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Bovine Tuberculosis in Central Ethiopia: Epidemiology, BCG Efficacy in Reducing Transmission, and Performance of Defined Antigen Cocktails for Differentiating Infected from Vaccinated Crossbred Cattle

PhD Dissertation

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Bovine Tuberculosis in Central Ethiopia: Epidemiology, BCG Efficacy in Reducing Transmission, and Performance of Defined Antigen Cocktails for Differentiating Infected from Vaccinated Crossbred Cattle

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This is to certify that the dissertation prepared by Abebe Fromsa Merga, entitled "**Bovine Tuberculosis in Central Ethiopia: Epidemiology, BCG Efficacy in Reducing Transmission, and Performance of Defined Antigen Cocktails for Differentiating Infected from Vaccinated Crossbred Cattle**" and submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Tropical and Infectious Diseases complies with the regulation of the Addis Ababa University and meets the accepted standards with respect to originality and quality.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
Ad585A	Adenovirus type 5 expressing Ag85A
Ag85	Antigen 85
AFB	Acid-Fast Bacilli
AGP	Alpha-1 acid glycoprotein
AHI	Animal Health Institute
AhR	Aryl Hydrocarbon Receptor
AIM2	Absent in Melanoma 2/ Apoptosis inhibitor of macrophages 2
ALIPB	Aklilu Lemma Institute of Pathobiology
ALRs	AIM2-like receptors
AMBP	Alpha-1-microglobulin/ bikunin precursor protein
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
BCG	Bacillus Calmette-Guérin
BCL2	B-cell lymphoma 2
BoLA	Bovine leukocyte antigen
BSL-3	Biosafety level 3
bTB	Bovine Tuberculosis
CCT	Comparative Cervical Tuberculin Test
CD	Cluster of differentiation
CFP-10	Culture Filtrate Protein 10 kDa
CFT	Caudal Fold Test
CFU	Colony Forming Unit
CHI3L1	Chitinase-3-like protein 1
CRP	C - reactive protein
CXCL-9	C-X-C Motif Chemokine Ligand 9
cGAS	cyclic GMP-AMP synthase
CI	Confidence interval
CMI	Cell-mediated immunity
CTLRs	C-type lectin receptors
CTLA-4	Cytotoxic T lymphocyte antigen 4 (CD152)
CTLs	Cytotoxic T lymphocytes
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DC-SIGN	DC specific intercellular adhesion molecule 3-grabbing nonintegrin
DIVA	Differentiate Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
DST	Defined Skin Test
DTH	Delayed type hypersensitivity

ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot Assay
ESAT-6	Early secretory antigenic target 6 kDa
EU	European Union
GMP-AMP	Guanosine monophosphate–Adenosine monophosphate
ICs	Immunological checkpoints
IFN-γ	Interferon Gamma
IGRA	Interferon Gamma Release Assay
IL-2	Interleukin 2
IL-2R	Interleukin-2 receptor
IP-10	Interferon gamma-induced protein 10
IS	Insertion sequence
LAG-3	Lymphocyte activation gene 3
LAM	Lipoarabinomannan
LM	Lipomannan
LJ	Löwenstein–Jensen
LMIC	Low- and middle-income country
LOSs	Lipooligosaccharides
ManLAM	Mannosylated lipoarabinomannan
MAP	*Mycobacterium avium* subspecies paratuberculosis
MCE	Mammalian Cell Entry
MHC I&II	Major histocompatibility complex class I and class II
Mincle	Macrophage Inducible C - Type Lectin
MIG	Monokine induced by IFN-γ
m-qPCR	multiplex quantitative PCR
MPB70	Mycobacterium Protein Bovis 70 kDa
MPB83	Mycobacterium Protein Bovis 83 kDa
mRNA	Messenger Ribonucleic Acid
MTBC	*Mycobacterium tuberculosis* Complex
MVA85A	Modified vaccine virus Ankara expressing Ag85A
NLRs	NOD-like receptors
NLRs	Nucleotide-binding domain and leucine-rich repeat-containing receptors
NOD	Nucleotide-binding oligomerization domain
NTM	Nontuberculous mycobacteria
OD	Optical density
OR	Odds ratio
OTF	Officially tuberculosis-free
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death 1
PD-L1/2	Programmed cell death ligand 1/2
PGLs	Phenolic glycolipids
PIMs	Phosphatidylinositol mannosides

Pks5	Polyketide synthase 5
PM	Post-mortem
PPD	Purified protein derivative
PPD-A	Purified protein derivatives of Tuberculin – of *M. avium* origin
PPD-B	Purified protein derivatives of Tuberculin – of *M. bovis* origin
PRRs	Pattern recognition receptors
RD	Region of Difference
RIG	Retinoic acid-inducible gene
RLRs	RIG-I-like receptors
R_0	Basic reproduction ratio
SIT	Single Intradermal Tuberculin Test
SNPs	Single Nucleotide Polymorphisms
SR	Scavenger receptors
SSI	Staten's Serum Institute
STING	Stimulator of interferon genes
TB	Tuberculosis
TBL	Tuberculous Like Lesions
Tcm	Central memory T cells
TCR	T cell receptor
TDM	Trehalose-6,6'-dimycolate
TGF-β	Transforming growth factor β
TIM-3	T-cell immunoglobulin -3
Th1	T helper type 1
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
TST	Tuberculin skin test
UK	United Kingdom
US	United States
USD ($)	United States Dollar
VDBP	Vitamin D binding protein
WHO	World Health Organization
WOAH	World Organization for Animal Health
yrbE A and B	Genes encoding putative ATP-Binding Cassette transporter protein
ZN	Ziehl–Neelsen staining
zTB	Zoonotic tuberculosis

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ABSTRACT

Background: Bovine tuberculosis (bTB) is a threat to animal health, the livestock economy, food security, and human safety. Although animal level prevalence ranges from 25% to 39% and herd prevalence exceeds 50% in intensive dairy farms in central Ethiopia, control measures are lacking. The conventional test-and-slaughter strategy is resource-intensive and unaffordable for low- and middle-income countries (LMICs) such as Ethiopia. This necessitates a cost-effective alternative control strategy. Bacillus Calmette-Guérin (BCG) vaccination may constitute a cost-effective approach to control bTB in LMICs and regions with wildlife reservoirs. A meta-analysis of previous BCG vaccine evaluation studies demonstrated limited overall direct efficacy of 25% and additional partial protection by reducing the frequency and severity of disease pathology. Whether this partial protective effect translates into indirect protection through a reduction in the infectiousness of vaccinated animals has not been evaluated. Besides, since the interference of the BCG vaccine with the tuberculin skin testing is a major hindrance to the deployment of BCG vaccination for cattle, there is also a need to adopt a diagnostic test that differentiates infected from vaccinate animals (DIVA). On top of these, a slaughterhouse survey was conducted to identify hotspots of bTB in central Ethiopia to prioritize targeted control measures.

Objectives: The objectives of this study include to quantify the direct and indirect efficacies of BCG vaccination in crossbred cattle, evaluate the diagnostic performance of defined peptide antigen cocktails (ESAT-6, CFP10, and Rv3615c) in differentiating infected from vaccinated animals (DIVA), assess the practical implications of routine vaccination on the spread of bTB in the emerging dairy sector, and estimate the prevalence and identify the causative agents of bTB in cattle in central Ethiopia.

Methodology: A natural transmission experiment was conducted using a crossover design in two phases, each lasting for one year to evaluate both the direct and indirect effects of BCG vaccination in reducing the transmission of bTB. Sixty-seven unvaccinated control and 67 vaccinated calves were exposed to an equal number of respective seeders eight weeks post-vaccination and maintained in contact for twelve months during each phase. Sixty-five unvaccinated control and 62 vaccinated calves underwent postmortem tests. Skin and IGRA tests were conducted using tuberculin and DIVA antigen cocktails every four and two months, respectively. For efficacy evaluation, calves that ever tested positive for DIVA antigens in the IGRA test were considered infected and potentially infectious. Then a mechanistic transmission model calibrated using within-herd basic reproduction number (Ro) and empirical cattle movement data was used to explore the long-term effect of routine BCG vaccination in controlling bTB in the Ethiopian dairy sector. The performance of DIVA antigen cocktails was compared to tuberculin in both IGRA and skin test formats. A slaughterhouse survey was

conducted in Central Ethiopia to assess the prevalence, risk factors, and causative agents of bTB. The causative agents were identified using multiplex quantitative PCR (m-qPCR) in all culture-positive samples.

Result: The study found a significantly lower total visible lesion score in vaccinated animals compared to unvaccinated controls and a reduction in bTB transmission of 74% (95% CI: 46–89%) among vaccinated animals compared to unvaccinated controls. The direct efficacy was 58% (95% CI: 34%–73%). The combined direct and indirect effects resulted in a total vaccine efficacy of 89% (95% CI, 74–96%). The estimated Ro, representing the average number of secondary cases per infectious individual was 3.1 (95% CI: 2–4) in unvaccinated group while it was 0.3 (95% CI: 0.1–0.7) in the vaccinated group. A mechanistic transmission model suggests that routine neonatal calf vaccination has the potential to reduce the average population Ro to below 1 within 10 years, presenting significant opportunities for the control and elimination of bTB in cattle. Eight weeks after vaccination, but before exposure to infected seeder animals, the specificities of IGRA and skin test-based defined antigen cocktails were 98.5% and 94.0%, respectively. However, at the end of the experiment, the sensitivities of these tests were 46% and 47%, respectively, which were similar with 45% sensitivity of the comparative tuberculin test (CCT). A slaughterhouse survey revealed the ongoing challenge of bTB in Central Ethiopia, with a prevalence of 2.3% bTB-related lesions and an eleven-fold greater risk in crossbred cattle than in local zebu cattle. All mycobacterial species isolated from experimental and slaughterhouse cattle were identified as *M. bovis*, except for two cases from the slaughterhouse. One of these cases exhibited co-infection with *M. bovis* and *M. tuberculosis*, while the other was co-infected with *M. bovis and M. caprae*. However, no positive *M. orygis* was detected in any of culture-positive samples.

Conclusions: The study revealed previously unrecognized superior indirect effects of BCG on bTB transmission compared to its direct protective effect against infection. Scenario analyses using mechanistic models indicate that BCG vaccination may enable effective control of bTB and contribute to elimination efforts. DIVA antigens demonstrated high specificity in BCG-vaccinated cattle, highlighting their importance for potential integration into BCG vaccination programs for bTB control. These findings hold significant implications for resource-limited settings where the test-and-slaughter strategy is not feasible, while vaccination combined with DIVA testing remains a beneficial and effective approach. The higher risk of bTB in crossbred cattle underscores the necessity for targeted interventions, particularly in crossbred cattle predominantly raised in intensive dairy production systems.

Keywords: Bovine tuberculosis, Bacillus Calmette-Guérin, Vaccine, Direct efficacy, Indirect efficacy, Transmission, Basic reproduction number, DIVA, Slaughterhouse survey, Ethiopia

ማጠቃለያ በአማርኛ

ዳራ፡ የከብቶች የሳንባ ነቀርሳ (bovine TB) ለእንስሳት ጤና፣ ለከብት አርቢዎች ኢኮኖሚ፣ ለምግብ ዋስትና እና ለሰው ጤና ከፍተኛ ስጋት ደቅኗል። በማዕከላዊ ኢትዮጵያ በሚገኙ ከፍተኛ የወተት አምራች አካባቢዎች ከ25% እስከ 39% እንስሳት እና ከ50% በላይ የወተት እርባታዎች በበሽታው የተጠቁ ቢሆንም የተተገበረ የበሽታ ቁጥጥር እርምጃ የለም። በዓለም ዓቀፍ ደረጃ ምርመራ አደርጎ በበሽታው የተጠቁ ከብቶችን ለይቶ በእርድ የማስወገድ ቁጥጥር ዘዴ ሀብትን የሚጠይቅ እና ዝቅተኛ እና መካከለኛ ገቢ ባላቸው እንደ ኢትዮጵያ ባሉ ሀገራት ለመተግበር አስቸጋሪ ሆኗል። ስለዚህ ወጪ ቆጣቢ የሆነ አማራጭ የቁጥጥር ስትራቴጂ ያስፈልጋል። የ ቢሲጂ (ባሲለስ ካልሜት ጌራን) / Bacillus Calmette-Guérin (BCG) ክትባት ዝቅተኛ እና መካከለኛ ገቢ ላላቸው እንደ ኢትዮጵያ ላሉ ሀገራት ወጪ ቆጣቢ የበሽታ ቁጥጥር ዘዴ ሊሆን ይችላል። ከዚህ ቀደም በከብቶች ላይ የተሰሩ ጥናቶች የቢሲጂ ክትባት በበሽታው የሚያዙ ከብቶችን በ25% የሚቀንስ እና በሽታው የሚያስከትለውን ጉዳት እንዲሁም በበሽታው የሚጠቁ አካል ክፍሎችን በመቀነስ ተጨማሪ የመከላከል አቅም አሳይቷል። ይህ የክትባቱ ከፊል የመከላል አቅም የተከተቡ እንስሳትን በሽታ አስተላላፊነት በመቀነስ ረገድ ትርጉም ያለው አስተዋፅዖ አለው ወይስ የለውም የሚለው ግን ከዚህ ቀደም አልተጠናም። በተጨማሪም የቢሲጂ ክትባት በቲዩበርክሊን የቆዳ ምርመራ ላይ ተፅዕኖ ስላለውና የተከተቡ ከብቶች ከበሽታው ነፃ ቢሆኑም የተያዙ አስመስሎ ስለሚያሳይ የቢሲጂ ክትባትን ጥቅም ላይ ለማዋል እንቅፋት ሆኗል። ስለሆነም በበሽታው የተያዙ እንስሳትን ከተከተቡት የሚለይ የምርመራ ዘዴ አስፈላጊ ስለሆነ በዚህ ላይ ጥናት ተካሂዷል። በተጨማሪ በማዕከላዊ ኢትዮጵያ የሚገኙ የከብቶች የሳንባ ነቀርሳ የሚከሰትባቸውን ቦታዎች ለማወቅና የታለመ የበሽታ ቁጥጥር እርምጃዎች ቅድሚያ ሊሰጣቸው የሚገባቸውን ለመለየት የቁራ ጥናት ተካሂዷል።

የጥናቱ ዓላማዎች፡ የቢሲጂ ክትባት የተዳቀሉ ከብቶችን ከሳንባ ነቀርሳ የመከላከል እና ተከትበው በበሽታው የተያዙ ከብቶችን በሽታ እስተላላፊነት በመቀነስ ረገድ ያለውን ውጤታማነት ማጥናትና መለካት። የተመረጡ የፔፕታይድ አንቲጂን ቅይጦች (ESAT-6፣ CFP10፣ እና Rv3615c) የተከተቡ እንስሳትን በበሽታ ከተያዙ እንስሳት በመለየት ያለውን የምርመራ አፈፃፀም መገምገም። የወተት ከብቶች እርባታ ዘርፍ በማደግ ላይ ባለባቸው አካባቢዎች መደበኛ የቢሲጂ ክትባት በሳንባ ነቀርሳ ስርጭትና መስፋፋት ላይ ያለውን ተግባራዊ ጠቀሜታ መገምገም። እንዲሁም በመካከለኛ ኢትዮጵያ የከብቶች ሳንባ ነቀርሳ የመስፋፋት መጠንን መገምገም ፥ የበሽታውን መስፋፊያ መንስኤዎች መለየት እና የበሽታው አምጪ ተዋስንያንን ማወቅ ናቸው።

የጥናት ዘዴ፡- የቢሲጂ ክትባት ከብቶችን ከሳንባ ነቀርሳ በመከላከልና የበሽታወን ተላላፊነት በመቀነስ ረገድ ያለውን ጠቀሜታ ለመገምገም በሽታው ከታመሙ ከብቶች ነፃ ወደ ሆኑ እንስሳት በተፈጥሯዊ መተላለፊያ መንገድ እንዲተላለፍ የማድረግ እያንዳንዳቸው ለአንድ ዓመት የቆዩ ሁለት ተሻገሪ የጥናት ዲዛይን ሙከራዎች ተካሂደዋል። በእያንዳንዱ የሙከራ ግዜ 67 ያልተከተቡ እና 67 የተከተቡ ከበሽታ ነፃ የሆኑ ጥጆች ክትባት ከተሰጣቸው ከስምንት ሳምንታት በኋላ ተመሳሳይ ቁጥር ላላቸው በበሽታው ለተያዙ እንስሳት ተጋልጠው ለአስራ ሁለት ወራት በአንድ ጋጣ ውስጥ ቆይተዋል። ስልሳ አምስት ያልተከተቡ እና 62 የተከተቡና ሙሉውን የጥናት ጊዜ የጨረሱ ጥጆች የድህረ ሞት ምርመራዎችን ተደርጎላቸዋል። የቆዳ ምርመራዎች በየአራት ወሩ እንዲሁም በየሁለት ወሩ በቲዩበርክሊን እና በፔፕታይድ አንቲጂን ቅይጦች (ESAT-6፣ CFP10፣ እና Rv3615c) የደም ምርመራ ተደርጎላቸዋል። በፔፕታይድ አንቲጂን ቅይጦችን የደም ምርመራ አዎንታዊ ምላሽ ያሳዩ ጥጆች በበሽታው የተያዙ እና በሽታ አስተላላፊ እንደሆኑ ታሳቢ በማደረግ የቢሲጂ ክትባት ውጤታማነት ግምገማ ተደርጎል፡፡ የበሽታ ስርጭት ሞዴል በመጠቀም የወተት ከብቶች እርባታ ዘርፍ በማደግ ላይ ባለባቸው አካባበዎች የቢሲጂ ክትባት የከብቶችን የሳንባ ነቀርሳ ስርጭትን ለረጅም ጊዜ በመቆጣጠር ረገድ የሚኖረውን ውጤታማ አፈፃፀም በሽታው በመንጋው ውስጥ የሚያሳየውን መሰረታዊ የመራቢያ ቁጥር (Ro) እና ተጨባጭ የከብት እንቅስቃሴ መረጃን በመጠቀም

ገምግመናል። የፔፕታይድ አንቲጂ ቅይጦችን በደም እና በቆዳ ምርመራ ያላቸውን ውጤታማ አፈፃፀሞች ከቲዩበርክሊን የደም እና የቆዳ ምርመራ ውጤታማ አፈፃፀሞች ጋር የማነፃፀር ስራ ተሰርቷል። በማዕከላዊ ኢትዮጵያ የከብቶች የሳንባ ነቀርሳ ስርጭትን፣ አጋላጭ ምክንያቶችን እና መንስኤዎችን ለማወቅ የቄራ ጥናት ተካሂዷል። የበሽታ መንስኤዎች በPCR ተለይተዋል።

የጥናቱ ውጤት፡ ክትባት የወሰዱ እንስሳት ክትባት ካልወሰዱ እንስሳት ጋር ሲነጻጸር ክትባት በወሰዱ እንስሳት ላይ የታየው በሽታው የሚያስከትለው በዓይን የሚታይ ቁስለት በእጅጉ ያነሰ ሲሆን የተከተቡ ከብቶች የሳንባ ነቀርሳ በሽታን የማስተላለፍ አቅም በ74% (95% CI: 46-89%) የቀነሰ ሆኖ ታይቷል። ክትባቱ በበሽታው መያዝን በመከላከል ረገድ የ58% (95% CI: 34%-73%) ውጤታማነት አሳይቷል። ክትባቱ በበሽታው መያዝን በመከላከል እና የበሽታውን መተላለፍ አቅም በመቀነስ ያሳየው ውጠታማነት ተጣምሮ ሲታይ አጠቃላይ የክትባቱ ውጤታማነት 89% (95% CI: 74-96%) መሆኑን አሳይቷል። ክትባት ባልተሰጣቸው ቡድን ውስጥ በበሽታው የተያዘ አንድ እንስሳ በአማካይ ወደ 3.1 (95% CI: 2-4) ጤነኛ እንስሳት በሽታውን የሚያስተላልፍ ሲሆን ክትባት በተሰጣቸው ቡድን ውስጥ ግን ወደ ከአንድ በታች 0.3 (95% CI: 0.1-0.7) ብቻ መሆኑን አመልክቷል። የበሽታ መተላለፊያ ሞዴል ጥናታችን ጥጆች እንደተወለዱ ክትባት ቢሰጥ በአማካይ አንድ በበሽታው የተያዘ እንስሳ በበሽታ ወዳልተያዘ እንስሳ የሚያስተላለፍበትን መጠን በ10 ዓመታት ውስጥ ከአንድ በታች ለመቀነስ የሚያስችል አቅም እንዳለው አሳይቷል። ይህ ውጤት ክትባቱ የከብቶች ሳንባ ነቀርሳን ለመቆጣጠር እና ለማስወገድ ከፍተኛ እድሎችን የሚፈጥር መሆኑን ያመለክታል። ለበሽታ ያልተጋለጡት ጥጆች ከተከተቡ ከስምንት ሳምንታት በኋላ በፔፕታይድ አንቲጂን ቅይጦች (ESAT-6፣ CFP10፣ እና Rv3615c) የደም እና የቆዳ ምርመራ መሰረት በቅደም ተከተል 98.5% እና 94.0% አሉታዊ ምላሽ አሳይተዋል። በሙከራው መጨረሻ ላይ በበሽታ ለተያዙ የተዳቁሉ ከብቶች ከተጋለጡ በኋላ በቅደም ተከተል 46% እና 47% ለእነዚህ ምርመራዎች አዎንታዊ ምላሽ ሰጥተዋል፣ ይህም የቱበርኩሊን የቆዳ ምርመራ (CCT) ካሳየው 45% በሽታውን የመለየት አፈፃፀም ጋር ተመሳሳይና ተነፃፃሩ ነው። የቄራው ጥናት በማዕከላዊ ኢትዮጵያ የከብቶች ሳንባ ነቀርሳ ቀጣይነት ያለው ተግዳሮት መሆኑን ያሳየ ሲሆን ከአገር ውስጥ ዝርያ ከብቶች ይልቅ በተዳቀሉ ከብቶች ላይ በአስራ አንድ እጥፍ የመከሰት እድል የለው መሆኑን ያመላክታል። ከሁሉም ከብቶች የተለዩ ሁሉም የማይኮባክቴሪያ ዝርያዎች ከቄራ ከተገኙ ሁለት እንስሳት በስተቀር የከብቶች ሳንባ ነቀርሳ ዋና አምጪ የሆነው M. bovis መሆኑ ተለይቷል። ከተቀሩት ከሁለቱ አንዱ በሁለቱም በM. bovis እና M. tuberculosis በአንድላይ የተገኙበት ሲሆን፣ ሌላኛው ደግሞ በሁለቱም በM. bovis እና M. caprae የተያዘ መሆኑ ታውቋል። ምንም ናሙና በM. orygis መያዝን የሚያመላክት የምረመራ ውጤት አላሳየም።

መደምደሚያ፡ ይህ ጥናት በሳንባ ነቀርሳ መያዝን በመከላከል ረገድ ካለው ውጤታማነት ሲነፃፀር የቢሲጂ ክትባት የበሽታውን መተላለፍና እና ስርጭት በእጅጉ ለመቀነስ የሚያስችል ቀደም ሲል ያልታወቀ እምቅ አቅም እንዳለው አሳይቷል። የበሽታ መተላለፊያ ሞዴል የሁኔታ ትንተና የቢሲጂ ክትባት በሽታውን ለመቆጣጠር እንደሚያስችል እና ለማጥፋት የሚደረጉ ጥረቶች እንደሚረዳም ያሳያል። የፔፕታይድ አንቲጂን ቅይጦች በቢሲጂ የተከተቡ ከብቶች ላይ ሀሰተኛ አወንታዊ የበሽታ ምርመራ ውጤት ስለማያሳዩ ከከብቶች ሳንባ ነቀርሳ በሽታ ቁጥጥር ፕሮግራሞች ጋር ሊዋሃዱ የሚችሉና ጉለህ ጠቀሜታ ያላቸው መሆኑን ያሳያል። የዚህ ጥናት ግኝቶች ፣ በሳንባ ነቀርሳ የተያዙ እንስሳትን በእርድ በማስወገድ በሽታውን መቆጣጠር በማይቻልበትና የሀብት ውስንነት በሚገድባቸው አካባቢዎች ጉልህ ጠቀሜታ አላቸው። ጥናቱ ክትባትን ከፔፕታይድ አንቲጂን ቅይጦች የበሽታ ምረመራ ጋር አጣምሮ መጠቀም ውጤታማ አማራጭ መሆኑን ያሳያል። በዲቃላ የወተት ከብቶች ላይ በሽታው ከፍተኛ የመከሰት እድል ያለው መሆኑ በነዚህ ከብቶች ዝርያ ላይ ያተኮረ የበሽታ ቁጥጥር እርምጃዎችን አስፈላጊነት በጉልህ ያመለክታል።

CHAPTER ONE: INTRODUCTION

1.1 General Background

1.1.1 Bovine tuberculosis

Bovine tuberculosis (bTB), caused by *Mycobacterium tuberculosis* complex (MTBC), poses a threat to the health of livestock, humans, and wildlife (Ashford *et al.*, 2001; Malone and Gordon, 2017; Smith *et al.*, 2021). The MTBC is a group of extremely closely related mycobacterial species with average nucleotide identity of 99.2 - 99.9 % (Brites *et al.*, 2018; Riojas *et al.*, 2018). All cases of tuberculosis caused by MTBC in cattle are considered bTB (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023). The MTBC subspecies include *Mycobacterium tuberculosis sensu stricto (lineages 1–4 and lineage 7)*, *M. africanum* (subtypes I and II), *M. bovis*, *M. bovis* Bacillus Calmette-Guérin (BCG), *M. microti, M. pinnipedii, M. orygis, M. mungi, M. suricattae, M. caprae*, and Dassie Bacillus (Brites *et al.*, 2018; Fellag *et al.*, 2020). *M. bovis* BCG, the attenuated vaccine strain, shares more than 99.9% genomic identity with *M. bovis* and retains the conserved sequences characteristic of the MTBC (Riojas *et al.*, 2018). The MTBC members exhibit host-specific preferences, with *M. tuberculosis* sensu stricto and *M. africanum* being human-adapted strains, whereas the remaining MTBC species and *M. bovis* are animal-adapted, infecting a wide range of domesticated and wild animals (Malone and Gordon, 2017). A recent systematic review revealed geographic patterns of MTBC species distribution in livestock. *Mycobacterium bovis* was the predominant pathogen globally, while *M. orygis* was specific to Asia and *M. africanum* was restricted to Africa. European livestock were mainly affected by *M. bovis, M. caprae, M. microti*, and *M. pinnipedii*, while *M. tuberculosis* had the highest prevalence in Africa (Reis *et al.*, 2021a).

