium bovis* Isolates From Dairy Cattle Diagnosed With Clinical Tuberculosis

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Molecular diagnosis of bovine tuberculosis plays an essential role in the epidemiological knowledge of the disease. Bovine tuberculosis caused by *Mycobacterium bovis* represents a risk to human health. This study aimed to perform the genotypic characterization of *M. bovis* isolated from bovines diagnosed as tuberculosis from dairy herds in the state of Pernambuco, Brazil. Granulomas from 30 bovines were sent for microbiological culture, and colonies compatible with *Mycobacterium* spp. were obtained in at least one culture from 17/30 granulomas. All isolates were confirmed to be *M. bovis* by *spoligotyping* and 24*loci* MIRU-VNTR typing. While *spoligotyping* characterized the isolates as SB0121, SB0295, SB0852, SB0120, and an unclassified genotype, 24*loci* MIRU-VNTR rendered two clusters of two isolates each and 13 unique profiles. *Loci* ETR-A showed higher discriminatory power, and *loci* (ETR-B, ETR-C, MIRU16, MIRU27, and QUB26) showed moderate allelic diversity. This is the first study on the genetic variability of the infectious agent cause of bovine TB in Pernambuco and demonstrates variability of strains in the state. Thus, it corroborates the importance of this microorganism as agent of bovine tuberculosis and its zoonotic potential, this epidemiological tool being a determinant in the rigor of the sanitary practices of disease control in dairy herds.

Keywords: bovine tuberculosis, dairy cattle, genotyping, *Mycobacterium bovis*, pathology, spoligotyping, MIRU-VNTR

INTRODUCTION

Bovine tuberculosis is a chronic progressive disease caused by *Mycobacterium bovis* which affects mainly cattle and buffalo but also infects other mammalian species of mammals, including humans (1). The zoonotic potential of this disease is related to the consumption of raw milk and unpasteurized derivatives, representing the main route of transmission to humans, more pronounced in rural areas. In the state of Pernambuco, a prevalence of outbreaks of 2.87 and 0.62% of infected animals was reported in 2016, with a tendency to concentrate in the Agreste region of the state and with a predominance in dairy properties (2).

The interest in nucleic acid-based diagnostic procedures increased because of the limitations of conventional testing such as lack of sensitivity and specificity of the allergic-skin test and the long period for confirming the presence of the agent by bacteriological methods (3). In addition, molecular typing methods have provided a great impetus in the molecular epidemiology studies of the *M. tuberculosis* complex including comparing mycobacterial genome sequences. Among the most used genotyping techniques for the study of the *M. tuberculosis* complex are *Spoligotyping* and Variable Number of Interspersed Repetitive Units of Mycobacteria (MIRU-VNTR) (4, 5). MIRU-VNTR has higher discriminatory power and has currently been the method of choice in the genotyping studies of *Mycobacterium* spp. and, in particular related to *M. bovis*, allows the identification of prevalent strains circulating in a herd or geographic regions (4, 5).

M. bovis infection has an impact on both animal and human health; non-etheless, scarce are the studies in the region on molecular genotyping. Given the lack of data on the contribution and nature of the *Mycobacterium tuberculosis* complex (MTBC) to bovine TB in the state of Pernambuco, we performed the genotypic characterization of Mycobacteria isolated from bovines from dairy herds in this region that were diagnosed clinically with tuberculosis, coming from dairy herds in the state of Pernambuco.

MATERIALS AND METHODS

The study included 28 bovines and two buffaloes that had been attended at the Bovine Clinic of Garanhuns/UFRPE, presenting clinical symptoms suggestive for tuberculosis. The animals were submitted to clinical examination, with information, including epidemiological, that was annotated in clinical records. Among the information present in the anamnesis provided by the owners, common to most animals, were progressive weight loss, dry cough, and decreased milk production.

According to the evolution/severity of the clinical cases and the result of the allergic-skin test, the animals were euthanized according to the current legislation (Brazil, Ministry of Agriculture, Livestock and Supply. Normative Instruction n. 19, 10 of October, 2016) and submitted for anatomopathological examination.

Fragments of organs with lesions characteristic of granulomas were collected for histopathological examination and lymph nodes with lesions for microbiological culture. The samples for bacteriology were stored in a freezer (−80°C) for further processing, while for histopathological evaluation, fragments were fixed in 10% buffered formaldehyde, processed, and stained with hematoxylin and eosin (HE). Granulomas from all 30 animals were collected and sent for microbiological culture and sample processing, and culture conditions favoring isolation of *M. bovis* were carried out following the recommendations of Franco et al. (6). Samples were minced and decontaminated according to the Petroff method, inoculated on Löwenstein–Jensen and Stonebrink medium, and incubated at 37°C for 90 days.

Nucleic acid was obtained from the cells by thermolysis. Molecular identification to the *Mycobacterium* species was performed by PCR amplification of a 1,020-bp fragment of the *gyr*B gene, as described by Chimara et al. (7) and Franco et al. (6). In the reaction, 1 μl of DNA (20 ng) and 47 μl of Master Mix (1×) were used (Thermo Scientific, Waltham, MA, USA), as well as 10 pM of each of the primers MTUBf (5′ TCGGACGCGTATGCGATATC 3′) and MTUBr (5′ ACATACAGTTCGGACTTGCG 3′) [DNA Express Biotecnologia LTDA, Brazil]. The cycling profile consisted of denaturation at 95°C for 10 min, followed by 35 amplification cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The amplification and fragment size were confirmed by electrophoresis in agarose gel (1%) stained with GelRed™ (Biotium, Hayward, CA, USA) using a 100-bp molecular marker (DNA Express Biotecnologia LTDA). Then, 10 μl of the amplified product was submitted for restriction fragment length polymorphism (RFLP) through digestion by restriction enzymes *Rsa*I, *Taq*I, and *Sac*II (Thermo Scientific, Waltham, MA, USA), following the manufacturer's recommendations. The generated fragments were separated on 2% agarose gel stained with GelRed™ using 50- and 100-bp molecular markers (DNA Express Biotecnologia LTDA). After electrophoresis, the gels were photographed in photo-documentation equipment (2UV Transilluminator UVP) and restriction patterns compared to those described by Chimara et al. (7).

Spoligotyping was performed as described by Kamerbeek et al. (4), and the amplified products underwent membrane hybridization (manufacturing *in-house*) with 43 oligonucleotides. For amplification of the DR region, 20 μM of each of primers DRa 5′ GGTTTTGGGTCTGACGAC 3′ (5′ biotinylated) and DRb (5′ CCGAGAGGGGACGGAAAC 3′), MyTaq Mix (12.5 μl), 1 μl (20 ng) genomic DNA, and ultra-pure water (9.5 μl) were submitted to PCR in a final volume of 25 μl.

MIRU-VNTR typing using a combination of 24-*loci* was performed according to Supply et al. (5). In each PCR reaction, 10 μl MyTaq Mix (Bioline®), 0.4 μl of each *primer* (20 mM), 2 μl of DNA (20 ng), and 7.2 μl of ultra-pure water were used in the final volume of 20 μl. *Mycobacterium tuberculosis* H37Rv DNA and water were used as positive and negative controls, respectively.

The genetic profile based on *spoligotyping* of each isolate was compared to those present in the international databases http://www.mbovis.org/ and http://www.pasteur-guadeloupe.fr:8081/SITVITONLINE. The 24-MIRU-VNTR patterns were compared to those present in the MIRU-VNTRplus database deposited in the application: http://www.miru-vntrplus.org/MIRU/index. The Hunter–Gaston discriminatory index (HGDI) was performed to evaluate the variability of the genotypes obtained by spoligotyping, and each of the alleles of 24-MIRU-VNTR typing.

RESULTS

The 17 animals from which *M. bovis* was isolated came from 10 municipalities in the state of Pernambuco (Alagoinha, Bom Conselho, Chã Grande, Garanhuns, Ibirajuba, Jurema, Pedra,

Pesqueira, Ribeirão, and Venturosa), which were mostly raised in the semi-intensive management system. These municipalities belong to three geographic regions of the state, namely, Southern Agreste, Central Agreste, and South Agreste. Among the animals diagnosed with the disease, females were the most affected (16/17) and 64.7% (11/17) were older than 5 years; one calf 7 months old also yielded positive culture.

The clinical examination of cattle and buffaloes revealed apathy, lack of appetite, low body mass score, seromucous nasal discharge, dry cough, dyspnea, tachypnea, polyps, crackles, and areas of silence in the lung fields. Upon evaluation of the mammary gland, two (2/17) bovines were diagnosed with hypertrophied lymph nodes: one of these presented an enlarged posterior breast of firm consistency, hyperemia and hyperthermia, and physical changes in milk in one of the teats (lumps with serum). The other bovine had an anterior breast of firm consistency but with no visible changes of the milk. During rectal examination, some animals presented nodular structures of varying sizes and hardened consistency in the region of the mesentery, serous in the rumen, and uterus.

Macroscopic observation of lesions seen during *postmortem* examination revealed that 12/17 animals (70.6%) had miliary or protruding tuberculosis, distributed mainly in the lungs, mediastinal and tracheobronchial lymph nodes, liver, and mesenteric lymph nodes and less frequently in the kidneys, spleen, and greater omentum. Among the animals with generalized tuberculosis, two cattle also showed changes in the mammary gland and the uterus, characterized by granulomatous lesions with multifocal distribution and varied sizes, with areas of calcification and abscesses.

The granulomatous nodules observed in all animals were pleomorphic and had a caseous, thick, and yellowish content, with the formation of a fibrous capsule (**Figure 1**). In buffaloes, granulomas had a more whitish color when compared to cattle (**Figure 2**). In the young calf, in addition to lung lesions, small granulomas were observed in the central nervous system and lesions compatible with meningoencephalitis.

Histopathological analysis of the lesions revealed areas of central caseous necrosis and dystrophic calcification and intense inflammatory reaction in the regions adjacent to the necrosis areas, with a predominance of epithelioid macrophages and multinucleated giant cells, like *Langhans*.