These MTBC members are transmitted via the transcutaneous, gastrointestinal, and respiratory systems, leading to infection of the lymphatic system and secondary spread to various organs (Fellag *et al.*, 2020). In cattle, the principal method of transmission is through the inhalation of infected respiratory droplets. Additional routes of infection include consumption of contaminated milk, feed, or water, and contact with infectious excretions such as urine, feces, vaginal secretions, and sperm (Parlane *et al.*, 2015; WOAH, 2022). The main drivers of animal TB transmission include management strategies (herd type, husbandry system, and multi-

species cohabitation), livestock-wildlife interactions, pathogen characteristics (different MTBC members and strains), host factors (sex, age, breed), animal movements, livestock-human interactions, and environmental conditions—each with varying predominance and contribution across different continents (Reis *et al.*, 2021a).

1.1.2 Bovine tuberculosis in wild animal reservoirs

As in livestock, the most frequently reported infectious agent in wild animals was *M. bovis* (89.5%). *M. orygis* and *M. mungi* were reported exclusively in Asia and Africa, while *M. caprae* was found in Europe. *M. pinnipedii* was detected in both South America and Oceania. *M. tuberculosis* was reported in Africa, Asia, and Europe. Asia documented cases of both *M. tuberculosis* and *M. bovis*, whereas North America reported only *M. bovis*. Both South America and Oceania reported *M. pinnipedii* and *M. bovis* (Reis *et al.*, 2021b)**.**

Cattle are the main sources of *M. bovis* infection referred to as maintenance hosts. The key characteristics of maintenance hosts are susceptibility to *M. bovis*, prolonged survival after infection, the ability to reproduce during infection, and the capacity to shed the pathogen through multiple routes spreading the disease (Goodchild and Clifton-Hadley, 2001; Haydon *et al.*, 2002). *M. bovis* infection of several wild animals has been confirmed in recent studies (Constable *et al.*, 2017; Chang *et al.*, 2024), indicating the challenge wildlife is posing to bTB control and eradication in endemic settings. In South Africa alone, *M. bovis* has infected 24 wild animal species (Bernitz *et al.*, 2021), showing the potential role of wildlife in spreading the disease and complicating control.

The well-known maintenance wild animal hosts for *M. bovis* infection are brushtail possums (*Trichosurus vulpecula*) in New Zealand, European badgers (*Meles meles*) in Great Britain and Ireland, kudu (*Tragelaphus strepsiceros*) and African buffalo (*Syncerus caffer*) in South Africa, wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) in the Iberian Peninsula, white-tailed deer (*Odocoileus virginianus*) in Michigan, USA, and bison (*Bison bison*) and elk (*Cervus canadensis*) in Canada (Bengis *et al.*, 2001; Ali, 2013; Palmer, 2013; Buddle *et al.*, 2018). Depending on their abundance and densities as well as the composition and structure of natural communities of these species, the specific wildlife hosts can be perceived as TB reservoirs in some countries (badgers in the UK, red deer and wild boar in the Iberian Peninsula and Central Europe), but spillover species in others (Reis *et al.*, 2021b). Many other animals can also serve as spillover hosts that do not support transmission in the absence of maintenance hosts.

However, spillover hosts can temporarily transmit the infection to maintenance hosts and other species under certain conditions such as high population density (Ali, 2013). Generally, the role of wildlife in bTB spread might be less important in the urban, intensive dairy systems, but the risk of transmission can still occur in peripherally located herds with close proximity to wildlife, where the biosecurity is poor and barriers limiting wildlife-cattle interactions is lacking. However, despite the potential influence of interactions between cattle, sympatric domestic animals and wildlife reservoirs, their role on the transmission of bTB has not been sufficiently studied and remains unconfirmed in Ethiopia. Tschopp *et al.* (2010) investigated bTB across 133 mammals representing 28 wildlife species from five regions in Ethiopia, utilizing rapid serology tests (RT) and lymph node cultures. Their study found that 23% of the animals tested positive for bTB antibodies in the rapid serology tests, and 32.5% of cultured specimens exhibited acid-fast bacilli (AFB); however, no *M. bovis* or other members of the MTBC were isolated. The high bTB antibody test positive rate suggests the potential for bTB presence in wildlife in Ethiopia. However, several limitations of the study may have contributed to the inability to detect MTBC, including: (1) small sample sizes for each species, (2) lack of RT validation for most African wildlife (except for elephants), (3) the test's low sensitivity, which could result in missed early infections, and (4) incomplete culturing of tissues from seropositive animals. These gaps highlight the need for more sensitive and specific diagnostic methodologies in future studies on wildlife tuberculosis.

1.1.3 Zoonotic tuberculosis (zTB)

The World Health Organization (WHO) defines zoonotic tuberculosis as an infectious disease in humans caused by *M. bovis* that originates from animals (WHO, 2019). However, five members of the MTBC species, including *M. bovis, M. caprae, M. microti, M. orygis,* and *M. pinnipedii,* that cause tuberculosis in animals has already been reported as zoonoses, transmissible to humans (Fellag *et al.*, 2020). Farmers, abattoir workers, veterinarians, and butchers are at higher risk due to their regular contact with sick livestock or wildlife (WOAH, 2024).

The global level median proportion of zoonotic TB cases caused by *M. bovis* was ≤ 1.4%, whereas Africa bears the highest burden with a reported median proportion of 2.8% zoonotic TB (Müller *et al.*, 2013). Zoonotic TB due to *M. bovis* caused 147,000 new cases and killed 12,500 people worldwide in 2016 (WHO, FAO, OIE, & The Union, 2017). The most affected

region was Africa, followed by Southeast Asia. In 2016, the European Union (EU) documented 182 confirmed human cases of *M. bovis* and 11 confirmed human cases of *M. caprae*. By 2020, these figures had decreased to 86 confirmed human cases of *M. bovis* and 2 confirmed human cases of *M. caprae*. Notably, the majority of cases reported in 2020 (55.7%) were of EU origin (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023). These figures are likely to be underestimates due to the inability to distinguish *M. tuberculosis* from *M. bovis* (WHO, FAO, OIE, & The Union, 2017) and the lack of reporting of causative *Mycobacterium species*. In North America and Europe, *M. bovis* causes 0.5 to 1.0% of human infections while in South America, where bTB is common, but milk is routinely pasteurized or boiled, human infection ranges from 1 to 6%. In regions like Africa, where bTB prevalence is high and raw milk is commonly consumed, human infection rises, ranging from 10% to 15% (Ashford *et al.*, 2001; WOAH, 2022). *M. tuberculosis* primarily affects the lungs, though it can also cause extra-pulmonary TB in about one-third of cases, whereas zTB due to *M. bovis* may also involve the lungs but more often affects extra-pulmonary sites such as lymph nodes and other organs (WHO, FAO, OIE, & The Union, 2017). However, the role of zTB in the extra-pulmonary human TB cases is not well studied. In Ethiopia, human infections with *M. bovis* have been reported in previous studies (Firdessa *et al.*, 2013; Nuru *et al.*, 2017), whereas *M. tuberculosis* has been identified in a variety of livestock species, including cattle (Ameni *et al.*, 2011), pigs (Arega *et al.*, 2013), camels (Gumi *et al.*, 2012), sheep, and goats (Mamo *et al*., 2012; Deresa *et al*., 2013), indicating transmission between animals and humans, highlighting the need for control of the disease at its animal source to prevent transmission to humans.

1.2 Justification

The impact of bTB on human health, cattle production, and wildlife conservation requires practical and affordable alternative control strategies (Zinsstag *et al.* 2006; Malone and Gordon 2017). The high endemic prevalence of bTB in crossbred cattle used for milk production is posing a substantial threat to food security and the livelihood of the dairy farmers in Ethiopia. BCG vaccination was also granted regulatory approval for use in selected wildlife species, such as badgers (WOAH, 2022), reflecting its practical application for bTB control in wildlife reservoirs. Although no vaccine has been approved for cattle, there is an increasing interest to employ BCG as a viable alternative or complementary control measure in resource-limited settings like Ethiopia.

In many developed countries, bTB has been effectively controlled through test-and-slaughter programs, which have enabled the elimination or reduction of the prevalence to very low levels. The test-and-slaughter strategy involves tuberculin testing and removal of infected animals, accompanied by monetary compensation to affected farmers and movement restrictions of affected herds. However, this strategy is expensive and unacceptable in many developing countries for economic, cultural, and religious reasons (Srinivasan *et al.* 2021). As a result, it has never been implemented in the majority of low- and middle-income countries (LMICs), where the disease is endemic with high prevalence (WOAH, 2024). Moreover, transmission to and from wildlife, the persistence of *M. bovis* in the environment, imperfect diagnostic tests, residual infection within the herd (undetected infected cattle remaining in a herd), and the movement of infected cattle all contribute to the failure of the test-and-slaughter strategy for controlling bTB in some countries such as the UK and Ireland (Chang *et al.*, 2024).

Previous vaccine efficacy studies measured direct efficacy of BCG based on confirmation of lesion development and positive mycobacterial culture at the endpoint. Field evaluation have shown highly variable direct efficacy ranging from 22 to 86% (Nugent *et al.*, 2018; Abalos *et al.*, 2022). A single, low-dose BCG vaccination (3×10^5 CFU) administered via subcutaneous injection has been shown to protect free-ranging cattle against infection with virulent strains of *M. bovis* and development of detectable bTB lesions with an efficacy of 85.7% (Nugent *et al.*, 2018). Despite similar ratios of susceptible calves to reactor seeders were maintained in two natural transmission experiments, a lower 30% direct effect was observed in the second study compared to the 56% in the first study (Ameni *et al.*, 2010b; Ameni *et al.*, 2018). The differences are attributed to severe bTB pathology in the infected seeders used in the second study. A metanalysis showed a direct efficacy of 18% in experimental and 61% in natural transmission studies, with an overall mean efficacy of 25% (Srinivasan *et al.* 2021). Higher infection doses of 10^3 to 10^4 CFU directly delivered through trachea in the experimental studies compared to a natural force of *M. bovis* infection, may have contributed to these differences, suggesting the effect of the force of infection on direct BCG efficacy estimates.

However, BCG can potentially reduce transmission within host populations. Studies have demonstrated that BCG vaccination confers partial protection through reduced frequency and severity of pathology (Ameni *et al.*, 2018; Bayissa *et al.*, 2021). This partial protection is expected to minimize bTB transmission within vaccinated herds, but not yet measured. Given

the primary objective of any vaccination strategy is not only preventing the risk of infection, but also to achieve herd immunity and minimize disease transmission, understanding the overall effect of the vaccine is crucial. In closed herds with high vaccination coverage, the total vaccine efficacy of BCG (VE_T) includes an indirect effect (VE_I) that represents the rate at which infected vaccinates transmit the disease. Thus, the overall efficacy depends on whether it is effective in reducing transmission from vaccinated to susceptible animals in a herd (Halloran *et al.*, 1991). Combined with the capability to maintain closed herds and high vaccination coverage, indirect effects can reduce bTB transmission. Hence, there is a need to estimate the impact of BCG on transmission rates within herds to inform decisions about the practical deployment of the BCG vaccine. The role of partially protective or leaky vaccines in the control of viral infections has been explored previously (De Jong and Kimman, 1994; Orsel *et al.*, 2007). The effectiveness of partially protective Salmonella vaccines in reducing infection prevalence, infectiousness, and outbreak size has also been evaluated using a stochastic transmission model based on longitudinal Salmonella Cerro outbreak field data in adult dairy cattle (Lu et al., 2014). However, appropriate experimental methods to measure the impact of vaccination on disease transmission in chronic bacterial infections, such as bTB, are lacking. As a result, the critical benefit of BCG vaccine-induced reduction in transmission has never been quantified, and the true impact of the BCG vaccine in lowering the infectiousness of vaccinated animals remains unknown. In this study, we employed a novel experimental design to address this key knowledge gap (Conlan *et al.*, 2018).

Currently, the only licensed vaccine for humans tuberculosis is the BCG (Thom *et al.*, 2012), which has also shown promising protective efficacy against *M. bovis* infection in cattle in experimental and natural transmission studies. Currently, no other vaccine against tuberculosis has consistently demonstrated better efficacy and safety compared to BCG. However, improved immunogenicity has been achieved through heterologous prime-boost strategies by combining BCG prime vaccination with booster viral vector vaccines such as Modified Vaccinia Ankara (MVA), a non-replicating poxvirus expressing antigen 85A (Ag85A) or adenovirus type 5 (Ad5), which also express Ag85A (Ad85A) (Vordermeier *et al.*, 2009; Rizzi *et al.*, 2012). This highlights their potential to boost primary immunity induced by BCG against bTB. MVA85A and Ad85A are recombinant viral vector vaccines designed to boost immunity following BCG vaccination. However, the production of MVA85A and Ad85A requires advanced biotechnological processes increasing their costs compared to the relatively

inexpensive and widely available BCG vaccine, limiting their potential accessibility in low-resource settings (Rizzi *et al.*, 2012).

The attenuated BCG strain is safe across a broad spectrum of animal species that are susceptible to *M. bovis*. Even high bacillary doses and repeated administration of BCG are safe in calves, pregnant heifers, guinea pigs, rabbits, and non-human primates (Irvine *et al.*, 2021; Williams *et al.*, 2024). Research has indicated that the Danish 1331 strain of BCG is well tolerated by calves and lactating cows, eliciting mild and temporary local or systemic responses. Additionally, no evidence of BCG shedding in the raw milk, saliva, or excrement of vaccinated animals has been reported and no adverse effects on the milk yield of lactating cows were observed (Williams *et al.*, 2022), suggesting the absence of public health risk and negative impact on animal productivity from use of BCG in the livestock.

The primary barriers to using the BCG vaccine in cattle are incomplete direct protection and interference with tuberculin-based diagnostic tests used in the official bTB surveillance, international trade, and bTB control programs (WOAH, 2022). Non-specific tuberculin reactions to BCG can hinder international cattle trading with countries that demand a strict bTB-free status. Tuberculin tests are also compromised by exposure to environmental mycobacteria, which reduces their specificity and accuracy. This implies that the deployment of BCG for cattle vaccination requires a compatible diagnostic test that distinguishes vaccinated from infected animals (DIVA).

Interest in BCG vaccination for bTB control in cattle has increased due to the development of diagnostic tests with DIVA capability (Srinivasan *et al.*, 2020). For specific bTB diagnosis, ESAT-6, CFP-10, and Rv3615c have been used ether as a recombinant fusion proteins or as a combination of short and long peptides (20-to 40-mer) spanning the whole sequences of ESAT-6, CFP-10, and Rv3615c with a 10 to 20-residue overlap (Figure 1.1). Defined antigen peptide cocktails, are used in both skin and IGRA DIVA tests. The defined antigen cocktails are chemically synthesized peptides through an automated, software-controlled process that sequentially adds and anchors amino acids to a growing peptide chain on an insoluble resin, enabling accurate synthesis. However, whole ESAT-6, CFP-10, and Rv3615c proteins are biologically produced expressed in *Escherichia coli*, as histidine-tagged fusion recombinant proteins and purified by nickel affinity chromatography. The defined peptide antigens offer multiple advantages over whole recombinant proteins and tuberculin, including a defined

composition, simpler manufacturing without biosafety level 3 (BSL-3) requirements, and easier quality control using chemical methods instead of a bioassay. Unlike defined peptide cocktails, whole recombinant proteins can vary in folding, stability, and purity between batches, introducing unwanted variability into the diagnostic test. A complete set of overlapping peptides ensures that every possible epitope from the antigen is present across diverse bovine leukocyte antigen (BoLA) than the whole proteins, improving sensitivity. The defined peptide cocktails have high potential for use in countries where genetically modified or recombinant proteins with synthetic tags are either not permitted or face a high threshold for approval (Srinivasan *et al.*, 2019).

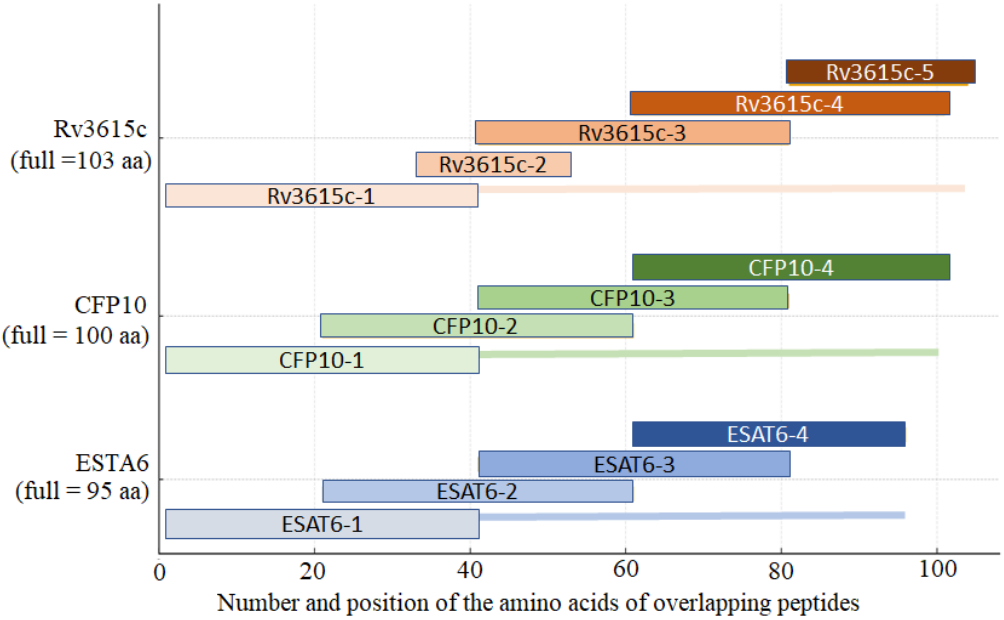


Figure 1.1: Schematic representation defined antigen peptide cocktails containing a combination of 13 immune-dominant overlapping long and short peptides covering the whole amino acid sequences of ESAT-6 (4 peptides), CFP-10 (4 peptides), and Rv3615c (5 peptides) (based on Srinivasan et al., 2019, Table S1).

All pathogenic mycobacteria secrete the ESAT-6 and CFP-10 encoded by the RD1 region, which is deleted from all BCG strains, presenting an opportunity to deploy along with BCG vaccination. The secretion of Rv3615c is dependent Esx-1, a secretion system located in the RD1 (Srinivasan *et al.*, 2019, 2020; Bayissa *et al.*, 2022; Subramanian *et al.*, 2022). These specific antigens demonstrated remarkable sensitivity and specificity in laboratory and field settings (Srinivasan *et al.*, 2019; Bayissa *et al.*, 2022). Their sensitivity of 78% is comparable or slightly better than CCT (Nuñez-Garcia *et al.*, 2017). The inclusion of combinations of immune-dominant long and short peptides has been shown to enhance antigenicity, offering

the opportunity to further optimize and refine the cocktail composition to elicit broad and promiscuous immune responses. A study that compared whole ESAT-6/CFP-10 proteins to overlapping peptide pools showed that mixtures of synthetic overlapping peptides elicited significantly stronger IFN-γ responses and superior performance to the recombinant fusion protein in IGRA and equivalent in inducing skin responses in infected animals while maintaining high specificity in naïve or BCG-vaccinated animals (Srinivasan *et al.*, 2019). Despite these encouraging findings, some issues remain. For example, except for a few studies conducted in the UK, most research evaluated the diagnostic performance of these antigens using tuberculin positive reactor status as a reference standard (Srinivasan *et al.*, 2020; Bayissa *et al.*, 2022).

Most non-tuberculous mycobacteria (NTM) do not express either ESAT-6 or CFP-10 homologues, providing an opportunity to improve test specificity in animals exposed to environmental mycobacteria that do not express these antigens. However, expression of ESAT-6 and/or CFP-10 homologues has been identified in NTM, including *M. kansasii, M. szulgai, M. marinum, M. riyadhense, M. persicum, M. gordonae,* and *M. lymphaticum* (Van Ingen et al., 2009; Scherrer et al., 2019), potentially raising non-specificity issues in bTB diagnosis that relies on the detection of immune reactivity to these defined antigens as well as tuberculins. Cross-reactivity has been observed between *M. kansasii*-expressed ESAT-6 and CFP-10 homologues and their *M. bovis* counterparts, leading to false-positive test results (Vordermeier et al., 2007). Thus, whenever possible, it is essential to use additional peptide antigens specific to MTBC other than ESAT-6 and CFP-10 such as Rv3615c followed by molecular confirmation from cultures of post-mortem or biopsy samples.

Tuberculin reactions may include false negatives in early stages of infection or anergy-related cases that compromises the accuracy of the findings. This underlines the importance of reassessing using confirmed infection status by *M. bovis* culture and PCR positive reference standard to provide better recommendations for use along with BCG vaccine for bTB control. Although BCG vaccine interferes with the tuberculin test, it is worth noting that regions affected the most by bTB lack national control programs and this test is not routinely used. In such cases, immunization remains beneficial. The effectiveness of vaccination programs can be monitored through DIVA testing complemented with slaughterhouse detection of lesions and BCG's interference with the tuberculin can be less relevant for LMICs where routine test-and-slaughter strategy is not employed.

1.3 Literature review

1.3.1 Distribution and epidemiology of bovine tuberculosis

1.3.1.1 Global distribution and epidemiology

Tuberculosis (TB) is globally distributed disease reported across six continents (Reis *et al.*, 2021b, 2021a). The prevalence varies substantially between countries and regions, depending on the presence or absence of control measures, animal movements, and wildlife reservoirs (Allen *et al.*, 2018). Bovine tuberculosis was endemic worldwide in the early 20th century, with herd prevalence rates reaching 63% and animal prevalence rates ranging from 20% to 45% (Constable *et al.*, 2017). However, the disease has been eliminated or nearly eliminated from domesticated animals in many developed nations with operational national bTB control using test-and-slaughter strategy. Many countries have achieved officially tuberculosis-free (OTF) status, which is defined as maintaining the percentage of bTB-infected herds below 0.1% per year for six consecutive years. Seventeen European members states including Austria, Belgium, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Latvia, Lithuania, Luxembourg, the Netherlands, Poland, Slovenia, Slovakia, Sweden and non-members states including Iceland, Liechtenstein, Norway, and Switzerland are OTF (Allen *et al.*, 2018; European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023). Other non-European nations classified as bovine tuberculosis-free include Australia, Barbados, Canada, Israel, Jamaica, Singapore, and USA (Ali, 2013). Bovine tuberculosis has been eradicated from most states of the USA, but some pockets of infection remain in wildlife, particularly in Michigan, where white-tailed deer serve as a free-ranging wildlife maintenance host (O'Brien *et al.*, 2023). Ten European member states (Bulgaria, Croatia, Cyprus, Greece, Ireland, Italy, Malta, Portugal, Romania and Spain) are non-OTF. Three of these non-OTF European member states (Italy, Portugal and Spain) had OTF regions or provinces. The prevalence of bTB is approximately 80 times higher in non-OTF states compared to OTF states within the European Union. An overall bTB-positive herd prevalence of 1.01% has been reported in eight non-OTF member states (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023).

Despite the insufficient surveillance of bTB in numerous regions, current evidence suggests that the disease is prevalent and uncontrolled in Africa, Asia, Latin America, and most countries in the Middle East (Ali, 2013; Kasir *et al.*, 2023). A review conducted by Reis *et al.* (2021a)

analyzed 443 articles on livestock tuberculosis published between 1981 and 2020 across six continents. The review identified 283 articles from Europe, 157 from Africa, 59 from South America, 55 from North America, 29 from Asia, and 21 from Oceania, highlighting the global significance of the disease in livestock. The review identified seven species of the MTBC infecting livestock, with a prevalence of 76.8% for *M. bovis*, 9.1% for *M. caprae*, 8.9% for *M. tuberculosis*, 2.5% for *M. pinnipedii*, 1.4% for *M. africanum*, 0.9% for *M. microti*, and 0.5% for *M. orygis*. Livestock tuberculosis was predominantly reported in farmed animals (90.4%), with lower prevalence observed in zoo animals (6.7%) and pets (2.9%). Among farmed species, Bovidae were most affected (82.1%), followed by Suidae (6.7%), Cervidae (6.2%), Camelidae (3.5%), and others (1.5%). Cattle accounted for 82.3% of cases in the Bovidae family, while small ruminants (goats, sheep, mouflon) represented 12.3%, buffalo 3.6%, and bison 1.8%, highlighting on the diversity of the livestock species infected with multispecies members of MTBC.

A meta-analysis encompassing 60 cross-sectional studies that evaluated prevalence using bovine and avian tuberculin alone estimated the global prevalence of bTB to be 3.3% at the animal level and 18.1% at the herd level (Neto *et al.*, 2024). This figure may likely be an underestimate. Notably, the overall pooled prevalence estimate for South America was the lowest at 0.36% as 15 of the 16 included studies were conducted in Brazil, where national animal tuberculosis control has been under implementation for two decades. Furthermore, the review solely considered tuberculin test-based reports, whereas South American countries conducted a higher proportion of nucleic acid-based studies (34.6%) compared to tuberculin-based studies (17.3%) (Reis *et al.*, 2021a). This exclusion of a significant number of studies and the low sensitivity of the CCT may have contributed to the lower global prevalence estimate in their study. Despite this, review revealed that both animal- and herd-level prevalences were highest in Asia, with estimates of 8.2% and 48.3%, respectively, followed by Africa, with estimates of 6.9% and 37.0%, respectively. From 1996 to 2011, 38 out of 54 African countries reported confirmed infections or clinical cases of bTB in livestock, demonstrating the widespread presence of disease across the continent (De Garine-Wichatitsky *et al.*, 2013). A recent meta-analysis indicated a pooled prevalence of 7.3% within the Indian cattle population, suggesting that approximately 21.8 million cattle may be affected (Srinivasan *et al.*, 2018). Between 2015 and 2020, 7.8% of individual animals and 61.7% of dairy farms in Fiji tested positive for bTB (Garcia *et al.*, 2022).

Despite being more prevalent in LMICs, the United Kingdom, and Ireland also experience considerable economic pressures from the bTB which continues to persist despite intensive and costly control programs (Allen *et al.*, 2018). In the UK, the reported herd prevalence of bTB was 14.4% in 2010 and 13.8% in 2011. In Ireland, the reported herd prevalence was 5.27% in 2009 and 4.37% in 2011 (European Commission, 2013). The annual prevalence of bTB-positive herds in the United Kingdom (Wales, England, and Northern Ireland) was consistently over 10% from 2010 to 2019. In 2019 and 2020, the UK reported *M. bovis* positive herd prevalence of 10.51% and 10.7% for Northern Ireland, 11.0% and 10.5% for Wales, and 11.7% and 10.72% for England, respectively, indicating the persistence of the disease despite the implementation of costly test-and slaughter control strategy for decades (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023).

Although the exact figures are not known, it is estimated that bTB infects 50 million cattle globally, resulting in $3 billion in annual economic losses due to decreased productivity, higher mortality, lower rates of reproduction, and trade restrictions (Schiller *et al.*, 2010). Bovine tuberculosis is estimated to cause a 5% loss in fertility, a 5% loss in carcass weight, and a 10% reduction in milk production (Meisinger, 1970).

Wildlife tuberculosis (TB) has been reported in Europe, Africa, North America, and Oceania. Most wildlife TB studies were conducted in Europe whereas less studied in Asia and South America. An analysis of a recent review identifies specific wildlife targets for bTB research across various continents. European studies primarily focus on badgers (35.7%), wild boar (37.5%), and red deer (26.2%), while 76.5% of African research is predominantly centered on buffalo, the majority of which are from South Africa, particularly from the Kruger National Park. In North America, 90.9% of wildlife TB research concentrate on white-tailed deer, whereas 66.7% of studies in Oceania focus on brushtail possums (Reis *et al.*, 2021b)**.** These trends highlight the different ecological and epidemiological elements of each region. The pooled prevalence was 21.98% in wild boar, 13.71% in red deer and 11.75% in European badger (Reis *et al.*, 2021b). The prevalence in wild boar was 31.68% in Spain, 23.84% in Italy, 18.12% in Hungary, 13.03% in Portugal, and 9.49% in France. A higher prevalence of wildlife tuberculosis has been observed in the southern regions of European countries, where both wild boar density and the incidence of tuberculosis in livestock are elevated, suggesting the importance of tackling the disease in both cattle and wildlife reservoirs. High prevalence of wildlife TB presents significant challenges for Southern European nations, impeding their

efforts to achieve OTF status. The estimated prevalence is 2.54% in wild boars in Asia, derived from only two studies both from South Korea. The prevalence of TB in red deer also varied across European countries, with higher prevalence rates of 31.6% in Austria and 27.8% in Portugal, moderate prevalence rates of 6.4% in France and 12.1% in Spain, and significantly lower prevalence rates of 0.2% in Switzerland, 0.2% in Italy, and 1.0% in the UK. The pooled prevalence of TB in red deer is estimated at 19.26% for New Zealand. The prevalence showed a decreasing temporal trend across study periods for European badgers and wild boar. Accordingly, pooled prevalence rates of 19.1% and 51.7% were observed during the 1991–2000 study period, 13.1% and 26.2% during 2001–2010, and 11.3% and 19.50% during 2011–2020 for European badgers and wild boar, respectively. A similar temporal decreasing trend was observed for bTB in cattle. In the Sicilian provinces of southern Italy, a higher prevalence of 6.74% was recorded during the 2010–2012 study period compared to 3.36% during 2017–2019 (Abbate *et al.*, 2020). The temporal decline in animal TB prevalence is likely attributable to enhanced control measures and/or the inclusion of lower prevalence areas adjacent to high burden TB zones in latter studies (Reis *et al.*, 2021b).

1.3.1.1 Distribution and epidemiology in Ethiopia

In Ethiopia where there is no national bTB control program, the disease is endemic. The pooled animal level prevalence was estimated at 5.8%, with the highest prevalence of 21.6% found in Holstein-Friesians, 9.9% in crosses and 4.1% in zebu cattle (Sibhat *et al.*, 2017). The animal level prevalence in the intensive dairy farms in central Ethiopia ranges from 25% to 39%, whereas herd prevalence surpasses 50% (Firdessa *et al.* 2012; Almaw *et al.* 2021; Tulu *et al.* 2021), posing substantial public health risk. However, the actual contribution of such a high prevalence of bTB to human TB cases is not well characterized due to the absence of routine surveillance and the possible misdiagnosis of the extra-pulmonary form of zoonotic TB caused by *M. bovis*. The prevalence is notably highest in the central region of the country, exhibiting a decreasing trend as one moves toward the peripheries in all geographic directions (Sibhat *et al.*, 2017). Current efforts in the genetic improvement and intensification of cattle farming aimed at increasing milk productivity are expected to further increase the risk of bTB transmission. In Ethiopia, bTB is estimated to cost the national improved dairy production system 132 million USD, or 219 USD per cow (Tschopp *et al.*, 2022). In addition to cattle, bTB also affects other livestock species including goats, sheep and camels. A recent systematic

review and meta-analysis reported an estimated pooled prevalence of 2.3% in goats, 0.8% in sheep and 8.2% in dromedary camels (Asmare *et al.*, 2020).

1.3.2 Immune response to mycobacterial infections

M. tuberculosis and *M. bovis* are closely related pathogens associated with chronic, granulomatous diseases in humans and animals, respectively (Mihret, 2012; Parlane and Buddle, 2015). This implies the immune response to *M. tuberculosis* can inform the immune response to *M. bovis* and vice versa. A variety of germline-encoded pattern recognition receptors (PRRs) expressed on antigen-presenting cells (APCs) recognize external and internal pathogen-associated molecular patterns (PAMPs), as well as endogenous molecules generated by damaged cells known as damage-associated molecular patterns (DAMPs). This detection triggers the release of proinflammatory cytokines, which facilitate the recruitment of immune cells responsible for eliminating the pathogens (Dorfleutner *et al.*, 2015; Gaudino and Kumar, 2019; Vlasova and Saif, 2021). A variety of PRRs that are expressed on APCs to recognize mycobacterial PAMPs include Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), C-type lectin receptors (CTLRs), scavenger receptors (SRs), cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING), CD14 receptors, AIM2, and AhR (Parlane and Buddle, 2015; Stamm *et al.*, 2015; Ravesloot-Chávez *et al.*, 2021; Li *et al.*, 2023). TLRs, scavenger receptors (SRs), and C-type lectin receptors recognize extracellular or vacuolar PAMPs and DAMPs, whereas RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and AIM2-like receptors (ALRs) recognize cytoplasmic PAMPs and DAMPs (Stamm *et al.*, 2015).

Mycobacterial antigens are presented by major histocompatibility complex (MHC) class I and class II molecules. All nucleated cells express MHC I while, only APCs express MHC II molecules. The main types of APCs are dendritic cells (DCs), macrophages and B lymphocytes (Bretou *et al.*, 2016). APCs bind to the appropriate PAMP or DAMP and then internalize it by phagocytosis, pinocytosis, or clathrin-mediated endocytosis. MHC class II molecules present peptide-derived antigens that are taken up by APCs via endocytosis. The process by which APCs display antigens bound to MHC molecules to T lymphocytes, resulting in their activation, is known as antigen presentation. The presentation of antigens to naive T cells in lymphoid tissues, leading to their activation, is known as T-cell priming (Gaudino and Kumar, 2019).

Contact between T cells and dendritic cells (DCs) initiates a T cell response, but one or more co-stimulatory signals from APC surface molecules and cytokines are necessary to prevent anergy and to enhance proper production and survival of effector and memory T cells (Schorer *et al.,* 2019). Full activation of naive CD4+ T cells and the lineage they will differentiate to requires three signals (Gaudino and Kumar, 2019). The binding of TCR to the antigen-MHC complex provides signal 1. Signal 2 comes from co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on APCs binding to CD28 on T cells. In the absence of signal 2, lymphocytes become anergic and fail to respond efficiently (Sharpe and Freeman, 2002). Signal 3 involves DC-derived cytokines such as IL-12 (Sharpe and Freeman, 2002; Ogawa and Abe, 2019). T lymphocytes are stimulated by various cytokines produced by dendritic cells and macrophages, including IL-2, IL-12, IL-18, and IL-23 (Lam *et al.*, 2017). Inflammatory cytokines act as a 'switch' that determines whether antigen and costimulatory signals lead to tolerance in the absence of inflammatory cytokines or a productive immune response leading to strong effector functions, survival, and memory formation in their presence (Curtsinger and Mescher, 2010). Though, induction of inflammation is a crucial component of an effective host response to infection, it must be tightly regulated to prevent severe tissue damage (Storek and Monack, 2015).

To prevent excessive T-cell activation and the subsequent immunopathology or autoimmunity, immune checkpoints (ICs), act as co-inhibitors functioning as brakes (Ahmed *et al.,* 2022). While B7-1 and B7-2 promote T cell activation via CD28, they can also bind CTLA-4 to suppress excessive T cell proliferation (Sharpe and Freeman, 2002; Ogawa and Abe, 2019), indicating a delicate balance in the immunological response involving CD28-mediated T-cell activation and CTLA-4-mediated inhibition. The engagement of CTLA-4 inhibits TCR and CD28-mediated signal transduction, suppresses IL-2 synthesis, blocks cell cycle progression and terminates T-cell responses (Sharpe & Freeman, 2002). Apart from CTLA-4, other immunological checkpoints (ICs), compounds that act as co-inhibitors of T-cell activation include lymphocyte activation gene 3 (LAG-3), programmed cell death 1 (PD-1, CD279), programmed cell death ligand 1/2 (PD-L1/2), and T-cell immunoglobulin 3 (TIM-3). These host pathogen interactions might result in latent infection, tuberculosis, or pathogen clearance (De Martino *et al.,* 2019) depending on the balance between proinflammatory and immunoregulatory cytokine and chemokine production (Averbakh and Ergeshow, 2018).

Once a cell-mediated immune (CMI) response is established, immune cells and cytokines form a granuloma around infected macrophages, the granuloma localizes the infection and prevent its spread. However, persistent immune activation and high frequencies of regulatory T cells (Tregs) predispose individuals to clinical tuberculosis. Hyperinflammation at the late stage of the disease causes cavitation of the granuloma and favors bacterial transmission (Chai *et al.*, 2020). Tregs suppress other T cells by producing inhibitory cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) (Romero-Adrian & Ndez, 2015).

1.3.3 Immune evasion and intracellular survival strategy of *M. bovis*

A Th1-biased response is required to eliminate intracellular *M. bovis* infections. This response is characterized by inflammatory cell infiltrates, enhanced phagocytosis, the release of IL-2, IFN-γ, and TNF-α, as well as the formation of reactive oxygen and nitrogen species, which kill pathogens inside cells (Romero-Adrian and Ndez, 2015). *M. bovis* and *M. tuberculosis* have both evolved diverse evasion strategies, including obstructing phagosome maturation, interfering with phagolysosome fusion, reducing autophagy and antigen presentation, causing phagosome damage to escape into the host cytosol from the hostile phagosome environment, and altering cytokine responses (Mihret, 2012; Parlane and Buddle, 2015; Chai *et al.*, 2020). It is hypothesized that one of the key strategies for *M. bovis* intracellular survival during the early stages of infection is inhibition of apoptosis by inducing BCL-2 (B-cell lymphoma 2) over-expression in order to evade host immune responses. BCL-2 is an anti-apoptotic member the Bcl-2 family proteins that play key role in cell survival by blocking mitochondrial outer membrane permeabilization, inhibiting the release of mitochondrial apoptogenic factors (Asimov, 2015). A study that compared bTB positive plus PCR positive to bTB positive, but PCR negative healthy cattle showed high BCL2 mRNA expression only in bTB positive and PCR-positive cattle (Fang *et al.*, 2020).

1.3.4 Brief historical overview of BCG vaccine

Large human populations have been safely immunized against tuberculosis using one of two live vaccines: BCG or *M. microti*, the vole bacillus (Pym *et al.*, 2002). The RD1 locus has been deleted from both BCG and *M. microti*. *M. microti* was used as a live vaccine until the 1970s but was discontinued because it was just as effective as the Danish BCG vaccine strain and not superior (Brodin *et al.*, 2004). BCG is named after Albert Calmette and Camille Guerin, who developed an attenuated *M. bovis* BCG vaccine strain between 1906 and 1919, founded on the

principle of the *similia similibus curentur* (like cures like) (*et al.* Lange *et al.*, 2022). It was developed through sequential passage of the *M. bovis* strain on cultures of potato slices immersed in ox bile and glycerol. Renewing their cultures every three weeks for four years, they made 230 passages by 1920. In this process, nine gene loci, collectively known as region of difference 1 (RD1), were deleted resulting in the loss of two powerful T cell antigens, ESAT-6 and CFP-10 and the attenuated *M. bovis* BCG strain (Pym *et al.*, 2002). The RD1 is now present in virulent *M. bovis* and *M. tuberculosis* strains, but absent in all BCG strains. The genes encoding for the peptide antigens ESAT-6, CFP-10, and the secretion system for Rv3615c are localized in this deleted region from BCG (Whelan *et al.*, 2010; Casal *et al.*, 2012). The deletion of RD1 from BCG has enabled the development of diagnostic tests based on these specific antigens (ESAT-6, CFP-10 and Rv3615c) to distinguish *M. bovis* infection from BCG vaccination (*et al.* Srinivasan *et al.*, 2020).

The first BCG efficacy evaluation was conducted in 1912 by Albert Calmette and Camille Guérin in newborn calves through intravenous injection. The early studies showed that BCG vaccination protected against *M. bovis* infection for up to 1.5 years. Calmette Guérin, and Benjamin Weill-Hallé gave the first BCG immunization to a neonate on July 18, 1921, providing three 2 mg doses suspended in milk without adverse reactions for 5.5 years. This oral route of BCG administration was discontinued following the 'Lübeck Disaster,' where the oral administration of the BCG vaccine contaminated with *M. tuberculosis* infected 228 out of the 251 vaccine recipient newborns (Fellag *et al.*, 2020). Oral delivery of BCG is still used to control bTB in wildlife reservoir hosts such as European badgers, brushtail possums, wild boars, and deer (Buddle *et al.*, 2018).

1.3.5 BCG induced immunity

The aim of vaccination is to prime the adaptive immune system with attenuated pathogens and induce memory cells capable of quickly and effectively responding to re-exposure to a virulent pathogen. *M. bovis* can be phagocytosed by both dendritic cells (DCs) and macrophages; however, the bacteria can proliferate in both cell types, though macrophages can kill more bacteria than DCs. BCG is more easily killed by both cell types compared to virulent *M. bovis* (Parlane and Buddle, 2015), enabling rapid and vigorous response upon re-exposure to pathogenic *M. bovis* infection.

The interaction of *M. bovis* BCG with APCs such as macrophages and dendritic cells at the inoculation site induces immune responses. Dendritic cells after engulfing BCG mature and upregulate MHC class I and II molecules, co-stimulatory molecules (e.g., CD80, CD86), and cytokines (e.g., IL-12), to effectively prime and initiate T-cell responses (Waters *et al.*, 2015). The dendritic cells present in the peripheral tissues are called immature dendritic cells, whereas those in lymph nodes are called mature dendritic cells or activated dendritic cells. Dendritic cells within the site of infection are active in antigen capture, intake, and processing, but when they move to secondary lymphoid tissue, they lose these properties while gaining the ability to activate naive T cells (Parham, 2015). DCs scan the tissue environment for danger-associated antigens and ingest extracellular components of the pathogen. This encounter initiates the DC maturation process, which results in decreased antigen internalization and increased expression of the chemokine receptor CCR7, directing the DCs to lymphatic vessels and lymph nodes, where they present the antigen to CD8+ and CD4+ T lymphocytes via MHC class I and class II molecules, respectively (Bretou *et al.*, 2016). Dendritic cells that engulf mycobacteria antigens, activate CD8+ T cells by cross-presenting the antigen via MHC class I molecules (Lam *et al.*, 2017). Activated BCG-specific CD8+ T cells develop into cytotoxic T cells that recognize and kill infected cells through the release of cytotoxic proteins such as granzymes, a family of five serine proteases, the proteoglycan serglycin, the membrane-disrupting protein perforin, and granulysin, a detergent-like protein that attaches to membranes (Parham, 2015).

BCG vaccination induces a strong Th1 response, characterized by increased production of IFN-γ. Increased antigen-specific IFN-γ secretion was observed for up to 90 weeks following BCG vaccination (Thom *et al.*, 2012). IFN-γ activates macrophages to enhance the intracellular *M. bovis* killing by increasing the lysosome fusion with phagosomes containing captured pathogens and increasing the synthesis of potent microbicidal agents such as oxygen radicals, nitric oxide (NO), and proteases to destroy the pathogens (Parham, 2015). The IFN-γ also promotes the differentiation of cytotoxic CD8+ T cells. The IFN-γ production induced by BCG is essential indicator effective immunization, but induction of IFN-γ alone is not a sufficient biomarker of protection since not all IFN-γ producing immunizations are protective. vaccines that do not produce any IFN-γ quite frequently fail to protect (Vordermeier, 2014).

BCG generates long-lived memory T cells (central and effector memory T cells), which provide rapid and robust response upon re-exposure to *M. bovis* (Schorer *et al.*, 2019). BCG vaccination of calves indicated higher number of Tcm (central memory T cells) in vaccinated

than in control animals at 12 weeks pos-vaccination and strongly associated with protection (Thom *et al.*, 2012).

1.3.6 Variable efficacy of BCG and influencing factors

Field evaluations have shown high variability in BCG vaccine efficacy. Several factors may influence vaccine efficacy in the field (Buddle, 2010). Explanations given for lower and variable efficacy of BCG include high doses of BCG (10^8 –10^{10} CFU), route of vaccination (SC, oral), breed, age at immunization, general health condition of calves, BCG strain (Tokyo, Danish, Russia, Pasteur), pre-exposure to NTM and consumption of milk from *M. bovis* infected cows before vaccination, helminth infections, test used to measure efficacy (pathology, mycobacterial culture, DIVA), and inadequate long-term protection (Thom *et al.*, 2012; Srinivasan *et al.*, 2021; Abalos *et al.*, 2022). Preexisting immune response against environmental mycobacterial antigens shared with BCG was shown to prevent BCG replication and vaccine take in animal models (Horwitz et al 2010), which has been associated with a lack of protective immunity, possibly due to rapid clearance of BCG resulting in insufficient release of immunogenic antigens (Pollock and Neill, 2002). A recent BCG vaccine efficacy evaluation, based on IFN-γ response to specific antigens ESAT-6, CFP-10 and Rv3615c, indicated 58.5% vaccine efficacy in Holstein Friesian × Swedish Red crossbred cattle. However, no evidence of protection was observed in pure Holstein Friesian in the same study, highlighting the influence of breed on efficacy of BCG vaccine (Contreras *et al.*, 2023). The BCG Danish vaccine at a lower dose equivalent to a 0.5 human dose, was shown to be as effective as a 10-fold-higher dose (Buddle *et al.*, 2013), dictating the use of high dose unnecessary. Neonatal calf vaccination has induced higher protection compared to vaccination at 6 months old (Buddle, 2010). Vaccination of neonatal calves provides significant protection against *M. bovis* challenge at 12 months, but protection decreases after 24 months due to a significant decrease in antigen-specific IFN-γ-secreting cells in long-term PBMC cultures (Thom *et al.*, 2012), highlighting the need for revaccination for long-term protection.

The distribution of the ancestral BCG strain to numerous institutions worldwide and further culturing over several decades resulted in genetic differences among current BCG strains. BCG strains used in vaccine trials includes BCG Pasteur (Hope *et al.*, 2023), BCG Danish (Ameni *et al.*, 2010b), Russian (Bayissa *et al.*, 2021). Although loss of some lipid virulence factors in certain BCG strains reduced protective efficacy in laboratory animal models (Tran *et al.*, 2016),

suggesting a positive correlation between virulence and efficacy, there is lack of definitive evidence on whether these differences contributed to the highly variable protective efficacy of BCG in cattle. Challenge method used such as experimental infection with virulent *M. bovis* through intranasal or endotracheal route (Hope *et al.*, 2005) and subcutaneous vaccination with exposure to reactors for natural transmission (Hope *et al.*, 2005; Ameni *et al.*, 2010; Lopez-Valencia *et al.*, 2010; Ameni *et al.*, 2018), might have contributed to the variability of reported BCG efficacies.

1.3.7 The omics overview of the MTBC: diagnostic biomarkers and immune signatures

High-throughput and integrated omics technologies have provided deeper insights into biology, virulence, and host interactions, allowing thorough analyses of gene sequence variations for generating molecular signature biomarkers (Brosch *et al*., 2002; Costa *et al*., 2014). Omics, defined as the comprehensive study of related sets of biological molecules, primarily focuses on identifying genes and genomic variants (genomics), mRNA expression levels (transcriptomics), proteins (proteomics), and low-molecular-weight metabolites (metabolomics) in specific cell or tissue (Nadeem and Javed, 2022). Biomarker is a broad term defined as any indicator of biological processes, either pathogen based or host related (Smith *et al*., 2021b) and subcategorized in various ways. The most common categorization of biomarkers has been based on their use as: diagnostic, staging, prognostic, prediction, monitoring, and surrogate endpoint (FDA-NIH Biomarker Working Group, 2016). For pragmatic reasons, biomarkers are classified as pathogen or host derived (Goletti *et al*., 2018). Host-derived biomarkers include transcripts, proteins, inflammation markers, and measures of immune cells responding to MTBC infections. Pathogen related biomarkers comprise mycobacterial DNA, peptides, lipids, and volatile molecules (Goletti *et al*., 2018).

Due to the dominance of CMI responses to mycobacterial infection, current bTB diagnostic biomarkers research emphasizes on gene expression and detection of cytokine and chemokine in livestock and wildlife (Smith *et al*., 2021b). The most studied TB biomarkers are host-derived consisting of transcriptomic, proteomic, metabolomics, cellular markers or marker combinations (Goletti *et al*., 2018). Host biomarkers are often used as a diagnostic tool when pathogen identification is not practical (Pedersen *et al*., 2019).

1.3.7.1 Genomics

Members of MTBC exhibit remarkable genetic similarity, exceeding 99% sequence homogeneity at the nucleotide level despite variation in pathogenicity, epidemiology and host preference (Huard *et al*., 2003). Comparative genomic analyses have demonstrated that the members have evolved from a common ancestor via sequential DNA deletions at specific genomic loci (Figure 1.2) (Malone and Gordon, 2017). Presence or absence of genomic regions such as TbD1, RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, and RD13 represent dominant candidate diagnostic markers capable unambiguously identifying MTBC members (Brosch *et al*., 2002).

The MTBC member species are classified into ancestral and modern strains based on the presence or absence of TbD1 deletion. The modern MTBC strains are defined by deletion of TbD1 genomic region and comprises Lineage 2 (Beijing genotype), Lineage 3 (Delhi/Central-Asian), and Lineage 4 (Euro-American), which are responsible for the majority of human tuberculosis cases worldwide (Brites and Gagneux, 2017). The loss of TbD1 is considered a key factor that enabled better adaptation to human-to-human transmission, leading to its global epidemic spread and evolutionary success (Bottai *et al*., 2020). The TbD1 region is present in the genomes of *M. bovis, M. canettii* and ancestral strains of *M. tuberculosis* including Lineage 1 (East African Indian), Lineage 5 (*M. africanum* West African 1), Lineage 6 (*M. africanum* West African 2) and Lineage 7 (East Africa) (Brites and Gagneux, 2017). The ancestral strains are geographically restricted and show a globally lower prevalence compared to modern strains.

The RD1 deletion differentiates *M. bovis* BCG from *M. bovis* and *M. caprae* (Reddington *et al*., 2011b). RD4 is a genotypic marker that is absent in *M. bovis* and *M. bovis* BCG vaccine strains used to differentiate them from other subspecies of MTBC (Taylor *et al*., 2007). RD7 differentiates *M. tuberculosis*, *M. africanum* subtype II, and *M. canetti* from the other MTBC subspecies (Huard *et al*., 2003). RD9 is present in *M. tuberculosis* and in *M. canettii* and commonly used for specific detection of *M. tuberculosis* (Reddington *et al*., 2011a). Like *M. tuberculosis*, *M. canettii* contains all the RDs with the exception of RD12 *M. canettii* ($RD12^{can}$), which has been deleted in *M. canettii* but present in *M. tuberculosis* and targeted for the specific detection of *M. canettii* (Reddington *et al*., 2011a). RD9 is absent in isolates of *M. africanum* type 1 whereas RD7, RD8, RD9 and RD10 are deleted in *M. africanum* type 2 (Parsons *et al*., 2002). The West African *Mycobacterium africanum* lineages can be differentiated from

modern *M. tuberculosis* by at least two variable genetic markers, namely the absence of RD9 and the presence of TbD1 (Brosch *et al*., 2002).

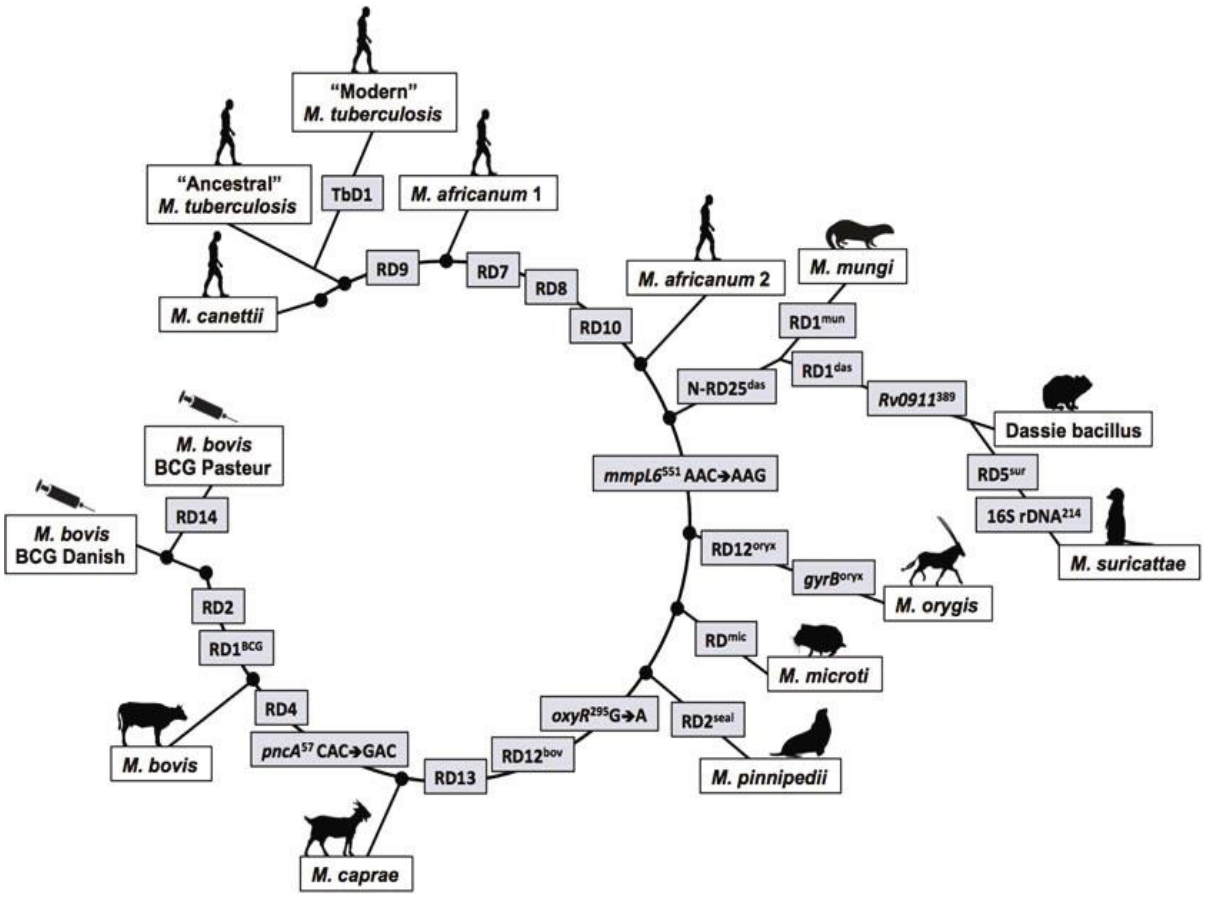


Figure 1.2 Phylogeny of the *Mycobacterium tuberculosis* complex (MTBC) based on genomic deletions and polymorphisms. The evolutionary relationships are defined by sequential regions of deletion (RD) and specific SNPs, tracing the lineage from an *M. canettii*-like ancestor to *M. bovis* BCG Pasteur. These DNA losses serve as unique molecular markers. Animal silhouettes indicate the primary host from which the associated MTBC species was originally or most frequently isolated, and a syringe marks the vaccine strain BCG (Malone and Gordon, 2017).