Microbiological cultivation presented growth of colonies in 17/30 (57%) samples that were confirmed to be *Mycobacterium* spp. and more specifically *M. bovis* by molecular techniques. In three samples, presence of *Trueperella pyogenes* and, in a single animal, *Nocardia* spp. was encountered. Of the 17 bacterial growths, 14 were classified by the enzymatic restriction analysis of the *gyr*B gene as *M. bovis*. However, due to the importance of bacterial isolation, recognized as a gold standard test, the 17 samples were submitted to molecular genotyping techniques by *Spoligotyping* and 24-*loci* MIRU-VNTR.

Spoligotyping revealed five spoligotypes classified as belonging to *M. bovis*, including SB0121, SB0295, SB0852, SB0120, and a *spoligotype* that was not yet present in the database (**Table 1**).

The analysis of 24-*loci* MIRU-VNTR identified 13 genetic profiles from the 17 isolates of *M. bovis* from 14 properties in the state of Pernambuco (**Table 1**).

The analysis of the discriminatory power (HGDI) of MIRU-VNTR in this study was higher, as expected, than *Spoligotyping*, respectively 0.980 and 0.713. Distribution of the isolates according to the number of alleles in each locus and the analysis of the allelic diversity of the 24-*loci* is summarized in **Table 2**. *Locus* ETR A showed the highest discriminatory power ($h = 0.69$), while five *loci* (ETR B, MIRU 16, ETR C, MIRU 27, and QUB 26) were classified as moderately discriminatory with h between 0.33 and 0.58. Eight *loci* (MIRU 20, MIRU 26, Mtub 04, Mtub 29, QUB 11b, QUB 4156, Mtub 21, Mtub 39) presented low discriminatory power ($h \leq 0.27$) while 10 *loci* showed absence of allelic diversity.

Isolates one and 10 showed failures in the amplification of some *loci* that are generally attributed to possible DNA mutations or degradation (5), thus preventing the *primers* from ringing. Given these results, the respective isolates started to be analyzed only in *Spoligotyping*, obtaining significant results.

DISCUSSION

It should be noted that the state of Pernambuco occupies a prominent place in milk production in the Northeast region, and the municipality of Garanhuns and its microregion are recognized as the state's milk basin (8). Dairy cattle and buffaloes are considered more vulnerable to *M. bovis* infection, as they have a longer life expectancy, stay longer on the properties, and are subjected to the rearing semi-intensive and intensive systems, very common in the region. During milking and other common management practices, animals cohabit, therefore increasing their likelihood of contact and the transmission of tuberculosis (2, 9), considered endemic in the State of Pernambuco (2, 10). The constant transit of animals between the properties within and between neighboring municipalities, the interstate cattle trade, and the absence of an effective sanitary control of the herds are factors that contribute to the spread of the disease in the region (2, 9).

In the present study, all animals presented clinical symptoms of tuberculosis with predominating respiratory impairment. In dairy farms, female animals generally remain for longer periods depending on the reproductive period, and this could be the main reason for having observed in this study the predominance of females over the age of 5 years to be exposed to *M. bovis* when compared to young cattle (9). Non-etheless, young animals also contract the infection and develop disease, as demonstrated by *M. bovis* isolation from a 7-month-old calf. The frequency of tuberculosis in cattle aged <12 months is generally associated with the ingestion of colostrum/milk from infected cows or transplacental infection (11, 12). The most evident clinical signs were observed in the advanced stages of the disease, as described by Izael et al. (10) and Waters (13), except for the calf that manifested the disease earlier in the form of cerebral tuberculosis combined with depression and paresis of the limbs. In addition to the predominant respiratory impairment in the animals in this study, two animals showed clinical changes in the mammary gland, resulting to be similar to that described by Waters (13). This observation reinforces the potential risk of the disease to

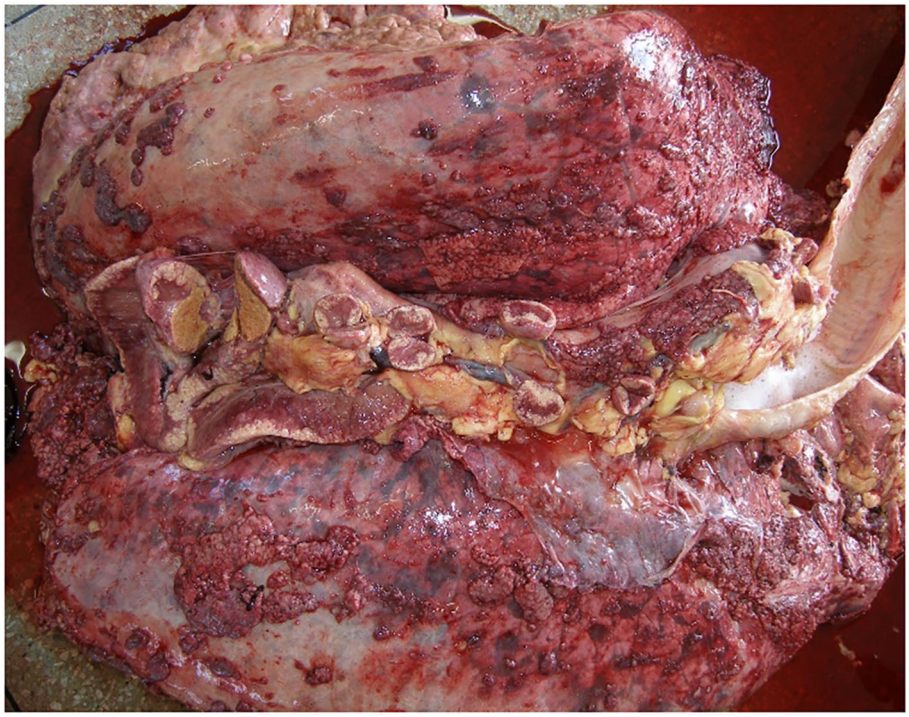

FIGURE 1 | Granulomatous lesions distributed in lung and mediastinal lymph nodes of bovines.

FIGURE 2 | Granulomatous lesions distributed in the liver of buffaloes.

TABLE 1 | Molecular characterization of *M. bovis* isolates from cattle in the state of pernambuco by *Spoligotyping* and MIRU-VNTR.

ID	Octal spoligotype	Cluster spoligotype	Profile of 24 MIRU-VNTR	Cluster MIRU-VNTR	SIT	Mbovis.org	Municipality
1	676773677777600	Cluster S1	2*63***313*3434253332*12	Orphan	481	SB0121	Bom conselho
5	676773677777600	Cluster S1	2263223323244142533326 22	Orphan	481	SB0121	Garanhuns
6	676773677777600	Cluster S1	2263223323544342533326 22	Orphan	481	SB0121	Chã grande
7	676773677777600	Cluster S1	22*322432364434253332632	Orphan	481	SB0121	Alagoinha
9	676773677777600	Cluster S1	*2*322332363444253332312	Orphan	481	SB0121	Pedra
10	676773677777600	Cluster S1	2*5******3*34*****3*2***	Orphan	481	SB0121	Pesqueira
15	676773677777600	Cluster S1	**5322*32353434253332*12	Orphan	481	SB0121	Bom conselho
26	676773677777600	Cluster S1	225322232353444253332512	Orphan	481	SB0121	Pesqueira
29	676773677777200	Cluster S2	224322232353444253332112	Orphan	698	SB0295	Jurema
34	676773677777200	Cluster S2	225322232353444251332512	Orphan	698	SB0295	Jurema
38	676773677777200	Cluster S2	2*5322332343444253332612	Orphan	698	SB0295	Ibirajuba
39	676773677777200	Cluster S2	2*432233234344425133 2512	Cluster M1	698	SB0295	Ribeirão
40	676773677777200	Cluster S2	2*432233234*444251332512	Cluster M1	698	SB0295	Ribeirão
35	676773777777200	Cluster S3	225322132323434253333512	Cluster M2	797	SB0852	Bom conselho
37	676773777777200	Cluster S3	2*5322132323434253333512	Cluster M2	797	SB0852	Bom conselho
12	676773777777600	Orphan	*1*32231342*434251332512	Orphan	482	SB0120	Garanhuns
2	New profile	Orphan	23*322331553414243332612	Orphan	New	New	Venturosa

Cluster S – cluster Spoligotyping Cluster M – cluster MIRU-VNTR.

*** Isolates that presented amplification failures in some loci.*

Order of the 24 loci of MIRU-VNTR patterns, MIRU 02–154; Mtub 04–424; ETR C–577; MIRU 04–580; MIRU 40–802; MIRU 10–960; MIRU 16–1,644; Mtub 21–1,955; MIRU 20–2059; QUB 11b-2163b; ETR A–2165; Mtub 29–2,347; Mtub 30–2,401; ETR B–2,461; MIRU 23–2,531; MIRU 24–2,687; MIRU 26–2,996; MIRU 27–3,007; Mtub 34–3,171; MIRU 31–3,192; Mtub 39 – 3,690; QUB 26 – 4052; QUB 4,156–4,156; MIRU 3–4,348.

TABLE 2 | Distribution and allele diversity (HGDI) of the 24-*loci* MIRU-VNTR.

Locus	**Number of repetitions**						**Allele diversity HGDI**
	1	**2**	**3**	**4**	**5**	**6**	
MIRU 02		14					0.000
Mtub 04	1	8	1				0.266
ETR C				3	7	3	0.570
MIRU 04			16				0.000
MIRU 40		15					0.000
MIRU 10		15					0.000
MIRU 16	2	3	8	1			0.571
Mtub 21	1		15				0.058
MIRU 20	2	13	1				0.275
QUB 11b			15	1	1		0.165
ETR A		4		3	6	2	0.690
Mtub 29			12	3			0.271
Mtub 30				17			0.000
ETR B	2		7	7			0.575
MIRU 23				16			0.000
MIRU 24		16					0.000
MIRU 26				1	15		0.058
MIRU 27	4		12				0.333
Mtub 34			17				0.000
MIRU 31			16				0.000
Mtub 39		15	2				0.158
QUB 26	1		1		7	5	0.582
QUB 4,156	13	2	1				0.275
MIRU 39		16					0.000

public health due to the consumption of raw milk and non-pasteurized derivatives, mainly observed in inland cities and rural areas, such as Garanhuns and the microregion (8).