In addition to RDs, other interspecies genetic differences including single-nucleotide polymorphisms (SNPs) at gyrA, katG, pncA, oxyR', hsp65, and gyrB genes have also been used to identify MTBC members (Huard *et al*., 2003). The pncA codon 57 SNP (pncA57; 2289071G→C) segregates *M. bovis* and *M. bovis* BCG from other MTBC subspecies, whereas the *oxyR'* pseudogene bears a position 285 SNP (oxyR285; 2725801C→T) that differentiates *M. bovis*, *M. bovis* BCG, and *M. africanum* isolates from other MTBC members. The genes such as MPB70 (Rv2875) and Rv0577 as well as insertion sequence IS6110 (formerly IS986) and IS1081 are reported to be highly specific to MTBC (Abe *et al*., 1993; Huard *et al*., 2003). The IS1561' element differentiates *M. microti* from the other MTBC

subspecies (Huard *et al*., 2003). The gene lpqT (Rv1016c), deleted in *M. bovis, M. caprae* and *M. bovis* BCG but present in *M. tuberculosis*, *M. canettii*, *M. africanum* and *M. microti* is used as a diagnostics target for collective identification of *M. bovis*, *M. bovis* BCG and *M. caprae* (Reddington *et al*., 2011b). The wbbl1 gene was used for the detection and differentiation of *M. tuberculosis* and *M. canettii* from the remainder of the complex (Reddington *et al*., 2011a).

1.3.7.2 Transcriptomics

Simultaneous expression studies of cytokines and chemokines gene have opened new possibilities for discovering diagnostic biomarkers capable of differentiating pulmonary, extrapulmonary, and latent forms of tuberculosis (Korma *et al.,* 2020). The availability of a well-annotated bovine genome sequence, together with advances in high-throughput functional genomics technologies such as gene expression microarrays and RNA-sequencing (RNA-seq), has enabled detailed analysis of the host transcriptome and better understanding of the immune response to *M. bovis* infection (McLoughlin *et al*., 2014). Several studies employed gene expression using microarray (Blanco *et al*., 2012), RNAsequencing (McLoughlin *et al*., 2014), RT-PCR (Korma *et al*., 2020) and proteomics (Wanzala *et al*., 2017; Smith *et al*., 2021a) for bTB biomarker discovery. Gene expression analysis of PBMCs from naturally infected animals using microarray has been used to identify a large panel of potential candidate genes, which are then verified by qPCR for detecting disease status (Blanco *et al*., 2012). Whole-genome microarray provides transcriptional profiling for identification of novel transcriptional changes in previously uncharacterized genes and revealing genome-wide expression patterns. RT-PCR is used as a confirmatory step in almost all microarray studies for being more accurate than whole-genome techniques (Ward *et al*., 2010). Genes encoding immune response-related proteins, such as IFNγ, interleukines were significantly upregulated whereas the expression of several matrix metallopeptidases was significantly downregulated in TB infected animals (Blanco *et al*., 2012). RT-PCR analysis has demonstrated high expression of IFN-γ, IP-10, IL2R, and CXCL-9 (MIG) in patients with latent and active tuberculosis, while IL-10 expression was decreased. Besides, the combination of IL-10 and CXCL-9 readouts differentiated patients with pulmonary tuberculosis from those with latent infection whereas IL-10 together with IP-10 and IL-4 differentiated pulmonary tuberculosis from latent cases (Korma *et al*., 2020). Evidence suggests that the systemic immune response in peripheral blood correlates with the local immune response at the primary site of bTB infection (Rhodes *et al*., 2000).

1.3.7.3 Proteomics

Foreign antigens recognition by T lymphocytes are the basis for adaptive immunity (Purcell *et al*., 2019). Immunization with protein antigens presented by MHC molecules generating Th1 response is known effective way for generating memory T-cells and immunity against *M. tuberculosis* infection (Van Rhijn *et al*., 2009). As a result, vaccine development has focused on identifying T-cell specific immunogenic proteins leading to production of macrophage-activating cytokines such as IFN-γ (Purcell *et al*., 2019). Many *M. tuberculosis* antigens have been discovered using genome-wide screening approaches. Culture filtrate proteins (CFP) and *M. tuberculosis* short-term culture filtrate (ST-CF) are among extensively studied antigens as targets for T-cell induced immune responses against *M. tuberculosis.* Several T-cell antigens including antigen 85 complex proteins (Ag85A/Rv3804c, Ag85B/Rv1886c and Ag85C/Rv0129c), 6-kDa secreted antigen (ESAT-6, Rv3875), CFP-10 (Rv3874) and MPT-64 (Rv1980c) were identified from *M. tuberculosis* ST-CF (Chugh *et al*., 2024). Several vaccine candidates under evaluation in clinical trials, such as H4-IC31, H56-IC31, and Crucell Ad35, include antigen 85 complex proteins as candidate antigens. ESAT-6 is a component of several TB vaccine candidates currently evaluated in clinical trials, such as H1-IC31, H56-IC31, and TB/FLU-04L. CFP-10 is being assessed as a component of the AEC-BCO2 vaccine currently under clinical trial (Chugh *et al*., 2024). Globally, 17 vaccine candidates are currently in Phase I, II, or III clinical trials. These vaccines fall into five categories and include viral vector-based, subunit, mRNA, live attenuated, and whole-cell vaccines (Chugh *et al*., 2024).

Several cytokines and chemokines have been identified as biomarkers of *M. bovis* infection and as potential surrogates of vaccine-induced protection in cattle (Vordermeier *et al*., 2009; Waters *et al*., 2016). Cytokines are signaling proteins that modulate the behavior or function of their source cell or neighboring cells. They are commonly categorized into interleukins (ILs), interferons (IFNs), chemokines, and tumor necrosis factors (TNFs). Given their role in stimulating and modulating the immune system, they are often employed as important biomarkers for assessing normal immune function and disease status (Romero-Adrian and Ndez, 2015).

The cytokine IFN-γ is the most commonly used biomarker to quantify *in vitro* CMI responses to MTBC infection in humans, livestock, and wildlife (Wei *et al*., 2015; Bernitz *et al*., 2021; Smith *et al*., 2021a). INF-γ was also evaluated in a range of wild animals and showed its

diagnostic capability in detecting *M. bovis* infected wild animals including white rhinoceros (Chileshe *et al*., 2019b, 2019a), African buffalo (Smith *et al*., 2021a) and warthogs (Roos *et al*., 2019). In addition to IFN-γ, the expression of various cytokines and chemokine were studied and some were suggested to serve as potential diagnostic markers to detect and differentiate between various tuberculosis infection statuses. Cytokines and chemokines involved in the host adaptive CMI such as IFN-γ-inducible protein 10 (IP-10) (Bernitz *et al*., 2019), IL-1β (Elnaggar *et al*., 2017) and IL-22 (Bernitz *et al*., 2019) showed capability to increase sensitivity of bTB detection. The IP-10 has been found significantly increased in bTB positive than in bTB negative animals and shown to be a biomarker of immune activation in African buffaloes and warthogs with bTB (Goosen *et al*., 2015; Roos *et al*., 2018). Measurement of the differential release of IP-10 in response to stimulation with bovine PPD versus avian PPD distinguished reactor cattle from nonreactors with a sensitivity of 100% and a specificity of 97% (Parsons *et al*., 2016).

A multidimensional proteomic analysis of sera from calves experimentally infected with *M bovis* identified an increase in 32 host and 16 pathogen specific peptides (Seth *et al*., 2009; Lamont *et al*., 2014a). Among three pathogen-specific peptide biomarkers discovered in calf serum and evaluated, MB2515c, a transmembrane family protein, and pks5 (polyketide synthase 5) were validated by indirect ELISA and found reliable for detecting tuberculosis infection in cattle and free-ranging deer sera, while the third hypothetical protein, MB1895c, was found less reliable (Wanzala *et al*., 2017).

Vitamin D binding protein (VDBP) distinguished confirmed bTB field samples from uninfected and exposed cattle and Circulating *M. bovis* proteins, specifically polyketide synthetase 5, detected M. bovis-infected cattle with little to no seroreactivity against M. kansasii- and M. avium subsp. paratuberculosis-infected animals (Seth *et al*., 2009). Serum levels of alpha-1-microglobulin/ bikunin precursor (AMBP) protein, alpha-1 acid glycoprotein (AGP), fetuin, and alpha-1B glycoprotein were elevated exclusively in *M. bovis* infected animals (Seth *et al*., 2009).

The levels of D-binding protein and alpha-1 B glycoprotein were reported to increase as *M. bovis* infection progressed (Seth *et al*., 2009). The plasma level of CRP increases during an inflammatory state and is widely used as a biomarker for pulmonary infections. PPD-B-stimulated C - reactive protein (CRP) has a potential to differentiate between different stages of *M. bovis* infection (Gao *et al*., 2019).

High levels of Chitinase-3-like protein 1 (CHI3L1/YKL-40), an extracellular matrix glycoprotein that plays a role in tissue inflammation and remodeling, have been observed in many lung diseases. CHI3L1 has been implicated in the pathogenesis of granulomatous and fibrotic interstitial lung diseases including bTB, explaining the why bTB positive, but PCR-cattle do not shed mycobacteria (Fang *et al*., 2020). Also significantly higher BCL2 was observed in bTB and PCR positive cattle than in bTB positive, but PCR negative healthy cattle (Fang *et al*., 2020), supporting the hypothesis that *M. bovis* uses inhibition of apoptosis as an intracellular survival strategy during infection. These findings suggest that BCL2 and CHI3L1 can be employed to differentiate bacteria shedding *M. bovis*-infected PCR positive cattle from *M. bovis*-infected but PCR negative cattle that are not shedding the bacteria.

Some biomarkers were evaluated in combination to improve bTB detection. Parallel measurement of IFN-γ and IP-10 has demonstrated improved detection of *M. bovis* infection with 100% specificity in cattle and buffaloes (Bernitz *et al*., 2019). Expression of CXCL10 and IL-22 identified bTB positive but TST and IGRA cattle, suggesting their potential valuable role in bTB diagnosis when TST and IGRA tests fail (Klepp *et al*., 2019). Research has demonstrated that using IL-1β as an additional readout can complement IFN-γ, potentially increasing the overall test sensitivity for detecting *M. bovis* infection in cattle. Using IFN-γ, IL-1β, and TNF-α in parallel increased the proportion of *M. bovis*-infected animals detected by any one of these cytokines (Jones *et al*., 2010). However, this parallel application also resulted in a drop in specificity. When TNF-α was omitted, the parallel use of only IFN-γ and IL-1β resulted in a 5% increase in sensitivity to ESAT-6/CFP-10 stimulation without a corresponding loss of specificity (Jones *et al*., 2010). Thus, incorporating an additional readout such as IL-1β can complement IFN-γ and improve overall test sensitivity.

The identification of predictors of protective immunity is also one of the crucial aspect of vaccine efficacy research (Thom *et al*., 2012). Quantifiable features of vaccine induced protection such as immunological surrogates and correlates of protection are considered helpful to select potentially effective vaccine before performing large scale efficacy trials (Lai *et al*., 2023). Immunological surrogates (predictors of vaccine efficacy) are defined as immunological markers detectable after vaccination and capable of predicting the protective efficacy of a vaccine before an exposure to infection. While correlates of protection refer to markers that predict vaccine success after *M. bovis* challenge. Vaccination induced IFN-γ production by T

cells was postulated to serve as a correlate of protection. Such correlates are usually elevated in infected, non-protected animals compared to infected vaccinated ones and may also serve as indicators of severity of pathology or bacterial burden (Vordermeier *et al*., 2016). Thus, IFN-γ responses cannot predict vaccine success, however, remain valuable for screening out vaccines that are unlikely to protect from further evaluation and avoid resources wastage.

Evidence also indicates that IL-17A and IL-22 can serve as both predictor of vaccine efficacy and correlate of protection. A pre-challenge Ag85A and PPD-B induced IL-17 responses and PPD induced IL-22 were elevated in protected, vaccinated cattle, similar to IFN-γ (Vordermeier *et al*., 2009, 2016). The pre-exposure expression of IL-17 mRNA after vaccination with an Ag85B-overexpressing BCG strain was inversely correlated with post-challenge disease severity (Rizzi *et al*., 2012). Furthermore, vaccine recipients showed reduced IL-17A responses 2.5 weeks after *M. bovis* challenge compared to non-vaccinated animals, which correlated with lower disease burdens (Waters *et al*., 2016).

1.3.7.4 Lipidomics

The mycobacterial cell wall is rich in lipids and glycolipids including mycolic acids, trehalose-6,6'-dimycolate (TDM), lipoarabinomannan (LAM), lipomannan (LM), phosphatidylinositol mannosides (PIMs), phenolic glycolipids (PGLs), and lipooligosaccharides (LOSs) (Chancellor *et al*., 2018; Mishra and Kapoor, 2022; Gong *et al*., 2023). PIMs, LM, and LAM are present in all mycobacteria, while glycopeptidolipids (GPLs) are specific to NTM but absent in *M. tuberculosis* (Liu and Larrouy-Maumus, 2022). The variety and diversity of mycobacterial cell wall lipids and glycolipids represents a potential reservoirs of specific biomarkers that can be used for detection of mycobacterial infections. LAM has been among extensively studied for its unique structural and immunomodulatory properties (Correia-Neves *et al*., 2019b).

The LAM present in body fluids during mycobacterial infection has been used as a diagnostic biomarker in a urine-based test (Goletti *et al*., 2018). Its urinary concentration was reported to range from 15 $pg \cdot mL^{-1}$ to several hundred $ng \cdot mL^{-1}$. However, inhibitory factors in patient urine have been implicated in reducing the sensitivity and accuracy of LAM-based urine tests (Correia-Neves *et al*., 2019a). Anti-LAM antibodies are also induced in *M. tuberculosis* infection and following BCG vaccination (Correia-Neves *et al*., 2019b). Commercial ELISA kits, available for detection of serum anti-LAM antibodies, differentiated bTB-positive from

exposed animals, with a reported sensitivity of 100% and specificity of 91.7% (Lamont *et al*., 2014b).

Although mycobacterial infection elicits both protein and lipid antigen specific T cell responses, extensive subunit vaccine studies and antigen selection have primarily focused on secreted proteins (Morgun *et al*., 2023; Chugh *et al*., 2024). The preparations and inclusion of non-protein antigens such as lipids and glycolipids into subunit vaccine strategies have not been well explored (Morgun *et al*., 2023). The major challenges were difficulty of formulating and administering lipid antigens due to their hydrophobic nature and lack of understanding on how they are presented and recognized by the immune system (Morgun *et al*., 2023).

However, more recent studies have described mycobacterial cell-wall lipid presentation to non-classical T cells through the CD1 molecules that are MHC class I like proteins.(Chancellor *et al*., 2018). Humans express group 1 (CD1a, b, and c) and group 2 (CD1d) and group 3 (CD1e) CD1 molecules whereas cattle expresses only CD1a, CD1e and multiple CD1b molecules (Van Rhijn *et al*., 2006). CD1a, CD1b and CD1c expression is restricted to professional APCs (Chancellor *et al*., 2018). Mycobacterial cell wall antigens presented by CD1 molecules are amphipathic, containing hydrophobic and polar regions. The hydrophobic moiety binds within the CD1 pocket, while the polar region interacts directly with the T-cell receptor (TCR) (De Libero *et al*., 2014). CD1-restricted TCRs recognize a broad range of lipids (Chancellor *et al*., 2018). Mycobacterial lipid antigens with CD1a restriction include dideoxymycobactin while CD1b binds lipids of different size ranging from C12 to C80 (De Libero *et al*., 2014). CD1b-restricted immunogenic lipid includes iacylsulfoglycolipid, glucose monomycolate, glycerol monomycolate, mycolic acid, LAM and LM. PIMs are CD1d restricted (De Libero *et al*., 2014). CD1 molecules exhibit limited polymorphism, suggesting that lipid antigens are more likely to be recognized by most individuals (Morgun *et al*., 2023). The development of subunit vaccines that simultaneously stimulate both lipid and peptide antigen-specific T cells was recently described (Morgun *et al*., 2023). A new nanocarrier system termed bicontinuous nanospheres allowed dual incorporation and co-encapsulation of mycolic acid and Ag85B (Morgun *et al*., 2023). However, although scientific and methodological advances have triggered a substantial progress in the investigation of immunogenic lipids for development of improved subunit vaccine strategy, substantial challenges remain. Primarily, most studies were conducted in mouse or guinea pig models and the role of immune responses to lipids in cattle and humans remain unknown. Additionally, the potential autoimmune reactivity to self-

phospholipids that are identical to pathogenic MTBC lipid components are unclear. Furthermore, the protective Th1 responses that do not trigger immune-evasive mechanisms requires a thorough investigation. For example, ManLAM internalized by DC-SIGN PRR is known to inhibit the release of pro-inflammatory cytokines (IL-12, TNF-α, IL-6) and induces IL-10 that blocks macrophage activation (Gong *et al*., 2023), highlighting a need for careful selection of lipid antigen components for vaccine. Thus, the current empirical evidence is insufficient to translate lipid antigens to an effective subunit vaccine strategy warranting further research.

1.3.8 Diagnosis of bovine tuberculosis

Bovine tuberculosis has no pathognomonic clinical symptoms, and only a limited number of diseased animals show clinical signs, making the detection of *M. bovis*-infected animals challenging (Bernitz *et al.*, 2021). The onset of the disease is typically slow and usually manifests late, allowing infected animals to remain in the herd undetected and transmit the disease despite exhibiting no overt symptoms. Although extensive bTB lesions are detectable at necropsy, some *M. bovis*-infected tuberculin reactor cattle may have no observable lesions (Menin *et al.*, 2013). Therefore, detection of infection requires the use of both pre- and post-mortem diagnostic methods, each with unique diagnostic capabilities and inherent limitations.

In cattle, bTB can be detected using direct or indirect diagnostic methods. Indirect (antemortem) tests rely on detecting host immunological responses to infection, including skin testing, IFN-γ release assays (IGRA), enzyme-linked immunosorbent assay (ELISA), and lateral flow antibody testing. Direct methods identify an organism in the host through positive bacterial culture or molecular detection of the bacterial genome.

1.3.8.1 Antemortem diagnostic tests

a) Skin tests

The tuberculin skin test is the OIE-recommended *in vivo* test for bTB diagnosis (WOAH, 2022). The test was first developed by Robert Koch in 1890 using tuberculin, purified protein derivatives (PPDs) from M. avium (avian PPD) and *M. bovis* (bovine PPD) (de la Rua-Domenech *et al.*, 2006). The intradermal tuberculin skin test is performed as a single intradermal tuberculin (SIT) test, or caudal fold tuberculin (CFT) test, and comparative cervical

tuberculin (CCT) test. The SIT test is applied as a single injection of bovine PPD whereas the CCT test requires both bovine and avian PPDs injected at adjacent sites in the mid-cervical region (de la Rua-Domenech *et al.*, 2006). The CFT is applied as a single injection of bovine PPD in the caudal fold or at the base of the tail.

Tuberculin induces a T-cell-mediated delayed-type hypersensitivity (DTH) reaction to MTBC or other mycobacterial infections. In a skin test, the local inflammatory response at the tuberculin injection site is evaluated by measuring the increase in skin thickness 72 hours later. Based on the WOAH's guidelines, ≥ 4 mm in skin thickness differences between the post- and pre-injection is considered positive in the SIT test. In the case of the standard interpretation of the CCT test, a >4 mm difference in skin induration between the bovine and avian PPD injection sites is considered positive, while in the severe interpretation the animal is positive if the skin induration difference is >2 mm. The CCT is the preferred test in regions where exposure to M. avium complex and other NTM species jeopardizes specificity. The reported specificity of the CCT test has been as high as 99.9% (de la Rua-Domenech *et al.*, 2006; Goodchild *et al.*, 2015). The CCT test is used in Ethiopia, the United Kingdom and Ireland, where cattle are frequently exposed to environmental mycobacteria (Ameni *et al.*, 2010a; Waters and Vordermeier, 2018). The CFT is currently used as a primary test for cattle in the New Zealand, South Africa and North America whereas the SIT is used in many European countries (de la Rua-Domenech *et al.*, 2006; Waters and Vordermeier, 2018).

b) Interferon gamma release assay (IGRA)

IGRA is an ex vivo assay that measures the interferon gamma (IFN-γ) released by T cells in response to PPDs, ESAT-6 and CFP-10 antigens in animals exposed to MTBC infection before the test. The IGRA test is an ancillary diagnostic test for bTB in cattle. The test is considered positive when the difference between the optical density (OD) value of the bovine PPD and avian PPD is >0.1 (Jones *et al.*, 2017; Srinivasan *et al.*, 2019). The IGRA is advantageous over the skin test for it does not require repeated handling of animals and long intervals to repeat the test. Its drawbacks include whole blood cells stimulation within 8 hours of collection, high cost, and extensive sample processing time, which makes testing large herds in remote areas difficult. Delay in processing the blood sample for more than eight hours results in a significant decrease in the IFN-γ responses. Delay in processing the blood sample for more than eight hours results in a significant decrease in the IFN-γ responses (de Lisle *et al.*, 2017), which

could lead to missed detection of infected animals. The IGRA test can also lead to false positive tests and cannot be applied in calves younger than six months old due to non-specific production of IFN-γ by NK cells, which adds to its limitation.

c) Serology or antibody tests

Serological methods assess the host's humoral antibody response to specific MTBC antigens when the disease progresses. Enzyme linked immunosorbent assays (ELISA) or lateral flow assays, are the most commonly employed. Since specific antibodies to MTBC infection are anticipated to form later during infection, serological or antibody testing is less sensitive than cell mediated immune response assays at early stage of the disease (WOAH, 2022). Prior injection of the skin test antigen within a specified period improves serological test sensitivity in cattle species. The sensitivity of serological antibody tests in detecting *M. bovis* infected animals before intradermal skin tests ranged from 23.9% to 32.6% in contrast to a significantly higher number of infected animal identification was achieved 15 days after intradermal skin tests, ranging from 66.7% to 85.2% (Casal *et al.*, 2014).

1.3.8.2 Postmortem diagnostic tests

Postmortem diagnosis include detection of bTB lesions, bacterial isolation, molecular detection of bacterial DNA and histopathological examination. The sensitivity of lesion detection at meat inspection for presumptive bTB diagnosis in cattle is approximately 46% (Nuñez-Garcia *et al.*, 2017). The sensitivity of lesions as a diagnostic test for *M. bovis* infected animals using culture as the reference standard was found to be 86% (95%CI: 81–90%), while the specificity stood at 80% (95%CI: 74–85%) (Egbe *et al.*, 2016). Many pathogens, such as *Actynomices bovis* and *Trueperella pyogenes* may induce granulomatous lesions similar to bTB contributing to lack of specificity to lesion detection (Ramos *et al.*, 2015).

a) Bacterial culture and histopathology

The gold standard test for confirmation of bTB is isolation of the causative MTBC species in mycobacterial culture. Although culture remains the gold standard with specificity of 100%, its sensitivity is 68.3% (Roy and Gupta, 2022). A report indicated that 58% of histological sections from 265 tissues with bTB lesions were paucibacillary while 15.7% were negative, explaining the reason for lower sensitivity of mycobacterial culture (Liebana et al., 2008).

Histopathological assessment of granuloma morphology and cellular characteristics has been used to gauge severity of pathology and vaccine efficacy (Palmer et al., 2022). The concurrent use of histopathology and bacteriology was also employed to maximize diagnostic sensitivity (Liebana et al., 2008). Tubercular granuloma displaying central necrosis with or without mineralization surrounded by macrophages, lymphocytes, plasma cells, neutrophils, epithelioid cells, and Langerhan's giant cells, and partly or completely enclosed by a thin capsule were defined as positive cases whereas irregular lesions characterized by non-capsulated clusters of epithelioid macrophages without multinucleated giant Langerhan's cells and absence of necrosis were defined as inconclusive cases. But lesions with inconsistent features of tubercular granuloma, including significant eosinophilic infiltrates and lymphoid hyperplasia were considered negative (Medeiros *et al*., 2012).

b) Molecular diagnosis of MTBC

Although the constituent MTBC species show a high degree of genetic similarity (>99%), there are also significant differences apparent in their genomes (Huard *et al.*, 2003). According to comparative genomic studies, members of the MTBC are evolved from a common ancestor via successive DNA deletions at specified genomic sites (Brosch *et al.*, 2002). The *M. bovis* has a smaller genome, with large regions missing compared to *M. tuberculosis*. Nine RD were deleted from *M. bovis* explaining its reduced genome size. These deleted sections are known as regions of difference (RD) (Gagneux and Small, 2007; Egbe *et al.*, 2016). The insertions or deletions of these RDs and single nucleotide polymorphism (SNP) in the genomes of MTBC members offers a genetic signature that can be used to differentiate between these mycobacteria (Brosch *et al.*, 2002; Costa *et al.*, 2014).

DNA amplification in the diagnosis of tuberculosis was first introduced by Hance *et al.* (1989) to avoid the detrimental delay in conventional culture procedures. PCR can differentiate the MTBC members to the level of species and subspecies (Reddington *et al.*, 2011b). The discrimination between MTBC members provides an accurate diagnosis and epidemiological assessment of mycobacterial disease, crucial for improved bTB control (Costa *et al.*, 2014). The widely applied MTBC genotyping methods such as standard 24-locus MIRU-VNTR typing, spoligotyping and IS6110-RFLP typing are all PCR based techniques (Merker *et al.*, 2017). Although these techniques have been extensively used for multiple applications, they require time-consuming and labor-intensive post-amplification product processing like gel

electrophoresis (Araújo *et al.*, 2014). These limitations stimulated the search for faster and alternative real-time PCR methods with more automation possibilities (Reddington *et al.*, 2011a, 2011b). As a result several studies have developed and tested multiplex qPCR assays targeting the variable genomic regions in MTBC for species-level identification (Reddington *et al.*, 2011a, 2011b; Dykema *et al.*, 2016; Duffy *et al.*, 2021).

c) Diagnostic molecular targets for MTBC identification

1) Gene encoding 16S rRNA

One of the most widely recognized strategies for improving mycobacterial strain identification is the analysis of the 16S rRNA gene. This gene is present in all mycobacteria and has been used to design both pan-mycobacterial and species-specific primer pairs (Huard *et al.*, 2003; Park *et al.*, 2000). The identity of *Mycobacterium species* has been determined using species-specific nucleotide polymorphisms in the 16S rRNA gene (Huard *et al.*, 2003). The internal transcribed spacer (ITS), located between the 16S and 23S rRNA genes, is approximately 270 to 360 base pairs (bp) long. Its size varies between species, making it suitable for differentiating mycobacterial species (Park *et al.*, 2000).

2) Insertion sequences (IS)

i) IS6110

IS6110 is present in all MTBC species genomic sequences and has become an important diagnostic target for identification of MTBC species (Noordhoek *et al.*, 1994; Figueiredo *et al.*, 2010). The host range of this transposable element is limited to species of the *M. tuberculosis* complex group of bacteria (Noordhoek *et al.*, 1994). It is the most commonly used genetic marker for DNA fingerprinting in outbreak investigations and tracking transmission of *M. tuberculosis* (Cousins and Dawson, 1999). It has high discriminatory power for strains that contains ≥6 copies, but low discrimination for strains with ≤5 IS6110 copies (Noordhoek *et al.*, 1994). In *M. bovis* BCG and most *M. bovis* strains, IS6110 element is present in only one or two copies, limiting its value for typing these species (van Soolingen *et al.*, 1994). Thirty-six (80%) of 45 isolates studied in Australia harbored a single copy of IS6110 whereas in New Zealand, 97% of 160 *M. bovis* strains investigated carried a single IS6110 element (Cousins and Dawson, 1999). Previous reports indicated that amplifying species-specific DNA

sequences like IS6110, and hybridizing the amplified sequence with a labeled probe can detect 5 fg of mycobacterial DNA, corresponding to one mycobacterium, in clinical samples (Vitale *et al.*, 1998).

ii) IS1081

It has been demonstrated that IS1081 is a multi-copy insertion element existing in six copies in the MTBC, providing an excellent sensitivity for quick identification of MTBC positive cases (Crawshaw *et al.,* 2014). All MTB complex strains, such as *M. canetti, M. tuberculosis* (H37Rv, H37Ra, CDC1551), all reference and field strains of *M. microti, M. bovis strain* AF2122/97, and BCG Pasteur were detectable using IS1081 (Taylor *et al.,* 2007). The IS1081 is sensitive enough to detect as little as 2.35 fg DNA (0.5 genome copies), whereas the RD4 is 10 times less sensitive, requiring 23.5 fg (5 genome copies) for positive case detection (Taylor *et al.*, 2007).

3) Mammalian cell entry (mce) proteins

The mce (mammalian cell entry) protein belongs to a protein family that plays a crucial role in the entrance and survival of the bacteria in the mammalian cells. Genomic analysis revealed eight mce3 genes only found in human isolates (Jia *et al.*, 2017), but absent from MAP, *M. bovis* BCG, *M. smegmatis, M. microti* and *M. leprae* genes (Hemati *et al.,* 2019). The MTBC genome has four dispersed but homologous sets of mce operons (mce1-mce4) arranged similarly. Each mce operon encodes two integral membrane proteins (yrbEA and yrbEB) and six mce proteins (MceA-F) (Hemati *et al.,* 2019). The mce3 operon is also absent in animal isolates in the two genes (yrbEA and yrbEB) located upstream of the eight mce3 genes encoding integral membrane proteins and the other four genes (Rv1974, Rv1975, Rv1976c, and Rv1977) located downstream of the eight mce3 genes encoding signal transduction (Jia *et al.*, 2017). The presence of mce3 only in the human isolates and the absence from the yrbE3A gene of mce3 operon in the animal isolates are serving as valuable genetic markers to identify and differentiate *M. tuberculosis* from animal-adapted MTBC members: *M. bovis, M. orygis, M. caprae*. The *M. tuberculosis* lineage 6 also lacks the yrbE3A gene.

4) Regions of difference (RD)

Molecular deletion typing had been found to be an important tool to differentiate *M. bovis* from the other strains of the MTBC (Yahyaoui-Azami *et al.*, 2017). Deletion analysis combined with investigation of SNPs has been demonstrated to be effective method for studying the evolution of the tubercle bacilli and subsequent discovery of evolutionary markers. These regions—most notably RD9 and RD4, but also RD1, RD2, RD7, RD8, RD10, RD12, and RD13—represent potent diagnostic tools for the quick and precise identification of *M. tuberculosis* complex members. The genetic deletion of RD9 distinguishes *M. tuberculosis* from the other MTC members. A strain is designated *M. tuberculosis* if RD9 is intact (Costa *et al.*, 2014). Similarly, deletion type of RD4 provides for *M. bovis* identification because *M. bovis* is deleted for RD4 but all other MTBC members are not (Costa *et al.*, 2014; Yahyaoui-Azami *et al.*, 2017). The RD4 deletion is a single target per genome and it is less sensitive than IS1081 (Crawshaw *et al.*, 2014).

5) Single nucleotide polymorphisms (SNPs)

i) SNP at the lepA (Rv2404c) gene

A characteristic C to T substitution at position 690 bp of the lepA gene is specific for *M. caprae* and is used as a diagnostic target for detection of the MTBC members as well as for specific identification of *M. caprae* (Reddington *et al.*, 2011a, 2011b). LepA is an essential bacterial elongation factor present in all currently sequenced bacteria. This specific SNP is conserved and shown to be a stable marker (Domogalla *et al.*, 2013).

ii) SNP at the RskA (Rv0444c) gene

The RskA (Rv0444c) gene has variable sequence in MTBC members due to mutations that result in low/high-level expression of two antigenic proteins MPB70 and MPB83. MTBC members with low-level production of MPB70 and MPB83 have the wild-type gene (H37Rv sequence) whereas members with high-level expression of these antigens have a mutated Rv0444c gene. A G698C mutation in the Rv0444c gene is specific for *M. orygis* (Saïd-Salim *et al.*, 2006). Both *M. caprae* and *M. bovis* have a C to T mutations at 320 and 551 positions.

1.4 Problem statement

Control and eradication of bTB is primarily based on regular skin testing, mandatory slaughter of positive reactors, movement restrictions of reactor herds, and abattoir surveillance that have allowed some developed countries to eradicate or nearly eliminate the disease from farmed animals. However, the test-and-slaughter strategy is not widely implemented in LMICs since it is resource intensive and financially infeasible for developing nations. Moreover, it proved ineffective in areas with wildlife reservoirs of infection. These situations lead to the continued disease transmission among livestock, causing economic losses, threatening food security, and endangering public health, highlighting the urgent need for alternative, feasible control strategies for resource-limited regions. BCG vaccine could provide an alternative bTB control strategy for LMICs. However, its efficacy in reducing bTB transmission in cattle is still poorly understood. Furthermore, the lack of diagnostic tools capable of distinguishing infected from vaccinated animals (DIVA) poses a challenge for widespread adoption of BCG. Thus, validating the utility of such diagnostic tools are critical for supporting vaccination programs.

Although, conventional vaccine efficacy evaluations demonstrated highly variable and limited direct protective effects of BCG, these conventional vaccine efficacy evaluation methods are unable to measure indirect vaccine effect on the reduction of onward transmission by vaccinated animals. This mode of action of a vaccine is critical since the primary effect of BCG is reducing the severity and frequency of lesions and disease progression, rather than conferring sterile immunity. Addressing these knowledge gaps is essential to develop sustainable BCG vaccine based bTB control strategies. Our study addressed this gap by performing a natural transmission experiment in cattle using a novel crossover design. This method enables a more realistic and robust assessment of the true impact of BCG on bTB transmission, by quantifying both the direct efficacy of BCG and its indirect effect on reducing disease transmission. We also developed and used a mechanistic transmission model to explore the potential long-term impact of BCG vaccination in Ethiopia, where the transmission risk of bTB varies considerably between herds, and cattle trading is projected to contribute to a gradual yet substantial increase in prevalence.

1.5 Research questions

The key research questions of this study were

- Does BCG vaccination decrease the onward transmission of bTB in crossbred cattle?
- How effective are the novel cocktail antigens in the DIVA test for distinguishing infected among vaccinated cattle?
- Can BCG vaccination mitigate the risk of bTB transmission among herds associated with unrestricted cattle trade and movement?
- What are the predominant MTBC species responsible for bTB and the key risk factors associated with infection in cattle in Central Ethiopia?

1.6 Objectives

1.6.1 General objective:

The main objective of this study is to investigate the effects of BCG vaccination on the transmission of bTB in cattle under a controlled natural transmission setting and to evaluate the diagnostic performance of DIVA antigen cocktails in crossbred cattle in Ethiopia.

1.6.2 Specific objectives:

- To estimate the direct and indirect efficacies of BCG vaccination in protecting bTB transmission in crossbred cattle in an intensive production system,
- To evaluate the diagnostic performance of a new defined peptide antigen cocktails in differentiating infected from vaccinated animals (DIVA),
- To assess the implications of BCG vaccination for bTB control strategies in dairy sector in Ethiopia.
- Identify the risk factors and MTBC species causing bTB in both the experimental and field cattle populations in central Ethiopia.

1.7 Significance of the study

This study examined the direct and indirect protective effects of BCG vaccine in reducing TB transmission cattle using a novel crossover study design to fill the existing knowledge gaps. The experimental study and stochastic metapopulation models show that BCG vaccination can

reduce the reproduction number (R_0) below one, paving the way for bTB elimination from Ethiopian cattle herds. This remarkable indirect effect of the vaccine beyond its direct protective effect has not been measured before. This is the first study to show that BCG-vaccinated cattle infected with TB are substantially less infectious to other cattle susceptible to infection. This finding has important implications for LMICs, where traditional test-and-cull strategies are challenging to implement owing to economic and socio-cultural restraints, despite the high endemic prevalence of the disease. The performance of the defined antigen cocktails in DIVA skin and IGRA tests exhibited high specificities of 98.5% and 94.0%, respectively, in vaccinated cattle, while both test formats were 100% specific in unvaccinated cattle. These findings underline the significance of these tests to prevent false positives in vaccinated cattle and highlight their potential for integration with BCG vaccination in bTB control programs. The cross-sectional slaughterhouse survey revealed that crossbred cattle have an 11-fold higher risk of developing bTB than local zebu cattle. This study improves our understanding of the prevalence and the risk factors of bTB in cattle in central Ethiopia, underscoring the need for targeted bTB control measures in intensive dairy production systems.

1.8 Scope of the study

This study evaluated the direct and indirect effects of BCG and predicted its long-term impact on bTB transmission between cattle herds. In addition, the study examined the performance of defined peptide cocktail antigens as components of DIVA tests in the *in vivo* and *in vitro* tests. This study also examined the main MTBC species that cause bTB and characterized the risk factors contributing to bTB infection in the central Ethiopia. Although the geographic focus is on central Ethiopia, the results have broader implications for bTB control strategies in LMICs, especially in regions where the prevalence is high (>5%) and test-and-slaughter methods are not feasible.

1.9 Limitations of the study

In this study, the experiment was conducted in a controlled setting, which may not fully capture the complexities of field conditions in diverse farming systems throughout Ethiopia and beyond. This study evaluated the short-term impacts of BCG vaccination on bTB transmission; however, it did not explore longer-term effects, such as potential waning of immunity. In addition, the research focused only on cattle and did not consider the role of wildlife or other domestic animal species in the transmission of bTB. Although the study examined the

components of the DIVA test, their performance and field accuracy are likely to be influenced by biological variations, which were not considered in this study. In addition, the slaughterhouse survey primarily targeted central Ethiopia, which may limit the applicability of the results to other regions with different livestock management practices, climatic conditions, prevalence levels of the disease and exposure to environmental mycobacterium.

1.10 Structure of the study

This dissertation is presented five main chapters. The first chapter serves as the introduction, while chapters two through four present published research articles detailing on BCG efficacy, defined antigen cocktail diagnostic antigens and slaughterhouse-based survey of bTB. The final chapter includes the discussion, conclusion, and recommendations.

Chapter One: Presents the Introduction and Concise Literature Review

Chapter Two: Presents the study titled "BCG vaccination reduces bovine tuberculosis transmission, improving prospects for elimination" published in "Science" (383, eadl3962, March 2024, DOI: 10.1126/science.adl3962)

Chapter Three: Presents the study titled "Comparative performance of tuberculin and defined-antigen cocktails for detecting bovine tuberculosis in BCG-vaccinated cattle in natural settings" published in "Scientific Reports" **(**15(1):4564, DOI: 10.1038/s41598-025-85389-1

Chapter Four: Presents the study titled "Bovine tuberculosis in Central Ethiopian slaughterhouses and the identification of causative mycobacteria by multiplex qPCR published in "BMC Microbiology" (24:394, 2024, DOI: 10.1186/s12866-024-03543-7)

Chapter Five: Discussion, Conclusion and Recommendations: interprets the results in the context of current knowledge, highlighting the implications and potential areas for future research.

CHAPTER TWO: BCG VACCINATION REDUCES BOVINE TUBERCULOSIS TRANSMISSION, IMPROVING PROSPECTS FOR ELIMINATION

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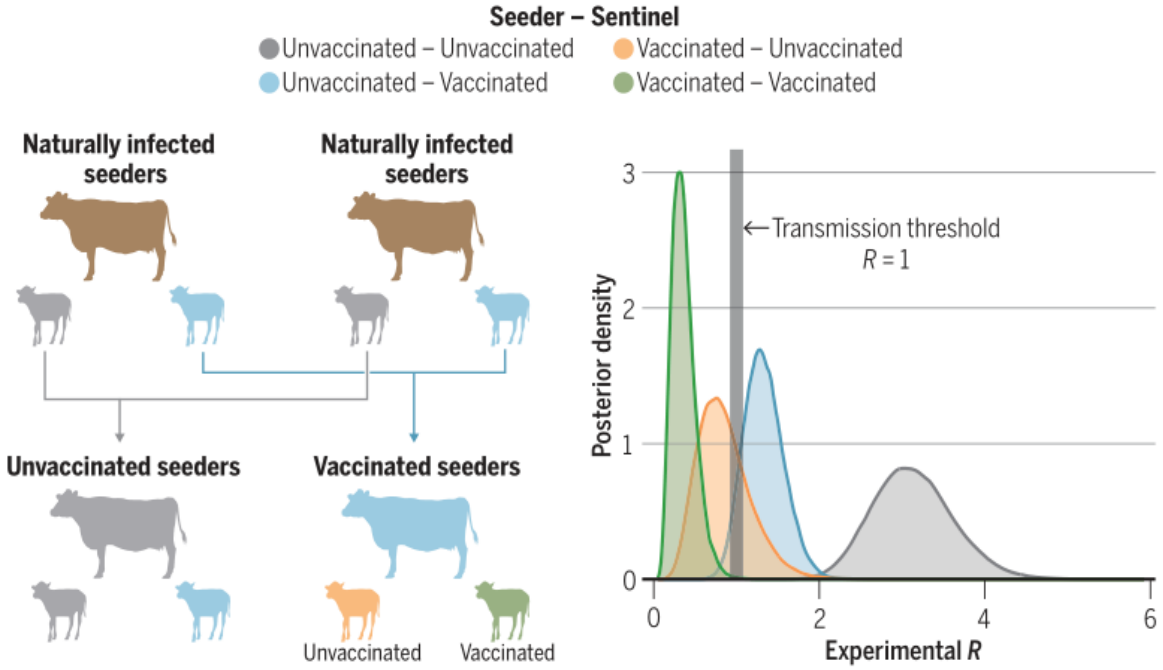
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2.1 Summary

Introduction: Bovine tuberculosis (bTB) poses a substantial global threat to animal health, food security, and human well-being. Although proven effective in many high-income countries, the traditional test-and-slaughter approach for bTB control is expensive and impractical for socioeconomic reasons in many regions where the disease remains endemic. This has necessitated a need for alternative bTB control strategies, with Bacille Calmette-Guérin (BCG) vaccination presenting a promising option. However, the effectiveness of BCG in controlling bTB by reducing onward transmission remains unclear. This study investigated both the direct and previously unexplored indirect effects of BCG vaccination on bTB transmission in cattle, thereby providing key missing insights for control.

Rationale: Traditional vaccine efficacy evaluations cannot measure the impact of vaccination on reducing onward transmission from infected individuals. This mode of action of vaccination is critical for the evaluation of BCG in cattle because the primary effect is to reduce the extent and rate of progression of lesions rather than to provide sterilizing protection. Our study addresses this gap by performing a natural transmission experiment with bTB in cattle, using a crossover design approach, where the sentinels from Phase-I were used as seeders in Phase-II. This method enabled a more realistic and robust assessment of BCG's true impact on bTB transmission, quantifying both the direct efficacy of BCG as well as its effect on reducing transmission. We developed a mechanistic transmission model to explore the potential of using BCG vaccination in Ethiopia, where the transmission risk of bTB varies considerably between herds and the relatively infrequent trading of animals is projected to contribute to a gradual yet substantial increase in prevalence.

Results: The natural transmission study showed a 74% reduction in bTB transmission [95% credible interval (CrI): 46 to 89%] in vaccinated as compared with unvaccinated animals. This substantial indirect effect of BCG vaccination exceeded the observed direct protection against infection (58%; 95% CrI: 34 to 73%), and the combined effects translated to a total vaccine efficacy of 89% (95% CrI: 74 to 96%). Vaccinated animals exhibited substantially lower total visible lesion scores compared with unvaccinated controls, which is consistent with the notion that BCG vaccination reduces disease severity and potentially infectiousness.



Quantifying BCG vaccination's total efficacy against bovine tuberculosis. Sentinel calves, both BCG-vaccinated and unvaccinated, were exposed to seeder cattle to measure direct efficacy through IGRA-conversion times over 12 months. Subsequently, these sentinels were used to determine BCG's indirect transmission-reducing effects, and the results helped inform development of models for evaluating TB elimination strategies.

A stochastic metapopulation transmission model, calibrated with data from Ethiopia, suggests that routine calfhood BCG vaccination has the potential to prevent the predicted expansion of bTB in dairy herds and bring the population average reproduction ratio below 1 within as few as 10 years, resulting in a substantial decrease in predicted bTB prevalence as compared with baseline scenarios without vaccination. The results highlight the critical importance of the combined direct and indirect effects of BCG vaccination in enabling bTB elimination. The findings suggest that BCG vaccination represents an important tool for bTB control, particularly in resource-limited settings where traditional methods are impractical. The results also suggest that achieving elimination through vaccination alone would require a long-term commitment, as the full benefits may take decades to be realized. Our studies highlight a need for further research on the duration of efficacy, including the potential for extending protection through revaccination, as well as the impact of vaccination age and cross-species transmission.

2.2 Abstract

Bacillus Calmette-Guérin (BCG) is a routinely used vaccine for protecting children against *Mycobacterium tuberculosis* that comprises attenuated *Mycobacterium bovis*. BCG can also be used to protect livestock against *M. bovis*; however, its effectiveness has not been quantified for this use. We performed a natural transmission experiment to directly estimate the rate of transmission to and from vaccinated and unvaccinated calves over a one-year exposure period. The results show a higher indirect efficacy of BCG to reduce transmission from vaccinated animals that subsequently become infected (74%; 95% CrI: 46-98%) compared to direct protection against infection (58%; 95% CrI: 34-73%) and an estimated total efficacy of 89% (95% CrI: 74-96%). A mechanistic transmission model of bTB spread within the Ethiopian dairy sector was developed and showed how the prospects for elimination may be enabled by routine BCG vaccination of cattle.

One-Sentence Summary: The protective benefits of BCG vaccination for reduction of transmission of tuberculosis in cattle are quantified.

2.3 Introduction

Bovine tuberculosis (bTB) is an economically important disease of livestock with the potential for zoonotic transmission (1). Statutory programs for elimination currently rely on intensive test-and-slaughter of animals that react to tuberculin, which provides a measure of exposure but not necessarily of infection. Test-and-slaughter based elimination programs have been successful in many countries including Australia (2) and the USA (3) but are unfeasible socioeconomically in most LMICs, where the disease is uncontrolled. Test-and-slaughter also continues to place considerable economic pressures in countries such as the UK (4), Ireland (5) and New Zealand (6) where the disease persists in cattle and wildlife despite intensive efforts. The development and deployment of vaccines such as BCG has thus long been seen as a solution for accelerating control and the ultimate elimination of bTB across the globe (4). However, the use of BCG vaccination as a supplement, rather than an alternative, poses economic challenges because of the ongoing costs associated with test-and-slaughter and the potential for BCG to undermine the effectiveness of existing bTB control programs (7,8).

Surveillance for bTB relies predominantly on tuberculin testing, a method that detects delayed hypersensitivity reactions to purified protein derivatives (PPDs, also known as tuberculins) from specific *Mycobacterium species*. The standard approach internationally is the skin test, where the response is assessed by the size of the swelling at the injection site. Complementing this, interferon-gamma release assays (IGRAs) offer a blood test alternative that utilize the same tuberculins but avoid the issue of desensitization that may be observed in animals repeatedly exposed to tuberculins. Animals vaccinated with BCG yield higher rates of false positive reactions to tuberculin in both formats of the test (9), which is the primary reason it has not yet been licensed for use in domestic livestock. Recent developments in diagnostic tests using defined antigens (ESAT-6, CFP10, Rv3615c, etc.) provide a promising avenue for the detection of infection in vaccinated animals (DIVA testing) (10–13) and potentially enable the use of BCG vaccination without compromising the ability to assess freedom from infection. These considerations are perhaps less relevant for endemically infected LMICs with major cattle populations throughout the world, such as Ethiopia or India, where the high, but variable prevalence of infection within herds (14–17) makes the introduction of test-and-slaughter economically prohibitive and the use of vaccines more attractive. Intensification of farming in these emerging dairy markets poses the risk that cattle movements will fuel a parallel increase in the prevalence of bTB. For instance, the animal-level prevalence in Ethiopia has been recently estimated at 5.8%; 95% CI: 4.5-7.5% (18), but with the prevalence within some heavily infected herds approaching 100% test positivity, this burden could dramatically increase.

While DIVA tests are being validated to pave the way for vaccination programs, the efficacy of BCG remains a subject of debate. Recent meta-analyses estimate its direct protection against infection to be as low as 18%; 95% CrI: 11-24% in experimental studies and 61%; 95% CrI: 40- 74%) in natural transmission models (19). This low and variable primary direct efficacy (VE_D) is consistent with BCG's performance in human populations (20). However, in the context of bTB control, these direct effects only partially capture the vaccine's impact on transmission dynamics. The primary claim for BCG is its ability to decelerate disease progression and minimize lesions. In closed herds with high vaccination rates, the total vaccine efficacy (VE_T) also encompasses an indirect effect (VE_I), which represents the reduced transmission rate from vaccinated, infected animals to others (21). This indirect effect is

increasingly recognized as a critical factor for assessing BCG's overall effectiveness in field conditions (19).

Existing literature primarily calculates BCG's efficacy based on the number of positive animals in both vaccinated and control groups at a fixed endpoint, often using the post-mortem presence of visible lesions or culture confirmation as this endpoint (19), and in one instance, by using multiple ante-mortem diagnostic tests (22). Such endpoint-based estimates are problematic for chronic diseases such as bTB in that they are highly sensitive to the force of infection within the study population and the duration of exposure (Supplementary Figure 2.1), hence making them unsuitable for predicting the effectiveness of vaccines or other interventions under field conditions. Hence, to accurately gauge BCG's utility in controlling bTB, there is a critical need to focus on its efficacy in reducing transmission rates. This is particularly crucial given the consistent evidence that BCG vaccination slows down disease progression and reduces lesion extent (19), implying that vaccinated animals, if infected, are less infectious. Therefore, the relevant metric for control is not the traditionally reported endpoint estimate, but rather the vaccine's efficacy in reducing transmission rates, especially so in closed herds with potential for high vaccination coverage (23). The comprehensive effects of vaccines, extending beyond mere reduction in susceptibility, have been previously explored for acute viral infections (23,24). However, their application to chronic bacterial infections, including bTB, are notably lacking. This knowledge gap is particularly relevant given the complex nature and extended course of chronic infections where an understanding of the full effect of vaccines, including impact on onward transmission, is key.

2.4 Methods

To quantify the efficacy of both the direct and indirect modes of action of BCG to reduce transmission in cattle, we carried out a natural transmission study under controlled conditions (Figure 2.1). In brief, four experimental groups of sentinel animals were included in the study, each exposed to different types of seeder animals across two distinct phases, with each phase lasting twelve-months of contact. In phase I, two groups (Groups 1 & 2) functioned as biological replicates. Each group included approximately equal numbers of seeder (~34) and sentinel animals, divided equally between vaccinated (~17 animals) and unvaccinated controls (~ 17 animals). The average age of the calves at vaccination was 133 (95% CI: 122-144) days. After an initial two-months post-vaccination, these sentinels were introduced to a separate barn,

joining older test-positive seeder animals sourced from local Ethiopian dairy herds. In phase II, sentinel animals from phase I were reassigned into two groups based on their vaccination status: vaccinated (34) and unvaccinated (33). These animals then served as seeder animals for a new set of approximately 34 sentinel calves (similar to the arrangement during phase I). We next developed a mechanistic transmission model calibrated using estimates of the within-herd reproduction ratio R_0 and empirical cattle movement data from four regions of Ethiopia to explore the potential for control of bTB in LMIC settings through routine BCG vaccination.