The generalized form of the disease was predominant both in cattle and in the two buffaloes, with lesions that had disseminated to several organs. All animals had granulomatous injuries in the thoracic organs (lungs, pleura, tracheobronchial, and mediastinal lymph nodes), causing respiratory impairment. This result is similar to those described by Ramos et al. (14), who reported a higher prevalence of lesions compatible with tuberculosis in tracheobronchial and mediastinal lymph nodes and lungs; such typical predominance of lesions in the respiratory tract is indicative for airborne transmission. On the other hand, Alzamora Filho et al. (15) identified the most evident lesions in the lymph nodes of the head (retropharyngeal and parotid) with pulmonary parenchyma. These results corroborate with the findings of the present study, due to the typical predominance of lesions in the respiratory tract, suggesting the airway, as the main gateway for *M. bovis* in bovines. The lower occurrence of mesenteric lymph node involvement here observed was also described by Ramos et al. (14) and justified by the fact that oral route infection is secondary to the respiratory route in adult cattle.

The granulomatous lesions observed in the mammary gland and uterus common to two animals in this study reinforce the potential risk of transmission of *M. bovis* to humans due to the consumption of raw milk and its products (16, 17). On the other hand, the granulomatous lesions located in the central nervous system in young cattle are probably related to the ingestion of colostrum/milk from infected cows and can be justified by ascending infection *via* hematogenic route. This form of cerebral tuberculosis in cattle was also reported by Konradt et al. (11) and Silveira et al. (12).

The histopathological characterization of lesions present in granulomas was similar to the findings described by França et al. (18) who found in some samples a marked process of calcification with mineralization, differing from the lesions observed by Ramos et al. (14) and Silva et al. (19) who presented a more caseous aspect, suggesting that the animals that had been slaughtered were suffering from a recent infection or disease development.

The frequency of isolation of *M. bovis*, of 57%, was observed presently in animals, with clinical tuberculosis. It has been described that some factors can interfere with the success of mycobacterial isolation and in particular of *M. bovis*, including the rigorous decontamination process of samples and the chronic character of the disease that confers intense calcification of the lesions, leading to low concentrations or absence of viable bacilli (20). This might have been influenced by the low isolation of *M. bovis* in the present sampling.

Besides *Mycobacterium* spp., we also observed bacteria belonging to other genera such as *Trueperella pyogenes* and *Nocardia* spp. It is worth mentioning that some microorganisms besides these, such as *Actinomyces* spp. and *Actinobacillus* spp., are also responsible for causing granulomatous lesions similar to tuberculosis lesions (21).

In the present study, 17 isolates compatible with *Mycobacterium* spp. were subjected to molecular diagnostics by RFLP of the *gyr*B gene. However, the analysis classified only 14/17 isolates as *M. bovis*, different from the study carried out by Franco et al. (6) that obtained 100% compatibility between the isolation of *Mycobacterium* spp. and the *gyr*B analysis. The result obtained in the RFLP is probably related to factors that interfere with molecular tests, such as the presence of inhibitors of PCR reactions, low amount of viable bacilli due to chronic lesions, contaminants in the samples, and failures in extraction processing or DNA degradation (22).

Spoligotype SB0121, the most frequently encountered, was described as the most prevalent in national territory with a frequency of 29.1% in a study conducted in Latin American countries (23). The fact that we identified this *spoligotype* in the three defined geographical region studies here could be caused by the constant movement of animals, due to the practice of interstate cattle trade and also strongly suggestive for recent infections (23, 24).

The SB0295 profile was the second most prevalent *spoligotype* in this study (29%) and has been referenced in Brazil with a prevalence of 24% (23). This is similar to that in the Midwest Region of the country, being identified in 16.2% of the total isolates (25). The two isolates identified in buffaloes as SB0295 were also recorded in the Amazon region in mixed buffalo and dairy cattle breeding areas under the same management condition as reported by Carneiro et al. (26). SB0295 was identified in buffaloe isolates in Argentina, highlighting the propagation of common *M. bovis* strains among bovines and buffaloes (23).

Spoligotype SB0852 was identified in two isolates. According to the international database, SB0852 has only been registered in Italy (27), suggesting a process of natural selection of these strains between geographic locations (25) or convergent evolution (23).

Finally, two *spoligotypes* were observed in this study single isolates only, with the case for SB0120 being similar to the low frequency of occurrence in other regions of the country (6, 23, 28). The other was from a bovine that presented a *spoligotype* not present in the international database; this could be due to some microevolutionary events in the DR regions of a strain with an existing pattern (29).

In the region of development of the study, bovine tuberculosis is characterized as endemic, and the practice of commercialization and consumption of milk and fresh products increases the risk of zoonotic transmission, increasing the risk of sharing *M. bovis* isolates common among dairy cattle and the human population of the region, as previously recorded in other studies in different areas of the world. Genomic diversity in the *M. tuberculosis* complex remains a significant factor in the pathogenesis of tuberculosis, which can affect the virulence, transmissibility, host response, and drug resistance (29).

The genotyping performed in this study from the set of 24-*loci* MIRU-VNTR is recommended for the comparative study of *M. bovis* profiles worldwide (5). Molecular genotyping identified 13 distinct genetic profiles, suggesting a diversity of *M. bovis* within and between the regions studied and considerable higher discriminatory power as compared to *Spoligotyping*. This is

according to earlier results obtained both in Brazil (25) and in other countries. This demonstrated that although a large cluster was observed by *spoligotyping* alone, there exists genetic diversity among the strains of *M. bovis* in Pernambuco, probably due to the movement of animals between different regions, states, and rural properties (23, 25).

The analysis of allelic diversity of the different MIRUs are similar to those found by Souza Filho et al. (30) and Carvalho et al. (25) and demonstrating that for this MTBC species, only six of 24 *loci* allowed good discrimination, different from *M. tuberculosis* (31). The HGDI of 24-MIRU-VNTR and *spoligotyping* in this study was 0.980 and 0.713, respectively, close to that observed by Carvalho et al. (25) with values 0.980 and 0.810 and the HGDI of 0.912 reported by Souza Filho et al. (30). Therefore, it seems that simultaneous consideration of both genotyping techniques for clustering might be more accurate for *M. bovis* transmission studies, also in the present study. However, the association between these techniques has been considered the best strategy for the molecular typing of *M. bovis* because they present better reproducibility and reliability, aiming at the analysis of strains mycobacterial (25).

This study is of great importance for the region as it is the first work carried out on molecular genotyping through the association between *Spoligotyping* and MIRU-VNTR aiming at the molecular characterization of *M. bovis* isolates and identification of circulating genotypes in the state of Pernambuco. The importance of *M. bovis* as a cause of human tuberculosis is worth mentioning, although sometimes neglected, especially in developing countries. The consumption of raw milk and dairy products and the constant exposure to reservoir animals are considered the main risk factors in the epidemiological chain of infection.

CONCLUSION

The consumption of raw milk and dairy products is a frequent habit in the region, which, together with data on the occurrence of bovine tuberculosis, increases the risk of zoonotic transmission, alerting the possibility of sharing common *M. bovis* strains between dairy cattle and the population. The genotypic characterization allowed the identification of different *M. bovis* genotypes circulating in the state of Pernambuco, presenting both two large clusters by *spoligotyping* but evidencing considerable heterogeneity when using 24-MIRU-VNTR. Considering the diversity of genotypes obtained by combining *spoligotyping* and 24-MIRU-VNTR in the present setting, this methodology could be additive during transmission studies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use Ethics Committee (CEUA), of the Federal Rural University of Pernambuco with license no. 09/2017 CEPE/ UFRPE in accordance with COBEA and National Institute of Health Guide for Care and Use of Laboratory Animals standards.

AUTHOR CONTRIBUTIONS

EM, CM, JA, MF, HL, and AP: conducted and performed the microbiological diagnostic design of mycobacterial culture procedures. HG, PS, CM, and JA: constructed the molecular diagnostic methodology. EM, HG, PS, IS, ML, and RF: conducted and performed the molecular tests of genotyping and molecular typing. EM, CM, HG, and PS: accurately reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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Probing Differences in Gene Essentiality Between the Human and Animal Adapted Lineages of the *Mycobacterium tuberculosis* Complex Using TnSeq

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Members of the *Mycobacterium tuberculosis* complex (MTBC) show distinct host adaptations, preferences and phenotypes despite being >99% identical at the nucleic acid level. Previous studies have explored gene expression changes between the members, however few studies have probed differences in gene essentiality. To better understand the functional impacts of the nucleic acid differences between *Mycobacterium bovis* and *Mycobacterium tuberculosis*, we used the Mycomar T7 phagemid delivery system to generate whole genome transposon libraries in laboratory strains of both species and compared the essentiality status of genes during growth under identical *in vitro* conditions. Libraries contained insertions in 54% of possible TA sites in *M. bovis* and 40% of those present in *M. tuberculosis*, achieving similar saturation levels to those previously reported for the MTBC. The distributions of essentiality across the functional categories were similar in both species. 527 genes were found to be essential in *M. bovis* whereas 477 genes were essential in *M. tuberculosis* and 370 essential genes were common in both species. CRISPRi was successfully utilised in both species to determine the impacts of silencing genes including *wag31*, a gene involved in peptidoglycan synthesis and *Rv2182c/Mb2204c,* a gene involved in glycerophospholipid metabolism. We observed species specific differences in the response to gene silencing, with the inhibition of expression of *Mb2204c* in *M. bovis* showing significantly less growth impact than silencing its orthologue (*Rv2182c*) in *M. tuberculosis*. Given that glycerophospholipid metabolism is a validated pathway for antimicrobials, our observations suggest that target vulnerability in the animal adapted lineages cannot be assumed to be the same as the human counterpart. This is of relevance for zoonotic tuberculosis as it implies that the development of antimicrobials targeting the human adapted lineage might not necessarily be effective against the animal

adapted lineage. The generation of a transposon library and the first reported utilisation of CRISPRi in *M. bovis* will enable the use of these tools to further probe the genetic basis of survival under disease relevant conditions.