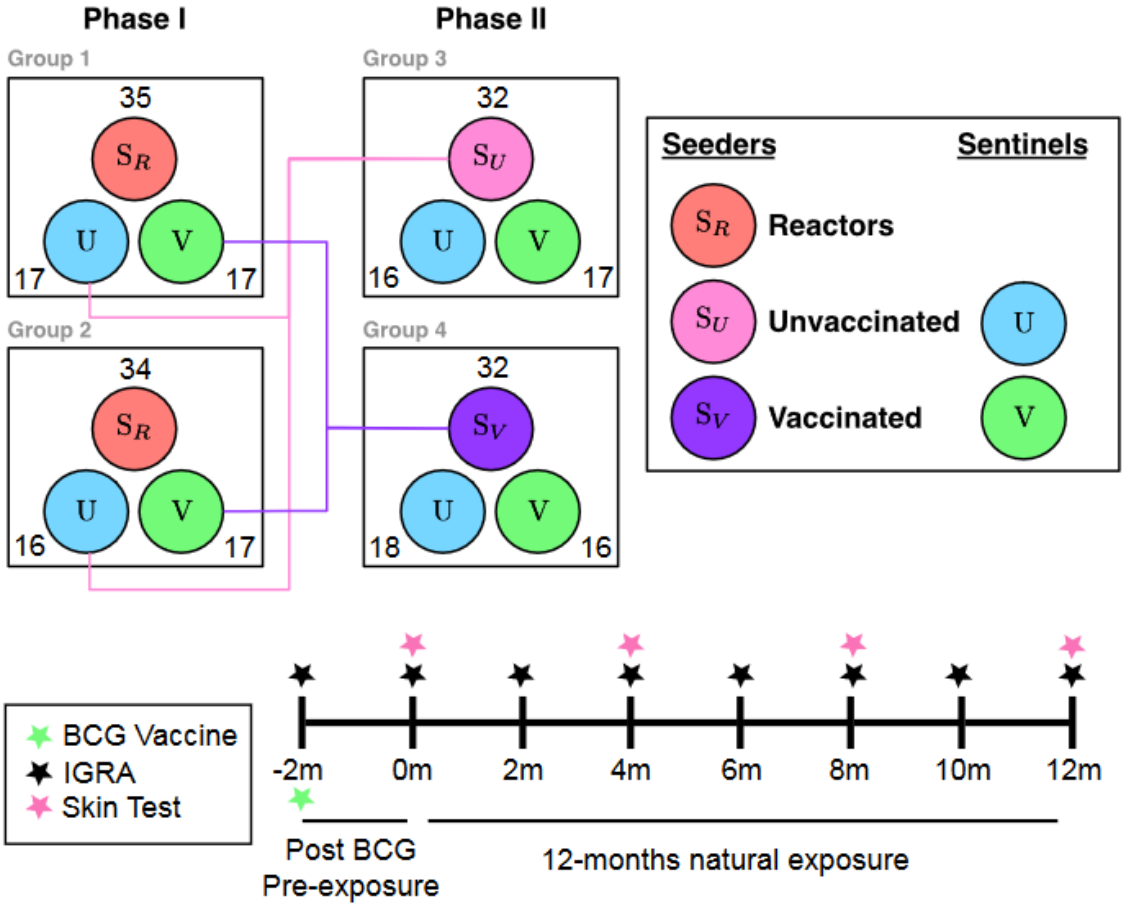


Figure 2.1. Schematic representation of study design. For Phase I sentinel animals (calves) were acclimatized for about 2 weeks post-recruitment, following which they were randomized (double-blind lottery system) into control and vaccinate experimental groups. At 2 months post-vaccination, blood was drawn for IGRA and a skin test was conducted to confirm vaccine response. After confirmation of vaccine response, two sets of ~34 adult infected (CCT and IGRA+) seeders were housed with a target of 17 unvaccinated and 17 BCG-vaccinated sentinel calves (2-6 months of age) in each group (1,2) with a range of between 16-18 animals in practice after the loss of animals after vaccination but before exposure. A total of 5, 4, 4, and 3 animals in total were removed early from groups 1, 2, 3, and 4, respectively. The majority of

removals in each group (except for group 3) were seeders consisting of 3/5, 3/4, 1/4 and 2/3, respectively. During the exposure period of 1 year, blood was drawn for IGRA every 2 months and skin test was conducted every 4 months. The surviving sentinel animals from Phase I were then used as seeders for two new experimental groups (3,4) with a fresh set of sentinel calves, which were then followed using the same schedule of tests for a second 12-month period of natural exposure.

Trajectories of cellular immune responses in BCG vaccinated cattle

Following acclimatization and prior to vaccination, sentinel animals negative bTB status was established with IGRA using PPDs from avian and bovine mycobacteria as the stimulating antigens. All sentinel animals entering the experiment showed no signs of infection and tested negative with no significant difference observed between responses elicited by controls and vaccinates to PPD (B-A) in IGRA prior to BCG vaccination (Supplementary Figure 2.1 A). At the eighth week post-BCG vaccination, and before exposure to seeder herds, both vaccinated and control calves were subjected to skin and IGRA blood tests using bovine and avian PPDs to confirm vaccine response.

The infection status of sentinel animals within each experimental group was then monitored using the same sequence of skin tests (bovine, avian and DIVA antigens) at four monthly intervals, with whole blood samples taken every two months for IGRA assays using the same set of antigens (Figure 2.1). In Phase II the sentinel animals exposed to naturally infected CCT test positive animals during Phase I were used as seeder animals in Phase II, split according to vaccination status (25). Thus, by comparing the rates of transmission experienced within the four experimental groups, we were able to directly compare the relative infectiousness of vaccinated and unvaccinated animals. Transmission rates were inferred by estimating a discrete time stochastic chain-binomial model (26,27) to logical infection histories imputed from the diagnostic test results (Figure 2.1).

Published evidence indicates that most *M. bovis* infected cattle are asymptomatic, indicating unreliability of clinical signs for identifying infectious animals. For example, 92% (228/247) of cattle presented with severe as well as disseminated bTB-lesions at post-mortem displayed no clinical signs (Menin *et al.*, 2013). According to a recent review, 87% (327/374) of bTB-positive bovines assessed for showing clinical signs are asymptomatic (Willgert *et al.*, 2025).

Thus, given the chronic nature of bTB and lack of reliable ante-mortem correlates of infectiousness, animals are assumed to be infected – and potentially infectious – from their first positive test through the duration of observation in order to avoid underestimating transmission. While the inferred transmission rates based on test positivity are influenced by the sensitivity of the diagnostic assay, for the purposes of estimating vaccine efficacy this may be treated as a nuisance parameter since the efficacy only depends on the relative magnitude of transmission rates between different treatment groups and not the absolute values. Traditional tuberculin-based tests are ill-suited for this purpose, as they reduce specificity for BCG-vaccinated animals, thereby inflating apparent transmission rates. In contrast, DIVA tests using peptide-based defined antigens offer more precise estimates with the key assumption that they perform equivalently in both vaccinated and control animals. Consistent with this assumption, even though the response magnitude for DIVA tests is generally lower than for tuberculin tests and they have higher specificity for BCG-vaccinated animals, prior evidence from multiple experimental trials suggests that the commonly used categorical cut-offs do not result in performance differences or misclassification of infected animals (28). For this study, we used antigens developed for the defined skin test (DST) in both a skin and IGRA format. Due to the risk of de-sensitization of animals from repeated exposure to antigens skin-testing was carried out at a lower frequency (4 monthly intervals) compared to IGRA. Given the higher temporal resolution, we focus on the DST1 (IGRA) results and compare estimates of vaccine efficacy to the more uncertain DST 10 (skin test) results.

2.5 Results and Discussion

Estimates of direct and indirect efficacy of BCG

Based on DST-based IGRA test history, the baseline experimental reproduction ratio (defined as the number of new infections expected where a single infectious individual is held in contact with a fully susceptible population for a 12-month period) was estimated to be 3; 95% CrI: 2-4%). The attack rate for both vaccinates and controls was consistent across all the experimental groups except for the group with vaccinated seeders (Group 4), indicating that high indirect protection of BCG reduced the infectiousness of vaccinates (Supplementary Figure 2.1). This effect is reflected in an estimated indirect vaccine efficacy for reduction in infectiousness (Figure 2.2C) of $VE_I = 74\%$; 95% CrI: 46-89%; this is higher than the estimated direct protection of $VE_D = 58\%$; 95% CrI: 34-73%), with a 86% posterior probability that VE_I is

greater than VE_D. Taken together, this corresponds to a total vaccine efficacy of BCG to reduce transmission of bTB of 89%; 95% CrI: 74-96%). Estimates from the DST 10 (skin test) are consistent but more uncertain in line with the lower temporal resolution of this data (Table 2.1).

The practical importance of the indirect vaccine effect is reflected in the pairwise experimental reproduction ratios (Figure 2.2B, Table 2.2) which show that the direct and indirect effects of BCG, when considered alone, are not sufficient to prevent transmission within the experimental system with posterior probability that R is less than the critical threshold value of 1 of only 0.075 for vaccinated sentinels placed in contact with unvaccinated seeders, rising to 0.72 for the combination of vaccinated seeder animals and unvaccinated sentinels (Table 2.2). In contrast, the posterior probability that R < 1 is 1.0 for the combination of vaccinated sentinel animals and seeders, implying that both direct and indirect modes of action of BCG could achieve elimination in our experimental system. This has important consequences for the utility and monitoring of BCG vaccination policies in endemic settings as the full protective benefits may take multiple generations to accrue.

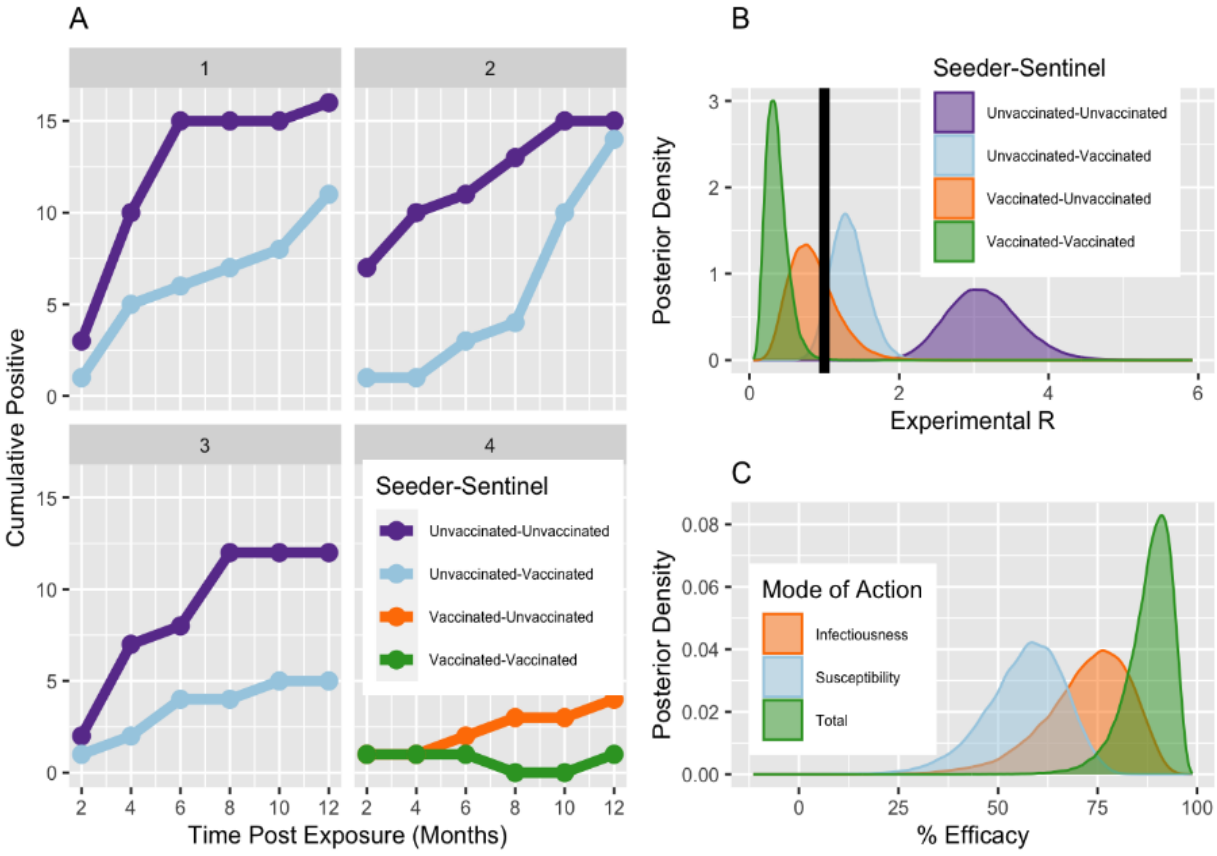


Figure 2.2. The two modes of action of BCG vaccination in cattle. (A) The infection history for unvaccinated (blue) and vaccinated (green) animals within each experimental group (1-4)

as represented by the cumulative number of animals that have ever tested positive by IGRA (DST1) and have survived to that time point. These curves represent the number of sentinel animals assumed infectious within each group at the test date, reflecting the animals removed on welfare grounds, as well as those that have tested positive. The lower attack rate in group 4, where all seeder animals were vaccinated, demonstrated the reduced infectiousness of these animals compared to the other groups with unvaccinated seeders. (B) The estimated transmission rate between the different combinations of vaccinated and unvaccinated sentinel and seeder animals can be expressed as an experimental reproduction ratio R, defined as the expected number of new infections in a fully susceptible population of the given sentinel type when exposed for a duration of one year to the respective seeder type. Posterior distributions for the four combinations are presented with the threshold value of R=1 indicated by the vertical line. Only the combination of vaccinated sentinels and seeders within our experimental unit shows a high posterior probability that R < 1, which increases from 0.72 to 1.0 with vaccination of both seeder and sentinel groups (C). Posterior estimates for the vaccine efficacy of BCG with respect to reducing infectiousness (VE_I,red), reducing susceptibility (VE_D,green), and the total efficacy (VE_T,blue). This total efficacy represents a combination of both direct (reducing susceptibility) and indirect (lowering infectiousness) effects of the vaccine.

Table 2.1. Estimated vaccine efficacy for BCG in cattle by mode of action.

Diagnostic Test	**Endpoint efficacy**	**Transmission rate efficacy**		
	Total Efficacy (95% CI)	Direct Efficacy ($\boldsymbol{VE_D}$) (95% CrI)	Indirect Efficacy ($\boldsymbol{VE_I}$) (95% CrI)	Total Efficacy ($\boldsymbol{VE_T}$) (95% CrI)
DST 1 (IGRA)	39 (22-54)	58 (34-73)	74 (46-89)	89 (74-96)
DST 10 (Skin)	40 (19-57)	46 (14-66)	67 (39-87)	82 (55-94)
Visible Lesions	25 (1-47)	-	-	-

Table 2.2. Estimated experimental reproduction numbers by the vaccination type of sentinel and seeder animals.

Seeder-Sentinel Pair	R (1-year contact) (95% CrI)	Posterior Probability that $R < 1$
Unvaccinated-Unvaccinated	3.1 (2.3-4.1)	0.0
Unvaccinated-Vaccinated	1.3 (0.9-1.8)	0.075
Vaccinated-Unvaccinated	0.8 (0.4-1.5)	0.72
Vaccinated-Vaccinated	0.3 (0.1-0.7)	1.0

The results of our investigations also corroborate the findings from previous studies on BCG vaccination's partial protective effects and lesion reduction in cattle (19). While lesions were still observed in vaccinated animals, the total visible lesion scores were notably lower compared to the control group across both seeder (phase 1 sentinels) and sentinel animals (Supplementary Figure 2.1). Given the study's design constraints that necessitated retaining Phase I sentinel animals as seeders for Phase II, it was not possible to obtain endpoint efficacy estimates based solely on visible lesion scores. However, when pooling all sentinel animals from Phase II groups 3 and 4, we observed a one-year endpoint efficacy of 25%; 95% CI: 1-47% (Table 2.1), consistent with earlier meta-analysis estimates (19). Importantly, while BCG's inability to fully prevent infection could raise questions about the efficacy estimates being influenced by reduced DIVA antigen detection sensitivity in vaccinated animals, the study design and results mitigate this concern. Specifically, if reduced test sensitivity amongst vaccinated animals were influencing the results, we would not expect to see the marked reduction in the attack rate among unvaccinated controls that was also seen in group 4 (Figure 2.2A).

Prospects for elimination of bTB by BCG vaccination

To explore the consequences of implementing BCG vaccination in a relevant context, we assessed the feasibility of eliminating bTB from the Ethiopian dairy sector through routine vaccination alone. Using tuberculin skin testing data from the Ethiopia Control of bTB (ETHICOBOTS) project (15,16) and historical data from the Animal Health Institute, Ethiopia (14) we estimated within herd reproduction ratios (R_0), defined as the expected number of new infections when a single infectious individual is introduced into a fully susceptible population,

ranging from 1.3 – 17.3 (Figure 2.3B), with a population average of 3.26; 95% CrI: 2.7 – 4.0%. Given that cattle rarely show clinical signs of bTB during the early stages of disease, cattle trade can facilitate long-distance transmission in the absence of systematic herd testing. Consequently, cattle movements were considered the primary mode of transmission between herds. Directed exponential random graph models (ERGM) were used to estimate new generative models for the frequency and pattern of cattle movements between intensive and semi-intensive herds in Ethiopia previously collected from three emerging dairy markets (29).

To predict the likely spread of bTB based on estimated networks of movements, and to assess the potential benefits of vaccination, a stochastic metapopulation transmission model was developed with four possible states: susceptible (S), infected (I), vaccinated (V), and infected vaccinated (Iv). Three transmission scenarios were simulated, motivated by the three regions around Gondar, Mekelle, and Hawassa for which movement data were available. For each scenario, a synthetic population was simulated with initial herd sizes sampled from the recorded herd sizes in Ethiopia, and initial herd-level prevalence sampled using point estimates from (15) (18.0% in Gondar, 38.9% in Mekelle, and 10.5% in Hawassa). The intrinsic within-herd R_0 was assumed to vary between herds and was sampled from our estimates. Affected herds were initialized at the endemic equilibrium and simulated forward for 50 years.

The baseline scenarios without vaccination (Figure 2.3C) demonstrate the scale of the potential risk posed by cattle trading with a predicted increase in the animal level prevalence across all scenarios to an endemic level of ~ 50%. However, this emergence is predicted to be slow – mediated by the relatively infrequent trades of animals – and taking the full 50-year span of our simulated scenario. This relatively slow progression – in contrast to the rapid potential for cattle-to-cattle transmission within herds as shown in our natural challenge system – offers a window of opportunity for vaccination, or indeed interventions targeted on the cattle movement networks (29).

The effectiveness of vaccination as a control measure for bTB was then evaluated by comparing the current baseline scenario to scenarios where all newborn calves are vaccinated (Figure 2.3C). The potential waning of vaccine protection over time was not considered, and it was assumed that animals would be revaccinated as needed to maintain levels of protection. To illustrate the importance of the additional indirect protection afforded by BCG we compare

two vaccine scenarios where animals are protected only by a direct effect or with the combined direct and indirect effects (sampled from our posterior estimates). Our model suggests that with the estimated total vaccine efficacy of 89%; 95% CrI: 74-96%) calfhood BCG vaccination has the potential to prevent the predicted expansion of bTB through the dairy sector in Ethiopia bringing the population average *R* below 1 within ten years of deployment. However, the path to elimination is slow with herd prevalence still above the international officially TB-free level of < 0.1% annually through the 50-year program simulation. Notably, in common with our experimental system, this impact can only be achieved as a result of both the direct and indirect effects of BCG (Figure 2.3C). Implementing vaccination alongside other control methods including movement restrictions, test-and-segregation, enhanced farm biosecurity, and best management practices such as barn ventilation would likely accelerate disease control by reducing both within herd and between herd transmission.

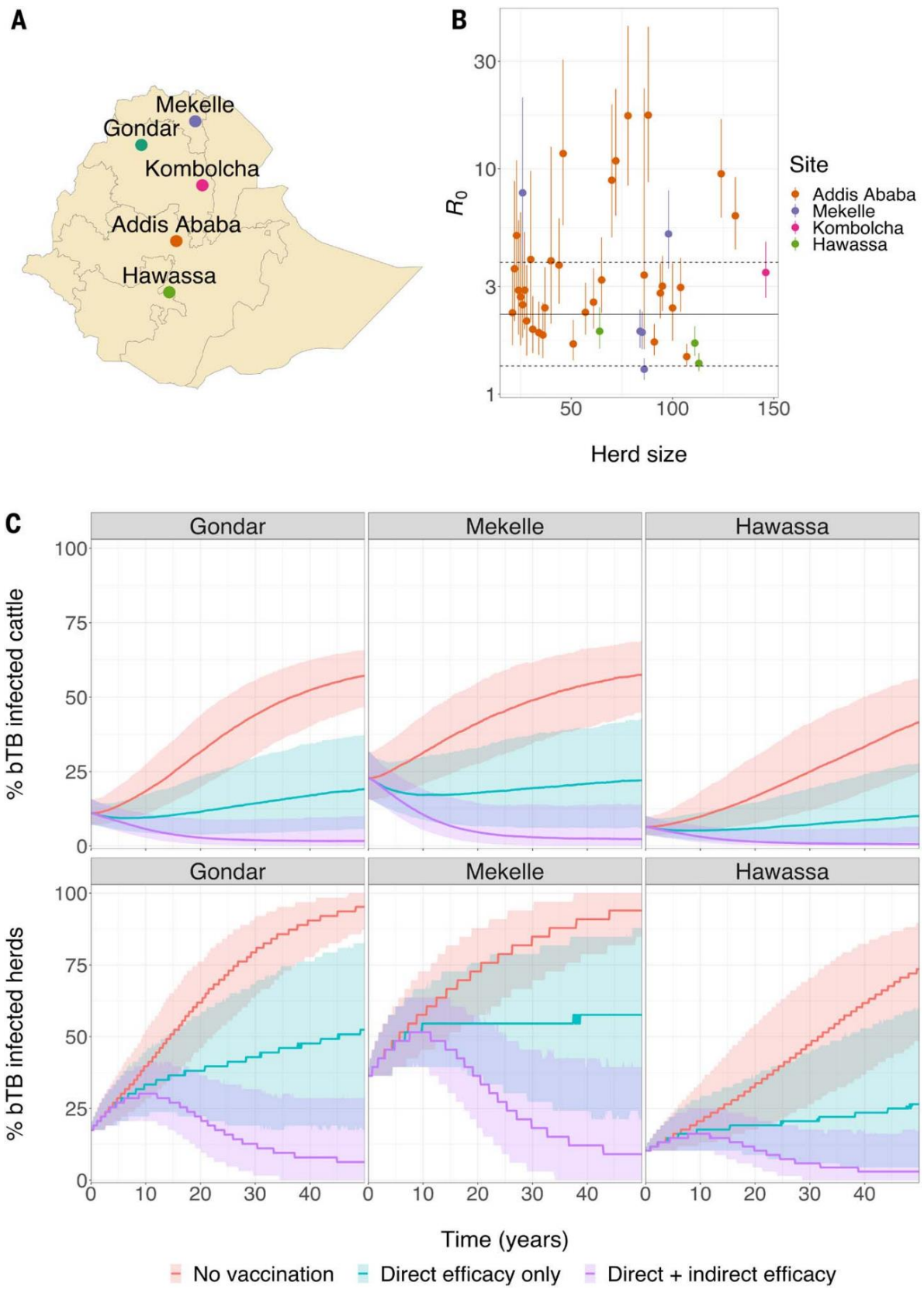


Figure 2.3. Scenario analyses for the prospective use of cattle vaccination within the Ethiopian dairy sector. (A) Within-herd rates of transmission of bTB were calibrated using herd level prevalence data collected from herds in the dairy belt surrounding Addis Ababa and

emerging markets in Hawassa, Mekelle and Kombolcha. Gondar, Hawassa, Mekelle are the only regions for which we also have between-herd cattle movement data. (B) The wide range of estimated animal level prevalence within herds ranging from (5.8-78%) corresponds to a similarly large heterogeneity in estimates of the basic reproduction ratio R_0 with no clear associations with either geography or herd size. (C) Cattle movement models estimated data from Gondar, Mekelle and Hawassa were used to project how the prevalence of bTB might be expected to evolve over the next fifty years. The projected median (solid line) and 95% prediction intervals (shaded area) for the percentage of herds infected with bTB for this baseline scenario (no vaccination, red) are compared to scenarios with routine vaccination coverage of all herds (with no assumed loss of immunity) with the estimated direct vaccine efficacy of 58%; 95% CrI: 34-73% only (blue) and with the additional indirect efficacy of 74%; 95% CrI: 46-89% (purple).

Implications for the deployment of BCG in the field

Our scenario analysis demonstrates the challenges of controlling a chronic infection such as bTB, where infected animals potentially remain infectious for life. Without the chance of recovery, the scale by which transmission is measured and control is manifested is determined by the life-expectancy of the host. Through this lens, even under the most favorable circumstances, routine BCG vaccination as the only control measure is expected to take decades to eliminate bTB infection. Even so, in endemic settings, vaccination could play an important role in limiting spread and reducing prevalence such that more rapid measures such as test-and-slaughter or test-and-segregate can become economically tractable. The long-term commitment to vaccination necessary to see epidemiological impacts must be factored into economic cost-benefit analyses to support the business case for specific regions. These benefits are likely to vary considerably between settings depending on the impact of bTB on animal production and productivity, both of which are remarkably poorly characterized outside of a single study from Germany in 1970 (30).

The evidence for substantial indirect protection provided by BCG in calves contrasts with recent findings from field trials in badgers in Ireland (31), but is consistent with a smaller scale trial within badger social groups in England (32). Since the evaluation of indirect vaccine effects in free-living animals, which depends on comparing the attack rates in sub-populations with varying levels of vaccine coverage (21), is exceptionally challenging and typically only

feasible once vaccines have been deployed at scale, the contradictory findings from the badger trials in England or Ireland are difficult to interpret in context. It is noteworthy that despite the widespread use of BCG vaccine globally, no similar indirect effects have been reported with respect to the transmission of human tuberculosis. The well-known geographic variation in BCG efficacy in human populations (20), more complex life-history of infection and the regular removal of infectious individuals through treatment (and mortality) would make such an effect more difficult to measure than in the managed settings of cattle production systems.