Keywords: TnSeq, mycobacteria, one health, essential genes, CRISPRi, mycobacterium bovis

INTRODUCTION

Mycobacterium bovis and *Mycobacterium tuberculosis* are closely related members of the *Mycobacterium tuberculosis* complex (MTBC). Although both species are >99% identical at the nucleotide level each species shows distinct host tropisms. *M. bovis*, the animal adapted species, is the main causative agent of bovine tuberculosis in cattle (1) while *M. tuberculosis* is the main cause of human tuberculosis (TB) and is responsible for ∼1.5 million deaths annually (1, 2). *M. bovis* exhibits a broader host range than *M. tuberculosis* and is also able to cause TB in humans through zoonotic transfer, representing a serious public health risk in countries without a control programme in domestic livestock (2, 3). The WHO recognises that zoonotic transfer of tuberculosis threatens the delivery of the end TB strategy, highlighting the importance of understanding the differences between the two species (3).

Many studies have explored the genotypic and phenotypic differences between *M. tuberculosis* and *M. bovis* to better understand host preference. Genome sequencing of the reference strains (H37Rv and AF2122/97) showed that the main genetic differences between these pathogens were several large-scale deletions, or regions of difference (RD), and over 2,000 single-nucleotide polymorphisms (SNPs) (4–7). More recently, studies that include clinically circulating strains have confirmed that all animal adapted lineages share deletions RD7, 8, 9, and 10 (8). Transcriptomic studies which have measured significant changes in gene expression between H37Rv and AF2122/97 have provided a functional insight into the impacts of some of these polymorphisms (9–11). For instance, a SNP in *rskA* (*Mb0452c*) an anti-sigma factor in *M. bovis,* prevents repression of *sigK* activity, leading to constitutively high levels of expression of *mpb70* and *mpb83,* genes that encode key immunogenic antigens; MPB70 and MPB83 (12, 13). Recent studies have shown that MPB70 mediates multi-nucleated giant cell formation in *M. bovis* infected bovine macrophages, but not in *M. bovis* (or *M. tuberculosis*) infected human macrophages, providing insight into bacterial effectors of the species-specific response (14). Transcriptomic studies have also indicated a differential response to *in vitro* mimics of host stresses such as acid shock and highlight the impact of SNPs in the signalling and response regulons in two-component systems such as PhoPR and DosSRT (15–18).

Genome-wide transposon mutagenesis coupled with next-generation sequencing (TnSeq) has allowed genome wide predictions of gene essentiality in *M. tuberculosis* (19–24). These studies have provided information on the genetic requirements for *in vitro* growth under a number of conditions and also for growth in disease relevant models such as macrophages (20). Most of these studies performed in the MTBC have used strain H37Rv. More recently Tnseq of different clinical strains of *M. tuberculosis* has shown that there are strain specific differences in fitness associated with Tn insertions in certain genes. The implication of this observation is that different strains can show different antibiotic sensitivities as a result (25). To date, there has been a single reported Tnseq study performed in *M. bovis* (AF2122/97) which focused on intra-cellular genetic requirements (26).

A direct comparison of gene essentiality in *M. bovis* and *M. tuberculosis* has not been reported. In this study, we created dense transposon libraries in both *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) generated on the same medium to enable direct comparisons between the two related species. We identified that there are key differences in gene essentiality in *M. bovis* compared to *M. tuberculosis*. We used CRISPRi to directly demonstrate that silencing the expression of a gene annotated to be involved in glycerophospholipid metabolism has different impacts on growth in the two species. This has implications for target discovery programmes as it implies that inhibition of therapeutically relevant pathways may have different impacts in the different species. This is important in the context of zoonotic tuberculosis.

MATERIALS AND METHODS

Bacterial Strains and Culture Methods

M. bovis AF2122/97 was maintained on modified Middlebrook 7H11 solid medium containing 0.5% lysed defibrinated sheep blood, 10% heat inactivated foetal bovine serum and 10% oleic acid-albumin-dextrose-catalase (OADC) (27). Liquid cultures of *M. bovis* were grown in Middlebrook 7H9 medium containing 75 mM sodium pyruvate, 0.05% Tween®80 and 10% albumin-dextrose-catalase (ADC). *M. tuberculosis* H37Rv and *Mycobacterium smegmatis* mc^2155 were maintained on Middlebrook 7H11 solid medium supplemented with 0.5% glycerol and 10% OADC. Liquid cultures were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween®80 and 10% ADC unless stated otherwise. MycomarT7 Phagemid was propagated on *M. smegmatis* mc^2155 lawns grown on Middlebrook 7H10 solid medium supplemented with 0.5% glycerol and 10% OADC in a 0.6% agar overlay. The strains and plasmids used or made in this study are given in **Table 1**.

Generation of Transposon Libraries

Transposon libraries in *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) were generated using the previously described MycomarT7 phagemid system as per Majumdar et al. with modifications (29). Briefly, 50 ml cultures of *M. bovis* and *M. tuberculosis* at OD_{600} ≅1 were washed twice with MP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgSO_4$ and

TABLE 1 | Strains and plasmids used in this study.

Strain/plasmid	Genotype/Description	Source
Strains		
E. coli DH5α	*SupE44 ΔlacU169 (lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
M. bovis AF2122/97	*M. bovis* reference strain	
M. tuberculosis H37Rv	*M. tuberculosis* reference strain	
$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$	*M. tuberculosis* or *M. bovis* with integrative plasmid containing $dCas9_{Spy}$(pRH2502), kan^R	This study
Mtb_{dCas9}_control/Mb_{dCas9}_control	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ with sgRNA –ve control plasmid (pRH2521), kan^R, hyg^R	This study
Mtb_{dCas9}_wag1/Mb_{dCas9}_wag1	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +26 bp to +45 bp downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. kan^R hyg^R	This study
Mtb_{dCas9}_wag2/Mb_{dCas9}_wag2	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +144 bp to +163 bp downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon kan^R, hyg^R	This study
Mtb_{dCas9}_ agpat1/Mb_{dCas9}_agpat1	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +2 bp to +21 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. kan^R hyg^R	This study
Mtb_{dCas9}_ agpat2/Mb_{dCas9}_agpat2	$Mtb_{\_dCas9}$/$Mb_{\_dCas9}$ expressing sgRNA targeting +40 bp to +59 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. kan^R hyg^R	This study
Plasmids		
pRH2502	Integrative plasmid derived from pTC-0X-1L, expressing $dCas9_{Spy}$ from an inducible tetRO promoter (uv15tetO). kan^R	(28)
pRH2521	Non-integrative plasmid derived from pTE-10M-0X, expressing sgRNA from an inducible tetRO promoter (Pmyc1tetO). hyg^R	(28)
pRH2521_wag1	pRH2521 with an sgRNA targeting +26 bp to +45 downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. hyg^R	(28)
pRH2521_wag2	pRH2521 with an sgRNA targeting +144 bp to +163 downstream of the $wag31_{Mtb}$/$wag31_{Mb}$ annotated start codon. hyg^R	(28)
pRH2521_agpat1	pRH2521 with an sgRNA targeting +2 bp to +21 bp over and downstream of the *Rv2182c/Mb2204c* annotated start codon. hyg^R	This study
pRH2521_agpat2	pRH2521 with an sgRNA targeting +40 bp to +59 bp downstream of the *Rv2182c/Mb2204c* annotated start codon. hyg^R	This study

2 mM $CaCl_2$) at 37°C, and then incubated with $\sim 10^{11}$ pfu of ϕMycoMarT7 phage for 16–18 h at 37°C without rolling. Transduced bacteria were washed in pre-warmed PBS + 0.05% Tween®80 to remove extra-cellular phage and plated on Middlebrook 7H11 solid medium containing 0.5% lysed defibrinated sheep blood, 10% heat inactivated foetal bovine serum, 10% OADC, 25 μg/ml kanamycin and 0.05% Tween®80. Cultures were allowed to grow for 5–6 weeks. Concurrent CFU plating was performed to estimate transduction efficiency. Approximately 15–20 colonies from each library were used for validation of random insertion using a nested PCR strategy followed by Sanger sequencing, method and data are shown in **Supplementary Material S1**. Libraries were scraped from the plates and incubated in liquid medium at 37°C with hourly vortexing for 3 h to homogenise. Homogenised mutants were distributed to cryovials and stored at −80°C for further selection or gDNA extraction.

DNA Extraction

Unless stated otherwise, reagents were acquired from Sigma Aldrich. Genomic DNA from harvested libraries was isolated by a bead beating procedure (mechanical lysis) or using de-lipidation followed by enzymatic lysis as previously described by Long et al. (30) and Belisle et al. (31). Briefly, for mechanical lysis, library aliquots were disrupted using 0.1 mm glass beads and bead-beating by 3 × 15 s bursts (5000 rpm) interspersed with 2 mins on ice using a beat-beater (Biospec). For enzymatic lysis, libraries were de-lipidated with equal volumes chloroform-methanol (2:1) for 1 h with agitation every 15 mins, suspension was centrifuged at 3,488 × g for 10 mins the bacterial pellet allowed to dry for 2 h after removal of both solvent layers. De-lipidated bacteria were suspended in TE buffer and incubated with 100 μg/ml lysozyme in the presence of 100 mM TrisBase (pH 9.0) at 37°C for 12–16 h. Bacterial lysis was completed by incubating for 3 h at 55°C in the presence of 1% SDS and 100 μg/ml proteinase K (NEB). Lysates from both methods were extracted twice with equal volumes of phenol–chloroform–isoamyl alcohol (25:24:1). The aqueous layer was harvested by centrifugation at 12,000 × g for 30 mins and DNA was precipitated with 0.1 volumes of 3M sodium acetate (pH 5.2) and one volume of ice-cold isopropanol overnight at −20°C. DNA pellets were washed several times in ethanol. DNA was re-suspended in water and quantity and quality were determined using a DeNovix Spectrophotometer (DeNovix Inc, USA), agarose gel electrophoresis and fluorometry using Qubit4 (Invitrogen).

Library Preparation for Transposon Directed Inserted Sequencing

Two μg of extracted DNA libraries were resuspended in purified water and sheared to approximately 550 bp fragments using a S220 focussed-ultrasonicator (Covaris), according to the manufacturer's protocol. Sheared DNA was repaired using NEBNext blunt-end repair kit (New England Biolabs) and

TABLE 2 | Oligonucleotides used in this study.