Despite the evidence for indirect effects of BCG in cattle and the promising implications of its use in accelerating bTB control programs, there are several limitations to our study. A key limitation is the relatively short duration of one-year contact with infected animals as part of these controlled trials. This is relevant since experimental studies using a revaccination approach indicate that the duration of direct protection of BCG in cattle is limited to between one and two years (33). Hence, the high efficacy of BCG to reduced infectiousness seen in our trial may not be maintained for the average lifetime of a dairy cow in the herd ~ 7 years in Ethiopia (34). Future investigations to assess the duration of indirect effects on infectiousness, as well as the potential to maintain or extend these effects through revaccination remain key knowledge gaps, both of which will likely only be practical to measure once the vaccine is widely deployed in the field. Another limitation is that our current models do not account for cross-species transmission from other livestock held on the same premises as cattle, such as pigs, goats and sheep or sympatric free-living wildlife species or even humans that may act as potential reservoirs of spillover infection to cattle. Future studies are needed to assess the potential benefits of cattle vaccination in the face of risk of cross-species transmission from other sympatric hosts (35). Finally, our model only projects the expected increase in prevalence of bTB based on current patterns of movements and rates of transmission within dairy herds. Intensification and changes in husbandry are likely to change both of these particularly over such a long period of projection, our scenarios should therefore only be considered as projections to illustrate the potential benefits of vaccination rather than as forecasts.

The implications of our studies for populations with established surveillance and control programs, such as the UK, are more nuanced as the costs associated with bTB are dominated by the impacts on the test-and-slaughter program itself (7) rather than the prevalence of disease. Concern over the potential for perverse consequences of vaccination, given the evidence for

limited direct protection and the compromised specificity of tuberculin diagnostics in vaccinated cattle, has been a major barrier for the willingness of statutory and governmental agencies to license BCG for use in cattle (8). These concerns should be somewhat alleviated by the results of our investigations that show that the indirect protection afforded by BCG vaccination is at least equal to, if not greater, than the direct protection typically measured by efficacy studies.

Our studies suggest that the effectiveness of BCG in the field is likely to be greater and the risks lower than previously understood and we provide key missing evidence of the potential benefits of a BCG-vaccination based approach to accelerate control of bTB in regions where the disease remains endemic.

2.6 Materials and Methods Summary

Experimental study location and subjects

The study took place at the Animal Health Institute, located in Sebeta, Ethiopia, about 20 km southwest of Addis Ababa. The premises had three separate barns, each capable of accommodating 80 adult cattle, with approximately 2,000 to 2,500 m^2 of fenced outdoor space. In Phase I 68 CCT and IGRA test positive cattle were recruited as seeder animals for groups 1 and 2 of the transmission experiment. 68 Holstein-Friesian x Zebu crossbred male calves, age < three months and tested negative for bTB, were recruited as naïve sentinels from low-prevalence herds split between the two experimental phases, with sentinel animals from Phase I (Groups 1 and 2) acting as seeder animals in the second phase (Groups 3 and 4).

Testing Procedures, Antigens and Vaccination

Eight weeks post-BCG vaccination, all sentinel animals were introduced to infected adult seeder herds. The seeders and sentinels were housed together in two separate barns during both Phase I and Phase II. Every four months, skin tests were conducted on sentinel animals using both Purified Protein Derivatives (PPDs) and the recently developed DST antigens. Blood was drawn for IGRA every two months. Bovine (PPD-B) and avian (PPD-A) tuberculin, were used as stimulating antigens in both skin and interferon-gamma (IFN-γ) release assays. A novel peptide cocktail representing the antigens ESAT-6, CFP-10 and Rv3615c was also utilized. We

refer to the skin test format of the DST as DST10 and the IGRA as DST1 (corresponding to the units of antigen used respectively for each test). Full details of the IGRA and skin test protocols are provided in the supplementary information. The calves of the vaccinate group were subcutaneously injected with 0.5 ml (1-4 x 106 CFU) of live BCG Danish Strain SSI 1331, while the controls received 0.5 ml of 0.9% normal saline (Supplementary Figure 2.4).

Definition of logical infection histories and chain binomial model to estimate vaccine efficacy

For the purpose of analysis, the infectious status for each animal is imputed through the response to each of the candidate DIVA (Differentiates Infected from Vaccinated Animals) tests. For each variant of the DIVA test (DST1, DST10) we construct a logical infection history where animals are considered to be infected - and potentially infectious - from the first positive test. The number of new infections per observation period is therefore calculated as the number of animals that switch diagnostic status within that interval.

From the imputed time-series of infection states we can estimate the rate of transmission to control and vaccinate animals respectively using a chain binomial model (27,36) where we assume that the rate of susceptible animals acquiring infection between two tests is constant and given by the number of infected (unvaccinated) animals I and infected vaccinates I_V at the earlier test:

$$R(S \rightarrow I) = \beta \frac{(I + \varepsilon_I I_V)}{N}$$

Where N is the total number of animals within the experimental group, β can be interpreted as the per capita transmission rate per infectious animal and ε_I measures the reduction in infectiousness of vaccinated animals that become infected (relative to unvaccinated controls). Under these assumptions the probability of transmission within a discrete time interval can be calculated and the number of new cases within that interval will be binomially distributed. As the probability of infection takes the same function functional form as the complimentary log-log transformation, this chain binomial model can be estimated using a generalized linear model (GLM) with binomial response (and complementary log-log link function). We use the rstanarm package (37) in R (38) to estimate the model within a Bayesian framework and obtain

posterior estimates for the direct vaccine efficacy to reduce susceptibility (1-ε_S), efficacy to reduce infectiousness (1-ε_I) and average base transmission rate (β) within the two experimental barns. Convergence was assessed using standard diagnostics (Rhat and effective sample size) and model fit was assessed using posterior predictive checks (specifically by forming Bayesian predictive p-values for the proportion of zeros, maximum and mean values).

Mechanistic transmission model for scenario analysis

A stochastic meta-population model with herds as the basic epidemiological unit was developed to carry out scenario analyses for the potential impacts of BCG vaccination in Ethiopia and implemented using the R package SimInf (39). This compartmental model tracks four possible epidemiological states: susceptible (*S*), infected (*I*), vaccinated (*V*), and infected vaccinated (I_v) and eight events corresponding to birth, transmission and mortality (Supplementary Figure 2.9). Full details of the model structure and calibration are presented in supplementary information along with raw data, R scripts and model code (40).

Transmission rates within herds were calibrated from estimates of the basic reproduction R_0 derived from tuberculin testing data from herds in five study sites across Ethiopia including the dairy belt surrounding Addis Ababa and emerging markets in Gondar, Hawassa, Mekelle and Kombolcha (14–16). Herds were assumed to be endemically infected with the value of R_0 inferred from the quasi-stationary distribution of the susceptible-infected-susceptible (SIS) model (41). To adjust for the imperfect sensitivity and specificity of tuberculin testing the true prevalence of infection within herds was inferred using a latent class model (42,43). The model was implemented in Stan and analyzed in R using the RStan package (37). Transmission between herds was assumed to be mediated by cattle movements. Using samples of movement records collected alongside tuberculin testing data for a subset of herds in Ethiopia we used exponential random graph models (ERGMs) to construct generative models for synthetic movement networks with the same statistical properties as the measured movement networks from Gondar, Hawassa and Mekelle (44,45).

The initial number of animals in a herd was randomly sampled from the recorded herd sizes from this subset of our study populations of herds with movement data. The population size of a herd was assumed to be constant and the birth rate in a herd was balanced against the mortality

rate and the average movement rate of cattle in the herd. The effectiveness of vaccination as a control measure for bTB was evaluated by comparing scenarios with no vaccination to those where all newborn calves were vaccinated. For all scenarios, we sample vaccine efficacy from the posterior estimates derived from our natural transmission study and carried out a further sensitivity analysis to the reduction in infectiousness (ε_i) for values of 0%, 25% and 50%. The range of vaccination scenarios explored are summarized in Figure 2.3, outputs were simulated from 1,500 replicates of the stochastic model.

2.5 References

1. F. Olea-Popelka *et al.*, Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*—a call for action. *Lancet Infect Dis*. **17**, e21–r25(2017). doi: 10.1016/S1473-3099(16)30139-6; pmid: 27697390
2. S. J. More, B. Radunz, R. J. Glanville, Lessons learned during the successful eradication of bovine tuberculosis from Australia. *Vet. Rec. 177, 224–232 (2015). doi: 10.1136/vr.103163; pmid: 26338937*
3. A. L. Olmstead, P. W. Rhode, An Impossible Undertaking: The Eradication of Bovine Tuberculosis in the United States. *J Econ Hist*. 2004 Sep;64(3):734–72.
4. J. McCormack, Horizon scanning: what next for bovine TB control in England? *Ir Vet J*. 2023 Jul 31;76(1):18.
5. More SJ. bTB eradication in Ireland: where to from here? *Ir Vet J*. 2023 Jul 4;76(1):11.
6. Livingstone P, Hancox N, Nugent G, de Lisle G. Toward eradication: the effect of *Mycobacterium bovis* infection in wildlife on the evolution and future direction of bovine tuberculosis management in New Zealand. N Z Vet J. 2015 Mar 25;63(sup1):4–18.
7. Conlan AJ, Pollock EB, McKinley TJ, Mitchell AP, Jones GJ, Vordermeier M, *et al.* Potential benefits of cattle vaccination as a supplementary control for bovine tuberculosis. PLoS Comput Biol. 2015;11(2):e1004038.
8. EFSA. Scientific Opinion on field trials for bovine Tuberculosis vaccination. EFSA J. 2013;11(12):3475.

9. Whelan AO, Coad M, Upadhyay BL, Clifford DJ, Hewinson RG, Vordermeier HM. Lack of correlation between BCG-induced tuberculin skin test sensitisation and protective immunity in cattle. Vaccine. 2011 Jul 26;29(33):5453–8.

10. Whelan AO, Clifford D, Upadhyay B, Breadon EL, McNair J, Hewinson GR, *et al.* Development of a Skin Test for Bovine Tuberculosis for Differentiating Infected from Vaccinated Animals. J Clin Microbiol. 2010 Jan 9;48(9):3176–81.

11. Bayissa B, Sirak A, Zewude A, Worku A, Gumi B, Berg S, *et al.* Field evaluation of specific mycobacterial protein-based skin test for the differentiation of *Mycobacterium bovis*-infected and Bacillus Calmette Guerin-vaccinated crossbred cattle in Ethiopia. Transbound Emerg Dis. 2022;69(4):e1–9.

12. Srinivasan S, Jones G, Veerasami M, Steinbach S, Holder T, Zewude A, *et al.* A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci Adv. 2019 Jul 17;5(7):eaax4899.

13. Srinivasan S, Subramanian S, Shankar Balakrishnan S, Ramaiyan Selvaraju K, Manomohan V, Selladurai S, *et al.* A Defined Antigen Skin Test That Enables Implementation of BCG Vaccination for Control of Bovine Tuberculosis: Proof of Concept. Front Vet Sci [Internet]. 2020 [cited 2023 Aug 7];7. Available from: https://www.frontiersin.org/articles/10.3389/fvets.2020.00391

14. Firdessa R, Tschopp R, Wubete A, Sombo M, Hailu E, Erenso G, *et al.* High Prevalence of Bovine Tuberculosis in Dairy Cattle in Central Ethiopia: Implications for the Dairy Industry and Public Health. PLOS ONE. 2012 Dec 28;7(12):e52851.

15. Mekonnen GA, Conlan AJK, Berg S, Ayele BT, Alemu A, Guta S, *et al.* Prevalence of bovine tuberculosis and its associated risk factors in the emerging dairy belts of regional cities in Ethiopia. Prev Vet Med. 2019 Jul 1;168:81–9.

16. Almaw G, Conlan AJK, Ameni G, Gumi B, Alemu A, Guta S, *et al.* The variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia provides opportunities for targeted intervention. PloS One. 2021;16(7):e0254091.

17. Srinivasan S, Easterling L, Rimal B, Niu XM, Conlan AJK, Dudas P, *et al.* Prevalence of Bovine Tuberculosis in India: A systematic review and meta-analysis. Transbound Emerg Dis [Internet]. [cited 2018 Aug 17];0(0). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/tbed.12915

18. Sibhat B, Asmare K, Demissie K, Ayelet G, Mamo G, Ameni G. Bovine tuberculosis in Ethiopia: A systematic review and meta-analysis. Prev Vet Med. 2017 Nov 1;147(Supplement C):149–57.

19. Srinivasan S, Conlan AJK, Easterling LA, Herrera C, Dandapat P, Veerasami M, *et al.* A Meta-Analysis of the Effect of Bacillus Calmette-Guérin Vaccination Against Bovine Tuberculosis: Is Perfect the Enemy of Good? Front Vet Sci [Internet]. 2021 [cited 2023 Aug 7];8. Available from: https://www.frontiersin.org/articles/10.3389/fvets.2021.637580

20. Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, *et al.* Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials. Clin Infect Dis. 2014 Feb 15;58(4):470–80.

21. Shim E, Galvani AP. Distinguishing vaccine efficacy and effectiveness. Vaccine. 2012 Oct 19;30(47):6700–5.

22. Lopez-Valencia G, Renteria-Evangelista T, Williams J de J, Licea-Navarro A, Mora-Valle AD la, Medina-Basulto G. Field evaluation of the protective efficacy of *Mycobacterium bovis* BCG vaccine against bovine tuberculosis. Res Vet Sci. 2010 Feb;88(1):44–9.

23. De Jong MCM, Kimman TG. Experimental quantification of vaccine-induced reduction in virus transmission. Vaccine. 1994 Jan 1;12(8):761–6.

24. Orsel K, de Jong MCM, Bouma A, Stegeman JA, Dekker A. Foot and mouth disease virus transmission among vaccinated pigs after exposure to virus shedding pigs. Vaccine. 2007 Aug 21;25(34):6381–91.

25. Conlan AJK, Vordermeier M, Jong MC de, Wood JL. The intractable challenge of evaluating cattle vaccination as a control for bovine Tuberculosis. eLife. 2018 Jun 5;7:e27694.

26. Velthuis AGJ, De Jong MCM, Kamp EM, Stockhofe N, Verheijden JHM. Design and analysis of an Actinobacillus pleuropneumoniae transmission experiment. Prev Vet Med. 2003 Jul 30;60(1):53–68.

27. Velthuis AGJ, Bouma A, Katsma WEA, Nodelijk G, De Jong MCM. Design and analysis of small-scale transmission experiments with animals. Epidemiol Infect. 2007 Feb;135(02):202–17.

28. Vordermeier HM, Jones GJ, Buddle BM, Hewinson RG, Villarreal-Ramos B. Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. Annu Rev Anim Biosci. 2016;4(1):87–109.

29. Mekonnen GA, Ameni G, Wood JLN, Berg S, Conlan AJK, The ETHICOBOTS consortium. Network analysis of dairy cattle movement and associations with bovine tuberculosis spread and control in emerging dairy belts of Ethiopia. BMC Vet Res. 2019 Jul 26;15(1):262.

30. Meisinger G. [Economic effects of the elimination of bovine tuberculosis on the productivity of cattle herds. 2. Effect on meat production. Monatsh Veterinarmed. 1970 Jan 1;25(1):7–13.

31. Aznar I, Frankena K, More SJ, O'Keeffe J, McGrath G, de Jong MCM. Quantification of *Mycobacterium bovis* transmission in a badger vaccine field trial. Prev Vet Med. 2018 Jan 1;149:29–37.

32. Carter SP, Chambers MA, Rushton SP, Shirley MDF, Schuchert P, Pietravalle S, *et al.* BCG Vaccination Reduces Risk of Tuberculosis Infection in Vaccinated Badgers and Unvaccinated Badger Cubs. PLOS ONE. 2012 Dec 12;7(12):e49833.

33. Parlane NA, Shu D, Subharat S, Wedlock DN, Rehm BHA, Lisle GW de, *et al.* Revaccination of Cattle with Bacille Calmette-Guérin Two Years after First Vaccination when Immunity Has Waned, Boosted Protection against Challenge with *Mycobacterium bovis*. PLOS ONE. 2014 Sep 2;9(9):e106519.

34. Lemma H, Belihu K, Sheferaw3 D. Study on the reproductive performance of Jersey cows at Wolaita Sodo dairy farm, Southern Ethiopia. Ethiop Vet J. 2010 Jan 1;14(1):53–70.

35. Brooks-Pollock E, Wood JLN. Eliminating bovine tuberculosis in cattle and badgers: insight from a dynamic model. Proc R Soc B. 2015 Jun 7;282(1808):20150374.

36. Velthuis AGJ, De Jong MCM, De Bree J. Comparing methods to quantify experimental transmission of infectious agents. Math Biosci. 2007 Nov;210(1):157–76.

37. Goodrich B, Gabry J, Ali I, Brilleman S. rstanarm: Bayesian applied regression modeling via Stan. R package version 2.21.3 [Internet]. 2022. Available from: https://mc-stan.org/rstanarm

38. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2019. Available from: https://www.R-project.org/

39. Widgren S, Bauer P, Eriksson R, Engblom S. SimInf: An R Package for Data-Driven Stochastic Disease Spread Simulations. J Stat Softw. 2019;91(12):1–42.

40. Willgert K, Conlan AJK. MonkeyMyshkin/BCGCrossover: BCG vaccination of cattle reduces transmission of bovine tuberculosis, improving the prospect of elimination [Internet]. Zenodo; 2023 [cited 2023 Dec 21]. Available from: https://zenodo.org/records/10417490

41. Allen LJS. An Introduction to Stochastic Epidemic Models. In: Brauer F, van den Driessche P, Wu J, editors. Mathematical Epidemiology [Internet]. Berlin, Heidelberg: Springer; 2008 [cited 2023 Mar 31]. p. 81–130. (Lecture Notes in Mathematics). Available from: https://doi.org/10.1007/978-3-540-78911-6_3

42. Hui SL, Walter SD. Estimating the Error Rates of Diagnostic Tests. Biometrics. 1980;36(1):167–71.

43. Collins J, Huynh M. Estimation of diagnostic test accuracy without full verification: a review of latent class methods. Stat Med. 2014 Oct 30;33(24):4141–69.

44. Schweinberger M, Krivitsky PN, Butts CT, Stewart J. Exponential-Family Models of Random Graphs: Inference in Finite-, Super-, and Infinite Population Scenarios. Stat Sci [Internet]. 2020 Nov 1 [cited 2023 Aug 4];35(4). Available from: http://arxiv.org/abs/1707.04800

45. Krivitsky PN, Hunter DR, Morris M, Klumb C. ergm 4: New features. J Stat Softw [Internet]. 2023 [cited 2023 Aug 9];105(6). Available from: http://arxiv.org/abs/2106.04997

46. Vordermeier H, Sidders B, Stoker N, Ewer K. Mycobacterium Antigens [Internet]. 2009 [cited 2023 Dec 21]. Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2009060184

47. Vordermeier H, Whelan A. Diagnostic Reagents [Internet]. 2011 [cited 2023 Dec 21]. Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2011135369

48. Jones G, Vordermeier H. Diagnostic Reagents [Internet]. 2012 [cited 2023 Dec 21]. Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO201201087

49. Kapur V, Srinivasan S, Vordermeier H, Jones G. Diagnostic Reagents [Internet]. 2020 [cited 2023 Dec 21]. Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2020208368

50. O'Hagan MJH, Ni H, Menzies FD, Pascual-Linaza AV, Georgaki A, Stegeman JA. Test characteristics of the tuberculin skin test and post-mortem examination for bovine tuberculosis diagnosis in cattle in Northern Ireland estimated by Bayesian latent class analysis with adjustments for covariates. Epidemiol Infect [Internet]. 2019 ed [cited 2022 Mar 7];147. Available from: https://www.cambridge.org/core/journals/epidemiology-and-infection/article/test-characteristics-of-the-tuberculin-skin-test-and-postmortem-examination-for-bovine-tuberculosis-diagnosis-in-cattle-in-northern-ireland-estimated-by-bayesian-latent-class-analysis-with-adjustments-for-covariates/3C45D3F3F5634138EE0DDE1F8762A127

51. Bronsvoort BM deC, Koterwas B, Land F, Handel IG, Tucker J, Morgan KL, *et al.* Comparison of a Flow Assay for Brucellosis Antibodies with the Reference cELISA Test in West African *Bos indicus*. *PLOS ONE*. 2009 Apr 20;4(4):e5221.

52. Dendukuri N, Hadgu A, Wang L. Modeling conditional dependence between diagnostic tests: a multiple latent variable model. *Stat Med*. 2009 Feb;28(3):441–61.

53. Irwin RJ, Hautus MJ. Lognormal Lorenz and normal receiver operating characteristic curves as mirror images. *R Soc Open Sci*. 2(2):140280.

54. Stan Development Team. RStan: the R interface to Stan [Internet]. 2023. Available from: https://mc-stan.org/

55. Krivitsky PN, Kuvelkar AR, Hunter DR. Likelihood-based Inference for Exponential-Family Random Graph Models via Linear Programming [Internet]. arXiv; 2022 [cited 2023 Aug 4]. Available from: http://arxiv.org/abs/2202.03572

56. Hunter DR, Handcock MS, Butts CT, Goodreau SM, Morris M. ergm: A Package to Fit, Simulate and Diagnose Exponential-Family Models for Networks. *J Stat Softw*. 2008 May 1;24(3):nihpa54860.

57. Hunter DR, Goodreau SM, Handcock MS. Goodness of Fit of Social Network Models. *J Am Stat Assoc*. 2008 Mar 1;103(481):248–58.

58. Clark DA, Handcock MS. Comparing the Real-World Performance of Exponential-Family Random Graph Models and Latent Order Logistic Models for Social Network Analysis. *J R Stat Soc Ser A Stat Soc*. 2022 Apr 1;185(2):566–87.

59. Csardi G, Nepusz T. The igraph software package for complex network research. *Inter Journal*. 2006;Complex Systems:1695.

60. Newman M. Networks, 2nd ed. [Internet]. Oxford University Press, Oxford, United Kingdom; 2016. Available from: https://doi.org/10.1093/oso/9780198805090.001.0001

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Competing interests: APHA (the former employer for H.M.V) holds three patents (patent numbers WO/2009/060184 (46), WO/2011/135369 (47), and WO/2012/010875 (48)) for the use of Rv3615c in diagnostic tests for bTB. In addition, APHA and Penn State (S.S. and V.K.) are in the process of intellectual property protection filings for a peptide based DIVA skin test (WO/2020/208368 (49)). SS is also affiliated with the Department of Epidemiology and Biostatistics, College of Human Medicine, Michigan State University, East Lansing, USA. The authors declare no other competing interests.

Animals: All relevant local biosecurity and safety procedures were adhered to.

Data and materials availability: All data and code are available in the main text or the supplementary materials and at: https://github.com/MonkeyMyshkin/BCGCrossover (40)

List of Supplementary Materials

Supplementary Materials and Methods
Supplementary Text
Supplementary Tables 2.1 to 2.6
Supplementary Figures 2.1 to 2.9

CHAPTER THREE: COMPARATIVE PERFORMANCE OF TUBERCULIN AND DEFINED-ANTIGEN COCKTAILS FOR DETECTING BOVINE TUBERCULOSIS IN BCG-VACCINATED CATTLE IN NATURAL SETTINGS

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3.1 Abstract

Background: Bovine tuberculosis (bTB) is a threat to cattle health and public safety. The current test-and-slaughter control programs implemented in developed countries are hampered by wildlife reservoirs while countries like Ethiopia remain without option due to socioeconomic barriers. Vaccinating cattle with Bacillus Calmette-Guérin (BCG) effectively reduces transmission, offering a potential solution for controlling bTB. A key requirement for vaccination strategies using BCG is the validation of defined antigens to differentiate infections among vaccinated animals (DIVA).

Methods: We compared tuberculin with DIVA peptide cocktails (ESAT-6, CFP-10, and Rv3615c) in 67 unvaccinated and 67 BCG-vaccinated cattle exposed to *M. bovis* in a natural setting. The cattle were tested every four months with a skin test and every two months with interferon-gamma (IFN-γ) release assays (IGRA) over a year of exposure.

Results: Before exposure, the DIVA skin, DIVA IGRA, and tuberculin tests showed 100% specificity in unvaccinated control calves. After exposure, the DIVA skin, DIVA IGRA, and comparative cervical tuberculin (CCT) tests had comparable sensitivities of 46% (95% CI: 36, 56), 45% (95% CI: 35, 55), and 47 (95% CI: 37, 57), respectively, when assessed against animals positive by *M. bovis* culture PCR.

Conclusion: The results suggest that test-and-slaughter control strategies using tests with low sensitivity are not expected to be effective in controlling bTB in high-prevalence herds, and highlight an urgent need to improve the sensitivity of DIVA diagnostic tests for bTB in these settings.

Keywords: BCG vaccination, bovine tuberculosis, defined antigen cocktails, tuberculin, IGRA and skin tests

3.2 Introduction

Bovine tuberculosis (bTB) is a chronic disease of cattle primarily caused by members of the *Mycobacterium tuberculosis* complex (MTBC), which can infect a wide range of domestic and wild animal species, including humans, posing a significant public health risk in regions with high prevalence [1]. The prevalence of bTB varies by country, influenced by control measures, animal movements and wildlife reservoirs [2]. In Ethiopia, bTB is more prevalent in genetically improved cattle raised for high milk production in intensive farming systems, with prevalence rates of 21.6% in Holstein-Friesians and 9.9% in crossbred cattle [3]. A recent study reported 54.4% of 299 dairy herds and 24.5% of 5,675 cattle were reactive to the comparative intradermal cervical tuberculin test [4]. In the absence of feasible bTB control options, efforts to increase milk productivity through cross-breeding and intensification of cattle farming are likely to increase disease incidence.

Currently, control of bTB relies on tuberculin testing of herds and culling positive test reactors. This approach has successfully reduced the prevalence of the disease in many countries and has led to elimination in Australia [5]. However, this strategy is extremely costly and unaffordable for resource-constrained countries like Ethiopia. It is also less effective in countries where wildlife reservoir hosts are present [6,7]. Bacillus Calmette-Guérin (BCG) vaccination offers a potentially practical and affordable alternative for countries lacking existing control programs [8–10]. Experimental investigations and field studies have demonstrated that while BCG does not protect all vaccinated animals from infection, it significantly reduces the severity of bTB pathology [7,8,10,11]. The BCG vaccine developed from attenuated *M. bovis* and approved for human tuberculosis, is considered a viable candidate vaccine for cattle. However, the World Organization for Animal Health (WOAH) does not recommend BCG for livestock due to its interference with tuberculin-based tests leading to false-positive reactions in official bTB control programs [1]. To overcome the issue of non-specific tuberculin reactions caused by BCG vaccination, researchers are exploring the use of defined antigens that can differentiate infected among vaccinated animals (DIVA), a crucial tool for effective implementation of BCG vaccination as a method of disease control[12,13].