Primer	Sequence
Primers used for Tn library	
Adaptor standard	GATCGGAAGAGCACAC
Adaptor P7+index[a]	CAAGCAGAAGACGGCATACGAGAT**XXXXXX XX**GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCT
ComP7 primer	CAAGCAGAAGACGGCATACG
ComP5 primer	AATGATACGGCGACCACCGAGATCTACACTC TTTCCCTACACGACGCTCTTCCGATCTCG GGGACTTATCAGCCAACCTG
Oligonucleotides used for nested PCR verification	
HiMar_Right_1	CCTCGTGCTTTACGGTATCG
Arb_primer_1c*	GCCAGCGAGCTAACGAGACNNNNN
HiMar_Tn_Jnct_PCR	ACTATAGGGGTCTAGAGACCGGG
Arb_primer_1*	GCCAGCGAGCTAACGAGAC
Oligonucleotides used for CRISPRi silencing sgRNA[b]	
agpat1_F	AAACTGTGGTACTACCTGTTCAAG
agpat1_R	GGGACTTGAACAGGTAGTACCACA
agpat2_F	AAACCTCTTTACGTTGCTTGGTCG
agpat2_R	GGGACGACCAAGCAACGTAAAGAG
Oligonucleotides used for RT-qPCR	
sigA_Fq	CCTACGCTACGTGGTGGATT
sigA_Rq	TGGATTTCCAGCACCTTCTC
agpat1_Fq	CTTTACGTTGCTTGGTCGCC
agpat1_Rq	AGAACCAGCGGTTGATCCAG
$dCas9_{Spy}$_Fq	AAGAAGTACAGCATCGGCCTGG
$dCas9_{Spy}$_Rq	TTCTTGCGCCGCGTGTATCG

[a]***XXXXXXX*** *in AdaptorP7+index primer denotes sequence of variable indices used.*
[b]*The four bases underlined at the start of each oligo were used for cloning into pRH2521.*

purified using Monarch PCR clean-up kit (New England Biolabs). Blunted DNA was A-tailed using NEBNext dA-tailing kit and column-purified. Custom transposon sequencing adaptors, or "TraDIS tags," (**Table 2**) were generated by heating an equimolar mix of adaptor standard primer and adaptor P7+index to 95°C for 7 mins and then allowed to cool to room temperature. Adaptors were ligated to A-tailed library fragments using NEBNext quick ligase kit. Transposon-containing fragments were enriched by PCR using ComP7 primers ComP5 using Phusion DNA polymerase (New England Biolabs) in a 20-cycle reaction. Library fragments were subsequently cleaned up with AMPureXP purification beads (Beckman).

Data Analysis

Indexed libraries were combined, spiked with 20% PhiX, and sequenced on the Illumina HiSeq 3000 platform, using v2 chemistry, generating single-end reads of 150 bp. Raw FASTQ sequence files were pre-processed using the TPP utility of TRANSIT python package (32) including removing TRADIS tags and adapter sequences and mapping using BWA-MEM algorithm [32], to generate insertion files in.wig format. Custom annotations, "prot tables," were created from the *M. bovis* strain AF2122/97 annotation file (NCBI Accession Number LT708304, version LT708304.1) and for the *M. tuberculosis* strain, H37Rv [NCBI Accession Number AL123456, version AL123456.3, assembly build GCA_000195955.2 (ENA)]. TRANSIT software was run on both *M.bovis* and *M. tuberculosis* files using the default normalisation (TTR), which normalises by trimming the top and bottom 5% of read counts and normalising to the mean read count. The TRANSIT HMM algorithm (33) was used to make calls of essentiality for each TA insertion site, and for each gene based on annotated gene boundaries. Data files (fastq) are deposited in SRA (PRJNA754037).

TABLE 3 | Summary statistics of the Tn libraries created in this study.

	M. bovis AF2122/97	*M. tuberculosis H37Rv*
Unique mutants	39,987 (of 73,536)	29,919 (of 74,604)
Saturation	54%	40%
Essential genes	527	477

CRISPRi Mediated Gene Silencing

We utilised dCas9 from *Streptococcus pyogenes* ($dCas9_{Spy}$) for silencing as previously described (28). sgRNA targeting $wag31_{Mtb/Mb}$ and *Rv2182c*/*Mb2204c* were designed according to the parameters derived from Larson et al. (34). Protospacer adjacent motif (PAM) sites, "NGG," were chosen and putative sgRNAs 20 bp downstream of the PAM were selected. All sgRNAs designed targeted the coding non-template strand. The probability of complementarity to any other region of the genome and predicted secondary structure of the sgRNA transcript was analysed using a basic local alignment search tool (BLAST) and M-fold, respectively (35, 36). Complementary forward and reverse primers using the sequence (without the PAM) with appropriate ends for ligation to the pRH2521 vector were designed (**Table 2**). Oligos were annealed and cloned into pRH2521 using BbsI as previously described (28, 37). One microgram of pRH2502 was electroporated at 25 kV, 25 μF with 1000 Ω resistance into electrocompetent *M. bovis* and *M. tuberculosis* to generate strains expressing $dCas9_{Spy}$ ($Mtb_{\_dCas9}$/$Mb_{\_dCas9}$). These strains were grown and further electroporated with 1 μg of pRH2521 expressing sgRNAs targeting $wag31_{Mtb/Mb}$ and *Rv2182c*/*Mb2204c* or pRH2521, the sgRNA –ve plasmid.

RNA Extraction and RT-qPCR

Cultures were grown to $OD_{600} \cong 0.1$–0.2 and the CRISPRi machinery induced with 200 ng/ml of anhydrotetrcycline (aTc) for 24 h. Total RNA was extracted as previously described (38). Briefly, cultures were centrifuged at 3,488 × g at 4°C for 10 mins. Pellets were resuspended in 1 ml of TRIzol containing 0.1 mm glass beads and were disrupted by three cycles of 30 s pulses at 6,000 rpm using a Precellys homogenizer. RNA was purified using a Qiagen RNeasy kit combined with on-column DNase digestion according to the manufacturer's instructions. Quantity and quality were determined using a DeNovix Spectrophotometer (DeNovix Inc, USA) and agarose gel electrophoresis.

To remove traces of contaminating DNA, RNA samples were treated with RNase-free DNase I (Invitrogen) according to the manufacturer's instructions. cDNA was synthesised from 100 ng of RNA using Superscript III Reverse transcriptase according to manufacturer instructions. qPCRs were performed using PowerUp SYBR Green Master Mix with 1 µl of cDNA and 0.3 µM of either *sigA* primers or gene specific primers (**Table 2**) in a final volume of 20 µl. Samples were run on a BioRad CFX96 analyser at 50°C for 2 mins, 95°C for 2 mins, followed by 40 cycles of 50°C for 2 mins, 95°C for 2 mins, followed by 40 cycles of 95°C for 15 s, 72°C for 1 min and 85°C for 5 s at which point fluorescence was captured. A melt curve analysis was also carried out for each run at 65–95°C in increments of 0.5°C. Gene expression data was analysed using the $2^{-\Delta\Delta CT}$ method (39). Reverse transcriptase –ve samples were used as a control to ensure removal of gDNA. All results were normalised against the house keeping gene *sigA*. Two or three biological replicates were run, with each measured in duplicate, unless otherwise stated.

RESULTS

Generation of High-Density Transposon Libraries in *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv

The Mycomar transposon inserts randomly into TA sites in bacterial genomes (40). There are 73,536 and 74,604 TA sites present in the *M. bovis* (AF2122/97) and *M. tuberculosis* (H37Rv) genomes, respectively. The smaller number of TA sites in *M. bovis* is likely to be reflective of a smaller genome. We successfully generated transposon libraries in *M. bovis* and *M. tuberculosis* containing 39,987 (*M. bovis*) and 29,919 (*M. tuberculosis*) unique mutants, representing 54 % (*M. bovis*) and 40 % (*M. tuberculosis*) saturation (**Table 3**). This corresponded to an insertion in 3,625/3,989 (91%) coding sequences in *M. bovis* and 3,554/4,018 (86%) coding sequences for *M. tuberculosis*. The distribution of transposon insertions in the two species is shown in **Figure 1**.

Himar1 transposase has been previously suggested to exhibit local sequence preferences rendering ∼9% of possible TA sites non-permissive to insertion (23) and others have also observed TA insertion cold spots within the *M. tuberculosis* genome. Using the non-permissive sequence pattern, "SGNTANCS" (where S is either G/C), we identified 6657 non-permissive sites in both *M. bovis* and *M. tuberculosis* genomes (data not shown). Taking a similar approach to Carey et al., we found that removing these sites prior to determining gene essentiality as described below did not affect the gene calls (25).

Comparisons of Essentiality Between *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv

We examined *in vitro* gene essentiality in *M. bovis* and *M. tuberculosis* using the TRANSIT HMM method (33). This approach classifies genes into four categories; those that are essential for growth and cannot sustain a transposon insertion (ES), those where the transposon insertion results in a growth defect (GD) and those where the transposon insertion results in a growth advantage (GA). Those that show no impact as a result of the transposon insertion are considered non-essential (NE). From this analysis, 527 genes were classified as ES (15.3%), 176 genes were classified as GD (5.1%) and 131

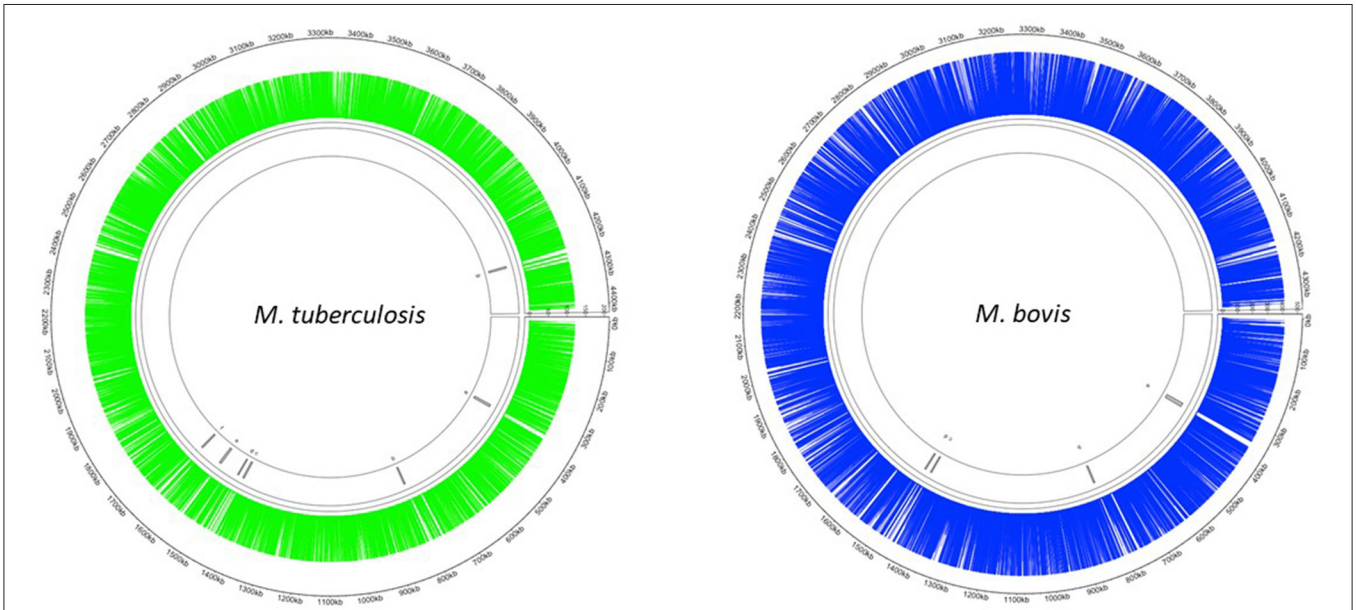

FIGURE 1 | Distribution of Tn insertions in both *M. tuberculosis* and *M. bovis*. Transposon libraries were created in *M. tuberculosis* and *M. bovis* using the *Himar1* system and sequenced on a HiSeq NGS platform (Illumina, UK) as described in the materials and methods. Insertion locations of *Himar1* across the *M. tuberculosis* genome (green) and *M. bovis* genome (blue) were visualised using Circlize (41).

TABLE 4 | Distribution of genes classified as ES across functional class.

Functional class	% ES *M. bovis*	% ES *M. tuberculosis*	% all
Cell wall and cell processes	19.7	18.66	18.71
Conserved hypotheticals	10.23	10.90	28.11
Information pathways	17.61	18.87	5.96
Insertion seqs and phages	0	0.63	2.54
Intermediary metabolism and respiration	42.23	38.57	23.1
Lipid metabolism	4.73	5.03	6.5
PE/PPE	0.95	1.68	4.44
Regulatory proteins	2.27	3.35	4.93
Virulence, detoxification and adaptation	2.27	2.31	5.71

as GA (3.8%) in the *M. bovis* genome. In *M. tuberculosis* 477 genes were classified as ES (13.7%), 179 genes were classified as GD (5.1%) and 1 gene as GA (0.03%). A complete list of calls for the genes that are conserved between both species is given in **Supplementary Table S1**. The status of the genes that are *M. bovis* specific are also included in the table.

Early sequencing and functional annotation of the genome of *M. tuberculosis* categorised genes into several different functional classes with an uneven distribution of genes across the classes (4, 5). We examined the distribution of the genes classified as ES in *M. tuberculosis* (477) and *M. bovis* (527) across the functional classes to determine if: (i) ES genes are over-represented in any particular functional class when compared to the genome as a whole; (ii) there are differences between the two species. The results are shown in **Table 4**. Chi squared testing showed that the distribution of ES genes across the functional classes was significantly different to the distribution of all orthologues ($p \leq 0.01$). ES genes in both species are over-represented in "information pathways" and "intermediary metabolism and respiration" and under-represented in "conserved hypotheticals" and "PE/PPE" functional classes. Our data are in line with previous reports; Griffin et al. noted that the distribution of ES genes across the different functional classes were different compared to the genome as a whole (22). DeJesus et al. also noted that insertions in PE/PPE genes were under-represented likely due to GC rich sequences and an increased proportion of non-permissive TA sites in the PE/PPE genes (23). There were no major differences in distribution of ES genes across the functional classes when *M. tuberculosis* and *M. bovis* were compared with each other except for "insertion sequences and phages" which did not contain any genes classified as ES in the *M. bovis* genome.

Genes categorised as ES in this study were compared between the two species and also compared to previously reported studies (21–23, 26, 42) (**Supplementary Table S1**). We found that the *M. bovis* dataset generated in our study shared 370 (70%) of genes classified as ES with *M. tuberculosis in vitro* (this study; **Figure 2A**) and up to 86% overlap with three key published *M. tuberculosis* data sets: DeJesus et al. (23) (71%), Griffin et al. (86%) and Minato et al. (79%) indicating good correlation with previous reports (**Figure 2D**). Similarly, the *M. tuberculosis* dataset generated in our study shared good overlap with other published datasets (**Figure 2C**). When comparing *M. bovis* genes classified as ES with those reported by Butler et al. (26) we found that 220 (42%) genes were shared between these data sets (**Figure 2B**). Butler et al. reported a total of 318 genes to be essential in *M. bovis in vitro* prior to selection in *Dictyostelium discoideum* compared to 527 reported in this study. Both libraries showed similar saturation levels (58 vs. 54% in this study) and use similar analysis methods, therefore differences might be due to the conditions under which the libraries were generated (although both studies used Middlebrook 7H11 solid medium supplemented with lysed sheep blood, heat inactivated foetal bovine serum and OADC) or between laboratory variation as might be expected for whole genome techniques such as Tnseq. It should also be noted that the similarities between the studies increases when GD genes are considered, for instance of the 307 genes that appear to be uniquely ES in our study, 212 of these are classified as GD in the study by Butler et al., indicating a debilitating impact of the transposon insertion.

Differences in Gene Essentiality Between *M. bovis* and *M. tuberculosis*

Genes uniquely classified as ES in either species are of interest to determine potential genetic insights for phenotypic differences between these closely related mycobacterial species. In this study 157 genes were uniquely ES in *M. bovis* when compared to the *M. tuberculosis* (**Figure 2A**), however, of these 157, 61 were classified as GD in *M. tuberculosis*. The remaining 96 were classified as NE in *M. tuberculosis* (**Supplementary Table S2**). The existence of multiple datasets allows for a robust meta-analysis and so we compared across datasets and found that there were 42 genes that were either ES or GD in this study and the study by Butler et al., and were classified as NE in *M. tuberculosis* in this study and the study by DeJesus et al. (**Supplementary Table S3**). Included in this subset of genes is *Rv3543c* (*fadE19*), *Rv3541c* and *Rv3540c* (*lpt2*), genes which are encoded on the same operon (*Rv3545c-Rv3540c*—based on intergenic gaps) regulated by *kstR* and involved in cholesterol catabolism. This study and the Butler et al., study indicates that insertional mutagenesis of this operon has a debilitating impact in *M. bovis* but not in *M. tuberculosis.*

Data for the entire *kstR* regulon is given in **Supplementary Table S4**. Interestingly, the media used in this study and the study by Butler et al., contains traces of cholesterol due to the presence of lysed sheep blood, although there is no evidence that cholesterol presented a selective pressure (for *M. tuberculosis*) in this study as there is little overlap of the *M. tuberculosis* dataset with the study by Griffin et al., In addition to the *Rv3545c-Rv3540c* operon considered above, several orthologues in the *kstR* regulon were classified as ES in *M. bovis*; *Mb3538* (*Rv3508*), *Mb3568* (*Rv3538*) and *Mb3581* (*Rv3551*), and *Mb3595* (*Rv3565*). Others such as *Mb3541* (*Rv3511*) and *Mb3574c* (*Rv3544c*) were classified as GD. Interestingly insertions in the genes belonging to the *mce4* operon and required for growth on cholesterol mostly confer a growth advantage for *M. bovis.* These observations might reflect

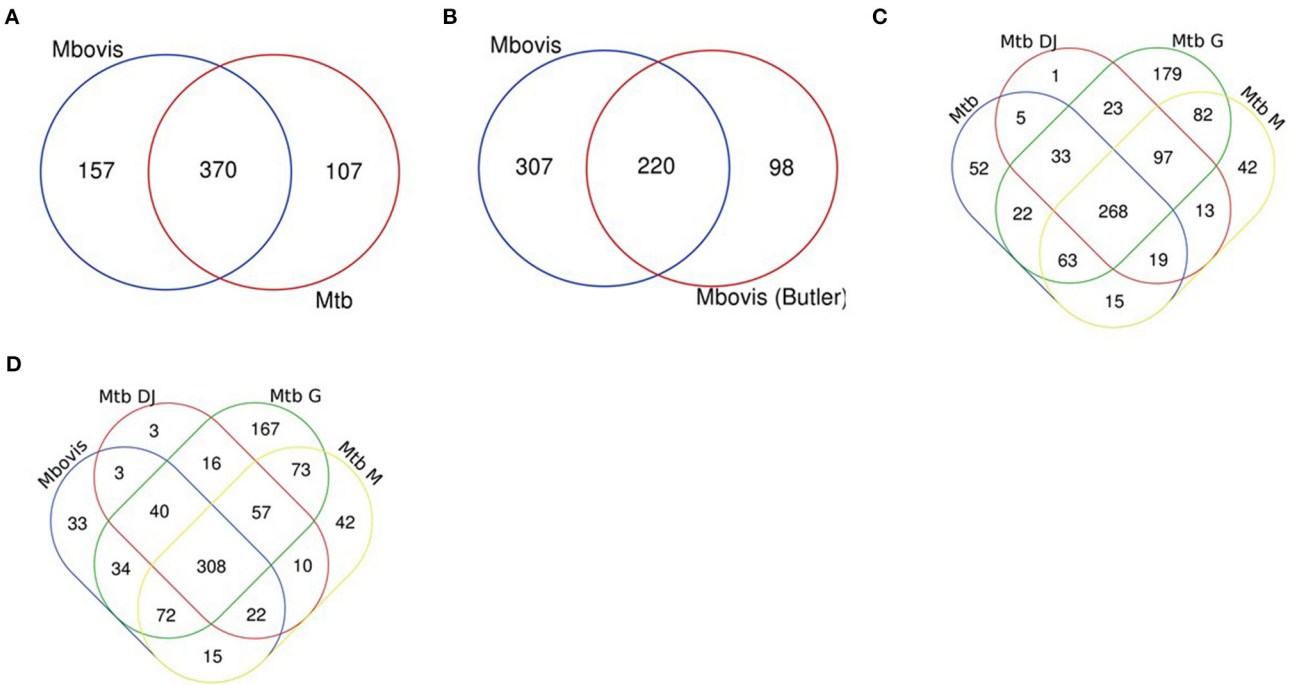

FIGURE 2 | Essential gene comparisons. Gene essentiality was determined for *M. bovis* and *M. tuberculosis* using custom HMM analyses with TRANSIT software and compared to previously published datasets. **(A)** *M. bovis* and *M. tb* (both this study), **(B)** *M. bovis* (this study) and *M. bovis* (26) **(C)** *M. tb* (this study) and *M. tb* DJ (23), *M. tb* G (22) and *M. tb* M (21) and **(D)** *M. bovis* (this study) and *M. tb* DJ (23), *M. tb* G (22) and *M. tb* M (21).

a difference in the requirement for cholesterol catabolism *in vitro* in a complex carbon mixture compared to *M. tuberculosis*.

One of the key metabolic differences between *M. bovis* and *M. tuberculosis* is the inability of *M. bovis* to utilise carbohydrates. Genes in the glycolytic pathway (**Supplementary Table S5**) such as, enolase (*eno*), pyruvate kinase (*pykA)* and pyruvate carboxylase (*pca*) might be expected to be NE in *M. bovis* as *pykA* is non-functional in *M. bovis* (43). The datasets show that *eno* is ES in *M. bovis* as well as *M. tuberculosis* perhaps indicating that its essentiality is linked to a role other than glycolysis. Similarly, the suggestion that a transposon insertion in *pykA* confers a growth advantage (this study only) is counter-intuitive and might suggest a non-glycolytic role for this enzyme. Only our dataset suggests that a transposon insertion in *icl1*, an enzyme required for growth on fatty acids, confers a growth advantage in *M. bovis*.

The two-component system PhoPR has been shown to control the biosynthesis of sulfolipid (SL-1) and di- and poly-acyltrehaloses (DAT and PAT) and also secretion of ESAT-6 by regulation of the *espACD* gene cluster (44, 45). It is of particular interest because a non-synonymous SNP in the sensor histidine kinase *phoR* in *M. bovis* renders signalling through the system defective and *M. bovis* lacks SL-1, however, the existence of compensatory mutations that restore ESAT-6 production obscures the role of the regulon in *M. bovis* (46). Additionally, it is known that genes associated with the synthesis of PDIM are over-expressed in *M. bovis* AF2122/97 (11) although the mechanism by which this occurs is not entirely known. There is some evidence that PhoPR indirectly controls the expression of PDIMs (16). Of the genes in the PhoPR regulon (**Supplementary Table S6**) only *Rv3778c* seems to be consistently required across species and studies. Genes in the redox sensing WhiB family are included in the regulon (*whiB1, whiB3* and *whiB6*) but only *whiB1* is ES in *M. bovis* in our study.

Genes encoding for the transport of inorganic sulphate; subI, *cysW*, *cysW* and *cysA*, were classified as ES in *M. bovis* in this study. These genes are involved in reductive sulphur assimilation and are conserved across the MTBC (47). This pathway was found to be essential *in vitro* for BCG Pasteur, but not BCG Danish, when grown on the same media (48). In our study, they were found to be essential *in vitro* for *M. bovis* but this was not corroborated in the study by Butler et al., Of the studies with *M. tuberculosis,* these genes have been found to be essential *in vitro* in some studies (22, 23) but not others (21). These discrepancies may reflect differences in the ability of the species (or strains) to acquire sulphate from organic sources such as methionine, different affinities of the transporter between species (or strains) or alternatively may reflect differences in the oxidative stress levels of the environment under which the screens are performed. Sulphated metabolites, such as mycothiol play a key role in the protection against oxidative stress (49). The metabolism of fatty acids can increase the cytoplasmic pool of reducing equivalents leading to a redox imbalance, therefore central carbon metabolism might also play a role in the differences observed across the studies.

Finally, as the electron transport chain and ATP synthesis is a relatively new therapeutic pathway, we chose to examine

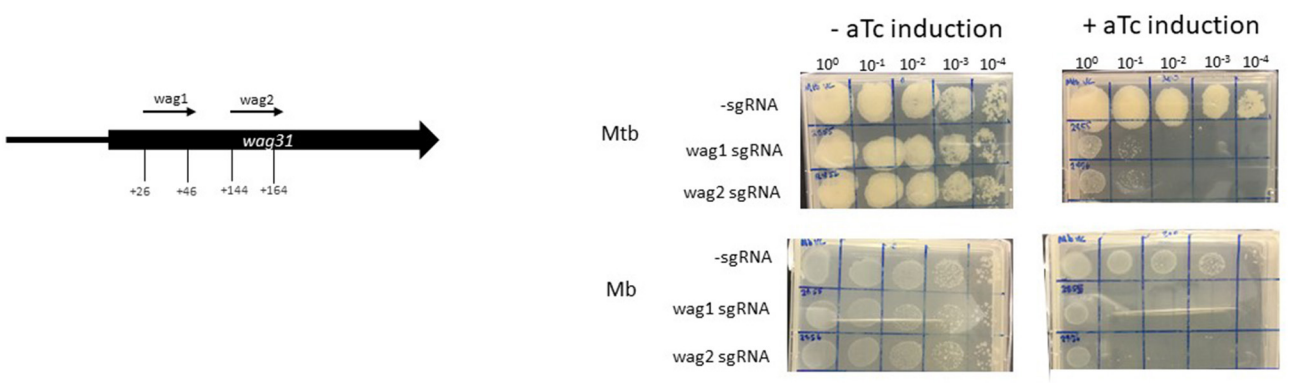

FIGURE 3 | Using CRISPRi/dCas9 to inhibit *wag31* expression in *M. bovis* and *M. tuberculosis*. **(A)** Schematic showing the target regions of two sgRNAs designed to target and inhibit *wag31* expression. The numbers show the nucleotide position of the sgRNA relative to the annotated start site. **(B)** CRISPRi strains were cultured in 10 ml of supplemented 7H9 medium to exponential phase and diluted to 2×10^7 CFU/ml. A 10-fold serial dilution to 10^{-4} was performed and 20 μl of each dilution was spotted onto 7H11 agar without aTc and with 200 ng/ml aTc to induce CRISPRi/dCas9 and the sgRNA in those strains that carried the guide. Two biological replicates were carried out.

ES more closely in these pathways (**Supplementary Table S7**). These pathways are targets of recently introduced drugs such as bedaquiline (ATP synthase) and those in development e.g., Q203 which targets the terminal cytochrome bc_1-aa_3 oxidase (50). Unsurprisingly, the genes encoding the ATP synthase are largely ES in both species in all studies (*Rv1304-Rv1311*) with the exception of *Rv1304* (*atpB*). The genes that encode a sub-unit of the terminal cytochrome bc1-aa3 oxidase complex (*qcrCAB*) the target of Q203 are classified as either ES or GD. One interesting observation is that both our study and the study by Butler et al., suggests that a growth defect occurs as a result of an insertion in *nuoG* but this is not observed in any of the *M. tuberculosis* studies. *nuoG* forms part of the multi-subunit NADH reductase-I complex in the respiratory chain and transfers electrons to the menaquinone pool while simultaneously contributing to the proton gradient through its proton pumping function. Menaquinone biosynthesis itself has been a long standing drug target in *M. tuberculosis* (51–53). Menaquinone is synthesised from chorismate by a series of enzymes (MenF, MenD, MenH, MenC, MenE, MenB, MenA). The enzyme that catalyses the first committed step in this biosynthetic pathway is encoded by *menD* and is essential in all datasets. Genes in this biosynthetic pathway (*menA*, *menB and menC)* are also ES in *M. bovis* in this study, illustrating that targets of this pathway might also be useful in the treatment of *M. bovis* infections.

Establishment of CRISPRi in *Mycobacterium bovis* Using *Wag31*

Wag31 is required for peptidoglycan synthesis and several Tnseq datasets have classified *wag31* in *M. tuberculosis* as ES *in vitro* (21–23). Additionally, essentiality of *wag31* in *M. tuberculosis* has been verified using conditional mutants (54). The Tnseq data obtained in this study classified *wag31* in *M. bovis* as ES, but NE in *M. tuberculosis*. The study by Butler et al., assigned *wag31* as NE in *M. bovis*. In order to probe this discrepancy with the literature and to establish CRISPRi silencing in *M. bovis* this gene was chosen for silencing. Early CRISPRi studies in *M. tuberculosis* performed by Singh et al. successfully utilised two plasmids encoding sgRNAs guides targeting +26 bp to +45 bp and +144 bp to +163 bp downstream of the annotated start codon of $wag31_{\text{Mtb}}$ (**Table 1**; **Figure 3A**). We utilised these plasmids to make strains of *M. tuberculosis* and *M. bovis* where *wag31* has been silenced. There are no differences in sequence in the area complementary to the sgRNAs between the two species, therefore the plasmids originally designed by Singh et al., for use in *M. tuberculosis* could be used in *M. bovis*. *M. bovis* AF2122/97 was transformed with pRH2502 to create a strain expressing $dcas9_{Spy}$(Mb_dCas9). Mb_dCas9 was then transformed with plasmids expressing the sgRNA guides. An identical strategy was used to make the equivalent strain in *M. tuberculosis*. Strains were cultured to exponential phase and serial dilutions were spotted onto agar containing 200 ng/ml aTc. Controls (without aTc, without sgRNA) were also included. The results, presented in **Figure 3B**, show that silencing $wag31_{Mb}$ in both *M. bovis* and *M. tuberculosis* results in a severe growth defect, visible at 10^{-1} dilution with complete cessation of growth at 10^{-2} dilution. This supports the consensus in the literature that *wag31* is an essential gene in *M. tuberculosis*. It also supports the classification of *wag31* as ES in *M. bovis* rather than NE as reported by Butler et al., Dysgonic growth of *M. bovis* on this medium can be observed in the figure. This has been previously reported (43, 55).

Silencing *Rv2182c* and Its Orthologue *Mb2204c* Shows a Species-Specific Growth Impact

Rv2182c/*Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-acyltransferase (agpat) and involved in glycerophospholipid metabolism. It is thought to synthesise diacylglycerol-3P through

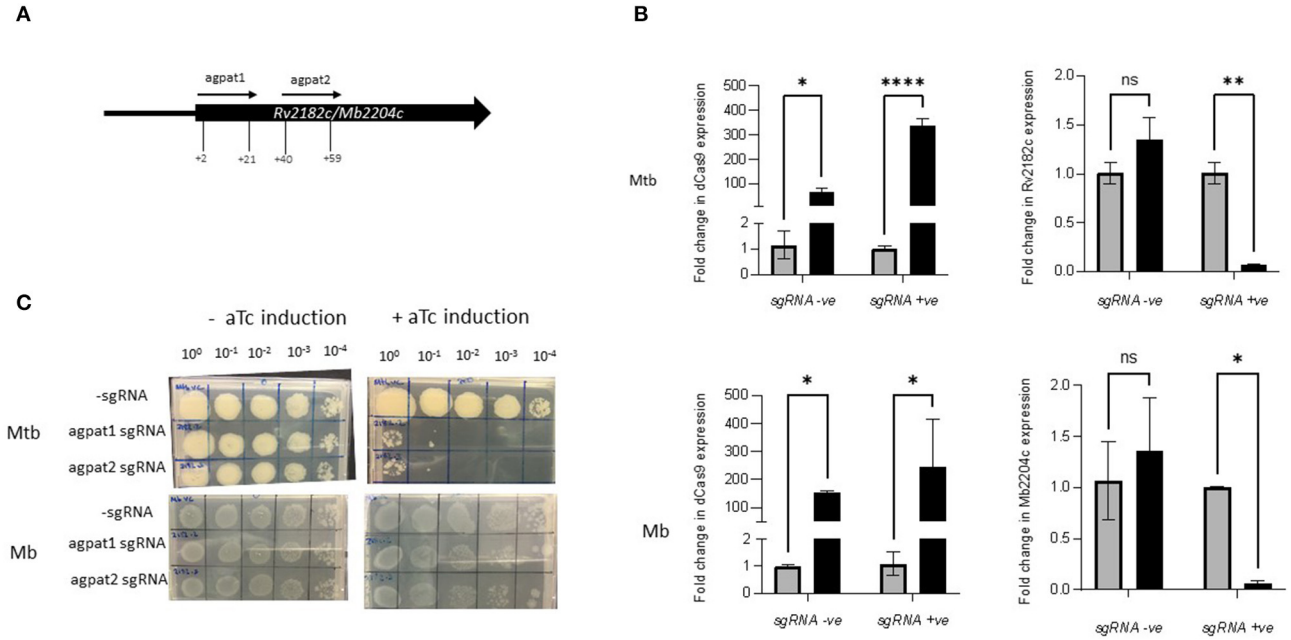

FIGURE 4 | Using CRISPRi/dCas9 to inhibit *Rv2182c/Mb2204c* expression in *M. bovis* and *M. tuberculosis*. **(A)** Schematic showing the target regions of two sgRNAs designed to target and inhibit *Rv2182c/Mb2204c* expression. The numbers show the nucleotide position of the sgRNA relative to the annotated start site **(B)** *dCas9* expression and *Rv2182c/Mb2204c* expression were measured by RT-qPCR as described in the methods section. Expression levels were measured in the strain expressing sgRNA agpat1. Gene expression was analysed using the $2^{-\Delta\Delta CT}$ method, normalised against *sigA*. Results represent two biological repeats with two technical repeats each. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ or not significant (ns), analysed using a 2-way ANOVA test. Black bars represent aTc treated cultures and grey bars untreated. **(C)** CRISPRi strains were cultured in 10 ml of supplemented 7H9 medium to exponential phase and diluted to 2×10^7 CFU/ml. A 10-fold serial dilution to 10^{-4} was performed and 20 μl of each dilution was spotted onto 7H11 agar without aTc and with 200 ng/ml aTc to induce CRISPRi/dCas9 and the sgRNA in those strains that carried the guide. Two biological replicates were carried out.

the addition of acyl chains to monoacylglycerol-3P. It is classified as ES in *M. tuberculosis* in this study and by others (22, 23, 56). It is classified as ES in *M. bovis* in this study but NE in the study by Butler et al., Strains of *M. tuberculosis* and *M. bovis* were constructed expressing sgRNAs targeting +2 bp to +21 bp and +40 bp to +59 bp downstream of the annotated start codon of *Rv2182c*/*Mb2204c* (**Table 2**; **Figure 4A**). The impact of inducing the system on expression of *Rv2182c/Mb2204c* was measured using RT-qPCR. The results, which are shown in **Figure 4B** show that $dCas9_{Spy}$ is similarly induced in both *M. tuberculosis* and *M. bovis* with 150 to 350-fold induction of expression in the presence of aTc. Additionally, the results show that, in the presence of the sgRNA, there is a clear reduction in expression of *Rv2182c/Mb2204c* in both species. These data demonstrate effective gene silencing of *Rv2182c/Mb2204c* in both *M. tuberculosis* and *M. bovis*, respectively.

To determine the impact of induction of the guides, strains were cultured to exponential phase and serial dilutions were spotted onto agar containing 200 ng/ml aTc. The results (**Figure 4C**) show that silencing *Rv2182c* in *M. tuberculosis* results in a severe growth defect, with almost complete cessation of growth at 10^{-1} dilution. However, the consequence of silencing *Mb2204c* on the growth of *M. bovis* is far less pronounced with a small reduction visible at the lowest dilution 10^{-4}. This demonstrates that, unlike *wag31*, silencing of *Rv2182c* and its orthologue *Mb2204c* in *M. tuberculosis* and *M. bovis* respectively, has a differential impact on growth, with *M. tuberculosis* being more vulnerable and showing a greater growth defect. These results do not support the classification of *Mb2204c* as an ES gene in *M. bovis* but they clearly highlight that there are different phenotypic consequences as a result of silencing the orthologue in both species.

DISCUSSION

The aim of this work was to directly compare gene essentiality in the human and animal adapted members of the MTBC. In order to do this, we generated transposon libraries in *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv using a rich medium that supported the growth of both species. We assessed gene essentiality using the TRANSIT HMM method to define 527 and 477 genes as ES for *M. bovis* and *M. tuberculosis*, respectively. Datasets from each species were compared with each other and with previously published datasets. Genes classified as ES were congruent between the species and also with existing studies of gene essentiality in *M. tuberculosis* (21–23, 42). Comparing this study with a previously published *M. bovis* dataset revealed a 42% overlap which increased when genes predicted to show a GD as a result of the transposon insertion were taken into account (26). There were some indications of differences between the species,

and a meta-analysis of the data indicated that 42 genes were differentially essential between the species. A recent study using whole genome CRISPRi screens showed that a similar number (80 genes) were differentially essential in two different strains of *M. tuberculosis* (H37Rv vs. HN878) (57). Genes that appear to show differential essentiality between the two species include those involved in cholesterol catabolism.

Whole-genome TnSeq provides a high-throughput assessment of fitness costs and has allowed the classification of genes based on essentiality but does not provide information on target vulnerability. More recent studies highlight the limitations of the (near) binary classification of genes into and ES/NE and utilise CRISPRi to assess vulnerability (57, 58). Additionally, datasets are prone to false calls of ES due to non-saturating mutagenesis. In this study CRISPRi was utilised to show that there are different impacts on bacterial growth as a result of silencing *Rv2182c*/*Mb2204c* in their respective species, despite achieving similar levels of gene silencing. Significant growth inhibition was seen as a result of silencing in *Rv2182c* in *M. tuberculosis* while only marginal impacts on growth were observed on silencing the orthologue *Mb2204c* in *M. bovis*. *Rv2182c*/*Mb2204c* is annotated as a 1-acylglycerol-3-phosphate O-acyltransferase and involved in glycerophospholipid metabolism. It is thought to synthesise diacylglycerol-3P through the addition of acyl chains to monoacylglycerol-3P. This pathway may be involved in detoxification and further work is required to fully understand the differential impact of silencing this gene in the two species. Given that *Rv2182c* was a predicted target in a recent compound screen (59), differential essentiality estimates in *M. bovis* and *M. tuberculosis* are important to predict if zoonotic TB caused by *M. bovis* can also be suitably treated with drugs designed to be effective against *M. tuberculosis*.

We have provided a comparative analysis of the genetic requirements for growth of two key MTBC members: *M. bovis* and *M. tuberculosis*. Genes which are uniquely ES for either *M. bovis* or *M. tuberculosis* have the potential to provide insights into niche specific aspects e.g., host tropism, survival in the environment, phenotype, and anti-tubercular drugs. Host tropism is of particular interest when considering the zoonotic nature of *M. bovis* and the involvement of wildlife hosts as reservoirs of infection for bovine TB. Use of *M. bovis* libraries in the context of the host i.e., through experimental infection of bovine TB will enable the study of the genetic requirements for survival *in vivo*. Further investigations exploring the role and function of ES genes between *M. bovis* and *M. tuberculosis* is necessary to better understand the physiological differences in these key MTBC species.

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: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA754037; SRA number Temporary Submission ID: SUB10190503.

AUTHOR CONTRIBUTIONS

AG, SW, IP, and SK designed the study. AG, IP, and VF carried out the experimental work. Data analysis was done by BS, TC, IN, JS, and DX. TC, SK, DW, BW, and BV-R did funding acquisition. AG and SK wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read, and 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.2021.760717/full#supplementary-material

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