Comparative genomic and transcriptomic studies have identified several antigens present in *M. bovis* field strains but either absent or not immunogenic in the BCG vaccine strain, enhancing the specificity of tuberculin-based bTB diagnostic tests[14,15]. Previous studies have shown that recombinant peptide antigens induce a greater skin response when used in combination than

when used alone [16]. Notable candidate antigens that demonstrated significant DIVA capability include ESAT-6, CFP-10, and Rv3615c, indicating a potential path to use along with BCG vaccination for bTB management and control [13,17].

The performance of these antigens is subject to variability depending on the geographical conditions, prevailing *Mycobacterium* lineages, dosage, cutoff values, and features of the examined population. Variability in the levels of interferon-gamma (IFN-γ) and innate immune response among different strains of *M. bovis* has been reported [18]. Therefore, prior to practical deployment, thorough validation of these DIVA antigens is required across a varied range of cattle breeds and geographic regions to determine their specificity and sensitivity. However, existing research data on the performance of DIVA tests in BCG-vaccinated animals are limited to studies primarily performed in the UK under experimental transmission [19,20], which might not reflect the performance of DIVA antigens in naturally infected cattle. Although other studies have also tested DIVA and tuberculin in BCG-vaccinated and unvaccinated cattle under natural transmission settings, they were limited to evaluating the efficacy of the BCG vaccine [7,8,21–23] or comparing the relative performance of antemortem tests without postmortem confirmation [13,17,24].

This study examined diagnostic data collected for the evaluation of BCG efficacy via a natural transmission experiment [10] for the assessment of the immunological responses of BCG-vaccinated cattle to mycobacterial antigens. The other similar studies published thus far have relied on the use of the DIVA skin test and IFN-γ release assay (IGRA) to determine the infection status of artificially challenged animals based on predetermined cutoff values. Here, we compared the quantitative responses of vaccinated and control groups to DIVA antigens against tuberculin. The study animals were followed longitudinally, with repeated tests and postmortem confirmation of infection status, providing a unique opportunity to assess the performance of bTB diagnostic tests in animals with confirmed (postmortem) disease status. Here, we evaluated the immune responses of DIVA peptide antigen cocktails on 67 BCG-vaccinated and 67 unvaccinated calves via both skin tests and *in vitro* IGRA formats and compared the performance of these peptides to that of tuberculin.

3.3 Materials and Methods

3.3.1 Study setting

The study took place at the Animal Health Institute (AHI) in Sebeta, which is located approximately 20 km southwest of the capital city, Addis Ababa, Ethiopia. At the AHI premises, three separate barns were constructed. Each barn had the capacity to accommodate 80 adult cattle and had a fenced outdoor area measuring approximately 2,000 to 2,500 m2. These barns were approximately 300 meters apart from each other. Two of the fenced barns were designated for housing the experimental replicate groups (seeders and sentinels) throughout the entire exposure period. This was done to control for any potential variations in exposure resulting from differences in infectiousness among the seeder animals. The third barn was used to house and acclimate the sentinel calves immediately after they were recruited and before they were exposed to the seeders. To ensure proper waste management, septic tanks were constructed behind each barn. The feces and urine were directed into the septic tanks through large pipes that connected the barns to the exterior premises. The animal attendants were all educated about the zoonotic risk of bTB. They were required to wear personal protective equipment, including N95 masks, disposable gloves, and rubber boots, whenever they handled animals or worked inside the barns. All relevant local biosecurity and safety procedures were strictly adhered to.

3.3.2 Study animals and design of the experiment

A total of 72 adult cattle, naturally infected and identified as reactors through skin and blood tests, were selected as seeders for the natural transmission experiment. The primary objective of our experimental study was to assess the efficacy of BCG vaccination through a natural transmission experiment. In this report, we conducted a secondary analysis of diagnostic data collected from the BCG efficacy study [10]. The experiment was divided into two phases, each involving an equal number of calves and comprising two biological replicate groups housed in separate barns. The reason for conducting the study in two phases was to measure both how well the vaccine prevented infection (direct effect) and how it reduces the infectiousness of infected vaccinated animals (indirect effect) for a more complete understanding of the total efficacy of the BCG vaccine. To find naive sentinels, dairy herds were screened using both skin tests and IGRA. For each phase, 68 male calves, less than three months old, were recruited from dairy herds that tested negative for bTB. These herds were located in Koka, Ziway, Alage,

Hawassa, Wondogenet, Jimma, and Ambo towns. The recruited sentinels were transported to AHI and housed separately from the seeders in an acclimatization barn to prevent early exposure. After an acclimatization period of three to twelve weeks, the sentinels that tested negative in the bovine (PPD-B) minus avian (PPD-A) tuberculin IGRA test were randomly assigned to either the control or vaccinated groups using a double-blind lottery system by drawing animal ID from one box and treatment group from the second box. Calves with a positive OD450 nm ≥0.1 on the bovine minus avian tuberculin IGRA test were excluded. In the first phase, 33 unvaccinated controls and 34 vaccinated calves were included. In the second phase, 34 unvaccinated controls and 33 vaccinated calves were included, resulting in a total of 67 calves per group across both phases. During the trial period, calves were fed boiled and pasteurized milk until they reached six months of age. Afterward, weaned calves and adult seeders were provided with concentrate feed, hay, green fodder, and water *ad libitum*. Throughout the twelve-month contact period, 5 vaccinated calves and 2 unvaccinated controls from both phases died, resulting in 127 (62 vaccinated and 65 unvaccinated controls) reaching the endpoint (Figure 3.1).

3.3.3 Test antigens

The PPD (purified protein derivative) tuberculin were procured from Prionics Lelystad BV, Lelystad, the Netherlands. DIVA peptide cocktails consisting of peptides representing ESAT-6, CFP-10, and Rv3615c were commercially synthesized and previously described [13].

3.3.4 BCG vaccination

The vaccine was supplied as freeze-dried preparation and reconstituted in Sauton's medium as per the manufacturer's instruction (Green Signal Bio Pharma, Pvt. Ltd., India) in the first phase and (AJ Vaccines A/S, Artillerivej 5, DK 2300 Copenhagen S, Denmark) in the second phase. The vaccinated group of calves were administered a subcutaneous injection of 0.5 ml (1-4 × 10^6 CFU) of live attenuated BCG Danish Strain SSI 1331 (Staten's Serum Institute [SSI], Copenhagen, Denmark). Conversely, the control group received 0.9% normal saline. A total of 67 calves received the BCG vaccination, while the remaining 67 sentinel calves were not vaccinated. The authors conducting the field work were blinded to the vaccination status of the animals until the conclusion of the experiment.

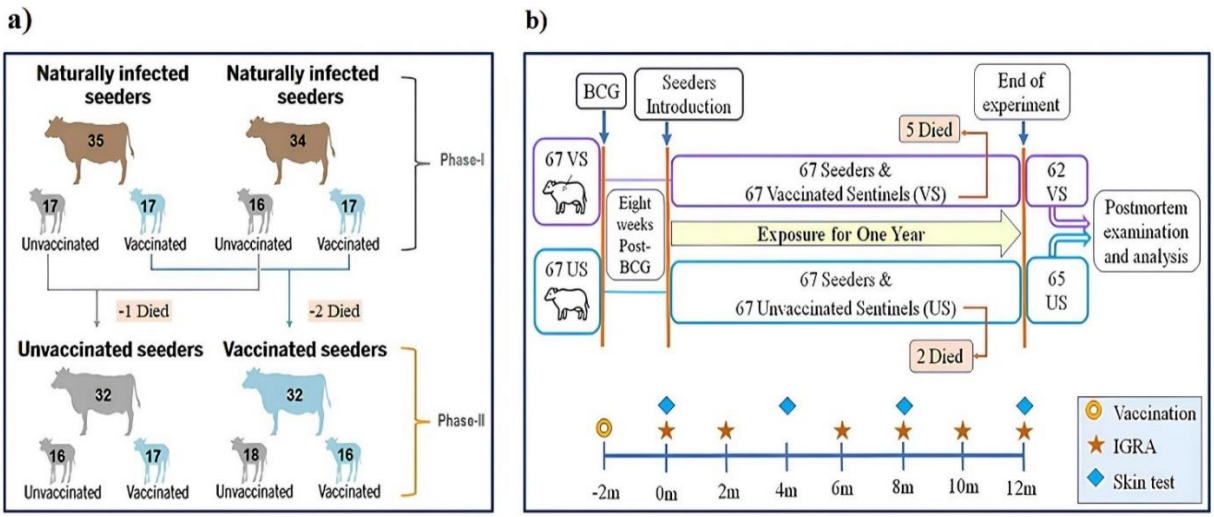


Figure 3.1: Schematic representation of the experimental protocol. **a)** The two-phase cross over design of the experiment and **b**) Testing schedules and postmortem examination. In phase I, two groups (Groups 1 & 2) functioned as biological replicates and were kept in a separate barn a) Phase I, Group 1 (top left): 17 unvaccinated controls and 17 vaccinated calves were exposed to 35 seeders. Phase I, Group 2 (top right): 16 unvaccinated controls and 17 vaccinated calves were exposed to 34 seeders. Phase II, Group 1 (lower left): the surviving 32 unvaccinated control sentinels from Phase I were pooled and became seeders for 16 unvaccinated controls and 17 vaccinated calves. Phase II, Group 2 (lower right): the surviving 32 vaccinated calves from Phase I were pooled and became seeders for 18 unvaccinated controls and 16 vaccinated calves. b) From both Phase-I and Phase-II, a total of 67 unvaccinated control (67 U) and 67 vaccinated (67 V) calves were exposed to their respective seeders eight weeks after vaccination and kept in contact for twelve months. During the twelve-month contact period, 5 vaccinated calves and 2 unvaccinated controls died across both phases, resulting in 62 vaccinated and 65 unvaccinated controls reaching the endpoint. IGRA tests were performed every two months, and skin tests were conducted every four months, starting just before exposure (0m) and continuing until the end of the experiment (12m). At the end of the experiment, all animals were slaughtered, and a postmortem examination was conducted to detect bTB lesions, followed by mycobacterial culture and PCR testing of culture-positive isolates.

3.3.5 Skin and IGRA Testing Schedules

The sentinel animals underwent skin testing every four months and IGRA every two months using DIVA, avian, and bovine tuberculin antigens in both the skin and IGRA test formats (Figure 3.1). The skin test and IGRA results analyzed and reported here are from the 12th-month post-exposure test, except for Table 3.1 and Figure 3.1, which are based on pre-exposure test result and the Supplementary Figure 3.1, which includes tests of all postexposure-time points.

3.3.6 Skin Testing Procedure

For the skin test, hair was shaved at two sites on the right and one site on the left mid-cervical area. Bovine tuberculin (30,000 IU/ml), avian tuberculin (25,000 IU/ml), and the DIVA peptide cocktail (100 µg/ml of each peptide) were injected intradermally in 0.1 mL volumes. Skin fold thickness was measured to the nearest millimeter using Irish calipers by the same person before and 72 hours after antigen administration. The change in skin thickness was determined by subtracting the pre-injection measurement from the 72-hour post-injection measurement for each antigen. The cut-offs used to determine test status were >4 mm for the Comparative Cervical Tuberculin (CCT), ≥4 mm for the Single Intradermal Tuberculin Test (SIT), and ≥2 mm for the DIVA antigens. The defined antigens induce considerably lower amplitude of skin reactions in reactor cattle with an average of 8 ± 1.5 mm as compared with 19.5 ± 3.5 mm for PPD-B without compromising sensitivity at the ≥2 mm cutoff for the DIVA established in previous study using ROC analyses (Srinivasan et al., 2020).

3.3.7 IGRA Testing Procedure

The IGRA test was conducted using the BOVIGAM® kit (Thermo Fisher Scientific Inc., USA). Approximately 5 ml of blood was collected from each animal's jugular vein in lithium heparin coated vacutainers as recommended by the BOVIGAM® kit based IFN-ɤ assay. Duplicate wells were stimulated with 25 µl of each antigen and 250 µl of whole blood, resulting in final concentrations of 1 µg/ml DIVA antigens, 250 IU/ml avian tuberculin (PPD-A), and 300 IU/ml bovine tuberculin (PPD-B). Positive and negative controls were stimulated with pokeweed mitogen (PWM) at 10 µg/ml and RPMI (Nil), respectively. Samples were incubated at 37°C in a humid atmosphere for 18–24 hours. The culture supernatants from duplicate samples were pooled and stored at -80°C until the IFN-γ test was performed. The results of the IGRA test were reported as the change in optical density (OD) at 450 nanometers (ΔOD450 nm). This

was determined by subtracting the OD450 nm of whole blood cultures stimulated with antigen from the OD450 nm of cultures without antigen. Optical Density (OD) refers to the amount of light absorbed by the sample, and 450 nm is the specific wavelength of light used for the measurement. The intensity of the color corresponds to the OD450 nm value and is directly related to the concentration of the target antigen in the sample.

3.3.8 Postmortem examination

After humanely euthanizing the animals using a captive bolt (CASH® special .22 caliber penetrating captive bolt) followed by immediate exsanguination, postmortem examinations were carried out by a skilled pathologist assisted by two veterinarians with extensive experience in diagnosing bTB lesions. At the end of the natural exposure to infection, all calves were euthanized and examined. Following visual inspection and palpation of each lymph node and tissue in the carcass, the relevant organs for sampling were separated and transferred to the postmortem examination table for detailed evaluation. To assess the true disease status, all the lymph nodes were sliced into 1-2 cm thick sections and meticulously examined. The animal was classified as bTB lesion positive (lesioned) if any of the examined tissues had gross visible lesions consistent with bTB and negative (non-lesioned) if no obvious lesions were found. The severity of the gross lesions was evaluated using the semiquantitative technique developed by Vordermeier *et al.*, [25] taking the number and size of the observed lesions into account.

Tissue sampling for mycobacterial culture

For bacterial culture, samples were collected from ten different tissues of each animal, including mandibular, lateral retropharyngeal, medial retropharyngeal, bronchial, cranial tracheobronchial, mediastinal, hepatic and mesenteric lymph nodes as well as from the left and right lung lobes, regardless of the presence or absence of lesions. Extra samples were collected when lesions were found in other examined lymph nodes and tissues. The lymph node and tissue samples were collected in sterile 50 mL Falcon tubes containing 10-15 mL of 0.9% saline solution and transported to the laboratory for bacterial culture and isolation. Prior to processing, the tissue samples were frozen at −20°C.

Mycobacterial culture

For mycobacterial isolation, lymph nodes or tissue samples were cut with sterile blades into thin slices. The pieces were then homogenized with a sterile pestle and mortar and transferred into a sterile plastic bag for further grinding in a stomacher. Then, 5 ml of the homogenate was transferred to a sterile 50 ml Falcon tube, decontaminated by adding an equal volume of 4% NaOH and mixing for 15 minutes. The mixture was neutralized by adding 40 ml of PBS, followed by centrifugation at 1,106 g for 15 minutes. The supernatant was discarded, and 0.1 ml of the sediment from each sample was spread onto duplicate slants of Lowenstein–Jensen (LJ) medium (Sigma Aldrich, Co, St. Louis, USA) [1], one enriched with sodium pyruvate and the other with glycerol. The cultures were then incubated aerobically at 37°C for up to 8 weeks, after which the growth of the mycobacterial colonies was observed weekly. Slants that did not show growth beyond the 8th week were considered to be negative. Ziehl–Neelsen (ZN) staining technique was used to confirm the presence of acid-fast bacilli (AFB) in the smears of culture-positive colonies.

DNA extraction

DNA was extracted from culture isolates using the DNeasy Blood and Tissue Kit (QIAGEN) with some modifications. First, loopfuls of cells were removed from culture-positive LJ media using a sterile loop and transferred to 2 ml Eppendorf tubes containing 300 μl of TE buffer. The tubes were then incubated at 85°C for 60 minutes on a heating block to inactivate the bacteria. Lysis buffer was prepared by adding 20 ml of 20 mm Tris-HCl, 12 ml of Triton X and 2 ml of EDTA to one liter of water, after which the mixture was stored at room temperature. Immediately before use, 80 mg of lysozyme was added per ml of lysis buffer to make a working lysis buffer solution. For each reconstituted culture sample, 100 µl of working lysis buffer solution was added. The samples were incubated at 37°C for 1 hour. Then, 180 µl of ATL buffer and 20 µl of proteinase K were added to each sample. The samples were incubated at 56°C for 3 hours and vortexed every hour to ensure complete mixing. Then, all downstream procedures were performed using the reagents provided by the manufacturer as described in QIAGEN [26], and the DNA was eluted in 100 µl of AE buffer.

Polymerase Chain Reaction (PCR)

The DNA samples were amplified by two-step real-time multiplex qPCR at the AHI, Sebeta, Ethiopia. In the first step of the multiplex qPCR cycle, three primer-probe sets targeting

IS1081, the yrbE3A gene and the 'RD7' deletion were used for the collective identification of both animal- and human-adapted, animal-adapted and human-adapted MTBC species. In the second step, three additional primer sets targeting the RD4 gene deletion SNP at the lepA locus and the SNP at the rskA locus were used for specific identification of *M. bovis, M. caprae and M. origis,* respectively. The target genes and primer/probe set sequences used in the current multiplex qPCR assay are as describe in our pervious study [27].

The amplification was carried out in a 20 μL final reaction mixture, which consisted of 5 μL of DNA template, 10 μL of prime gene expression master mix with ROX dye, 0.5 μL of each primer, and 3.5 μL of nuclease-free water. We used nuclease-free water as the negative control and synthetic gDNA targets (IDT, Iowa, USA) as the external positive controls in duplicate for each PCR run to ensure that the master mix and amplification conditions worked as expected. The IS1081 sequence, a common target for all MTBCs, was used as an internal amplification control. The results were considered invalid if the positive control was not detected or if the negative control tested positive. Amplification was performed using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The thermocycler protocol included one cycle of 3 minutes at 95°C for polymerase activation, 40 cycles of 15 seconds at 95°C for denaturation, and 40 cycles of 60 seconds each at 63°C for annealing/extension steps. For all primer-probe sets, a sample was considered PCR positive when the cycle threshold (Ct) value was ≤ 40.

Ethics statement

Prior to commencing the study, ethical approval was granted by the Institutional Review Board of Aklilu Lemma Institute of Pathobiology (reference number ALIPB IRB/44/2013/21). The experiment was conducted according to the Technical Guideline for National Research Ethics Review, ensuring compliance with the guidelines [28]. Additionally, the study adhered to and reported following the ARRIVE guidelines 2.0.

Statistical analyses

Data analysis was performed using GraphPad Prism version 9.4.0 for Windows (GraphPad Software, San Diego, California, USA), the R statistical language [29] and R Studio [30]. Plots were produced using the ggplot2 [31] and 'ggstatsplot' packages [32]. Normality tests were conducted using the Shapiro-Wilk test with the normality() function from the {dlookr} package in R.

Additionally, visual inspection of the distributions was conducted using the plot_histogram() and plot_density() functions from the {DataExplorer} package in R. Based on the results, the nonparametric statistical tests were used: the Wilcoxon rank-sum (Mann-Whitney U) test was applied to determine differences in skin and IFN-γ test responses between the two treatment groups; the nonparametric Friedman repeated measures ANOVA with Bonferroni correction for multiple comparisons was used to determine differences in skin and IFN-γ responses between different antigens within a group and the nonparametric Spearman's rank correlation coefficient was used to determine the strength and direction of association between the different test antigens IFN-γ and skin test responses. The rank biserial correlation coefficients were calculated using the ggbetweenstats() and ggwithinstats() functions from the {ggstatsplot} package in R. The ggbetweenstats() function was used for comparisons between two independent groups (vaccinate vs control response to each antigen) while the ggwithinstats() function was used for comparisons involving more than two dependent groups (animal immune response to different antigens). Sensitivity, specificity, positive likelihood ratio (LR+), negative likelihood ratio (LR-), and accuracy were calculated using 2 × 2 contingency tables (see Supplementary Table 3.1). In the contingency tables, columns indicate the disease status (positive or negative) determined by *M. bovis* PCR (Table 3.3) and visible lesions, culture or *M. bovis* PCR (Supplementary Table 3.2) used as a reference standard. The rows represent the positive or negative results obtained using the antemortem tests. The cells labeled (a) and (d) represent true positive and true negative test results, respectively while the cells labeled (b) and (c) represent false positive and false negative test results, respectively. The measures of diagnostic test performance were calculated as follows: sensitivity = a/(a + c); specificity = d/(b + d); LR+ = (a/(a + c))/(b/(b + d)); LR− = (c/(a + c))/(d/(b + d)); and overall diagnostic accuracy = (a + d)/N [33–35]. Cohen's kappa statistic, used to measure binary agreement between two tests for classifying animals into positive or negative groups, was calculated using the free online Graphpad web calculator. For statistical significance, $P < 0.05$ and 95% CI were used.

Definitions and interpretation of diagnostic test performance measures

Sensitivity refers to the proportion of infected animals (true positives) that are correctly identified by the test, while specificity refers to the proportion of animals without the disease that the test correctly identifies as negative. A high sensitivity indicates that the test effectively identifies infected animals, reducing the risk of false negatives, while a high specificity indicates that the test effectively excludes non-infected animals, reducing the risk of false

positives. The overall accuracy of a test refers to the proportion of correct identifications computed by dividing the sum of true positives and true negatives by the total number of animals tested [35]. The LR+ indicates the likelihood of observing a positive test in animals with the disease compared to those without the disease. A favorable test has a high positive likelihood ratio. The negative likelihood ratio (LR-) reflects how much less likely a negative test result is in diseased animals compared to healthy ones. A low LR- means that the disease is unlikely, with a negative test. The lower the value of (LR-), the more valuable that test is for increasing certainty about a negative diagnosis [35,36]. Both LR+ and LR- close to 1.0 suggest that the test does not distinguish between animals with or without the disease [35]. Kappa was interpreted using a scale as follows: kappa ≤ 0 as indicating no agreement, 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 [37].

3.4 Results

Skin test and IGRA responses of experimental calves' post BCG vaccination prior to exposure to infected animals

DIVA skin and all tuberculin skin test antigens elicited no positive responses in the unvaccinated control group, demonstrating 100% specificity within this sample (Figure 3.2**a** and Table 3.1). The DIVA skin test was positive for four vaccinated animals (specificity 94.0 (95% CI: 85.2, 98.1) (Table 3.1). Three DIVA skin test-positive vaccinated calves showed a 2 mm increase in skin thickness, while one showed a 3 mm increase at pre-exposure. However, at the fourth month post-exposure, only one showed a 2 mm increase, while the other three showed no change, with two consistently testing negative throughout the exposure period. Statistically no significant difference was observed between the proportions of vaccinated and control animals that responded positively to DIVA skin test ($p = 0.12$) (Table 3.1). However, only one vaccinated calf exhibited a positive response to the DIVA IFN-γ assay (specificity 98.5% [95% CI: 91.3, 100]). Although the OD450 nm value of the positive IFN-γ response in this vaccinated calf was 0.1, which falls on the positive cutoff value of the test (Figure 3.2**b**), the animal tested negative (ΔOD450 nm = 0.00) on the same assay at the two months after exposure. In contrast, the proportion of vaccinated animals that responded positively to the tuberculin skin test and IFN-γ assay was significantly greater than that of the controls ($p < 0.001$), confirming the lack of specificity of tuberculin antigens (Table 3.1).

Table 3.1. The specificity of the DIVA and tuberculin elicited IFN-γ and skin test responses eight-week post BCG vaccination. The cutoff value for all antigens tested in the IFN-γ assay was ≥0.1, whereas for the skin test, the cutoff values used were >4 mm for the CCT, >=4 mm for the SIT and ≥2 mm for the DIVA as established in previous study using ROC analyses (Srinivasan et al., 2020). Note: CCT (Comparative Cervical Tuberculin Test), PPD (purified protein derivative tuberculin, PPD-B (bovine tuberculin), PPD (B-A) (bovine minus avian tuberculin), SIT (Single Intradermal Tuberculin Test)

Test	Antigen	Group	Test Status		Specificity % (95%CI)	P value
			Positive	Negative		
IGRA	DIVA	Control	0	67	100 (93.5, 100)	**>0.99**
		Vaccinate	1	66	98.5 (91.3, 100)	
	PPD(B-A)	Control	7	60	89.6 (79.7, 95.1)	**<0.01**
		Vaccinate	30	37	**55.2** (43.4, 66.5)	
	PPD-B	Control	24	43	64.2 (52.2, 74.6)	**<0.01**
		Vaccinate	48	19	28.4 (18.9, 40.2)	
Skin	DIVA	Control	0	67	100 (93.5, 100)	**0.12**
		Vaccinate	4	63	94.0 (85.2, 98.1)	
	CCT	Control	0	67	100 (93.5, 100)	**<0.01**
		Vaccinate	22	45	**67.2** (55.2, 77.3)	
	SIT	Control	0	67	100 (93.5, 100)	**<0.01**
		Vaccinate	53	14	**20.9** (12.8, 32.2)	

Normality tests conducted on all measured antigen test responses resulted in p-values of <0.0001, and visual inspection of the distributions confirmed their non-normality with evident asymmetry, dictating non-parametric statistical tests for comparing responses between treatments and antigens. Eight weeks after vaccination, the BCG-vaccinated calves showed a significant increase in skin fold thickness and IFN-γ response to all tuberculin antigens compared to those of the unvaccinated controls (Figure 3.2**a** and 3.2**b**). However, for DIVA IGRA test, there was no statistically significant difference in IFN-γ production between the two groups, as indicated by a p value of 0.79. The rank biserial correlation coefficient (r) was close to zero (0.03), suggesting a negligible effect of BCG vaccination on IFN-γ production response to DIVA-specific antigen (Figure 3.2**a** and 3.2**b**).

Friedman repeated measure nonparametric ANOVA with Bonferroni correction for multiple comparisons used to compare the responses to different antigens showed that all tuberculin elicited significantly greater skin and IGRA ($p<0.001$) test responses than DIVA antigens in

BCG-vaccinated animals. In contrast, in the controls, the DIVA and all tuberculin antigens elicited similar and low skin test responses (Figure 3.2**a**). In the unvaccinated controls, avian and bovine tuberculin induced a significantly higher IFN-γ response than the DIVA. However, there was no significant difference between the DIVA and bovine minus avian tuberculin IFN-γ responses (P>0.05) (Figure 3.2**b**).

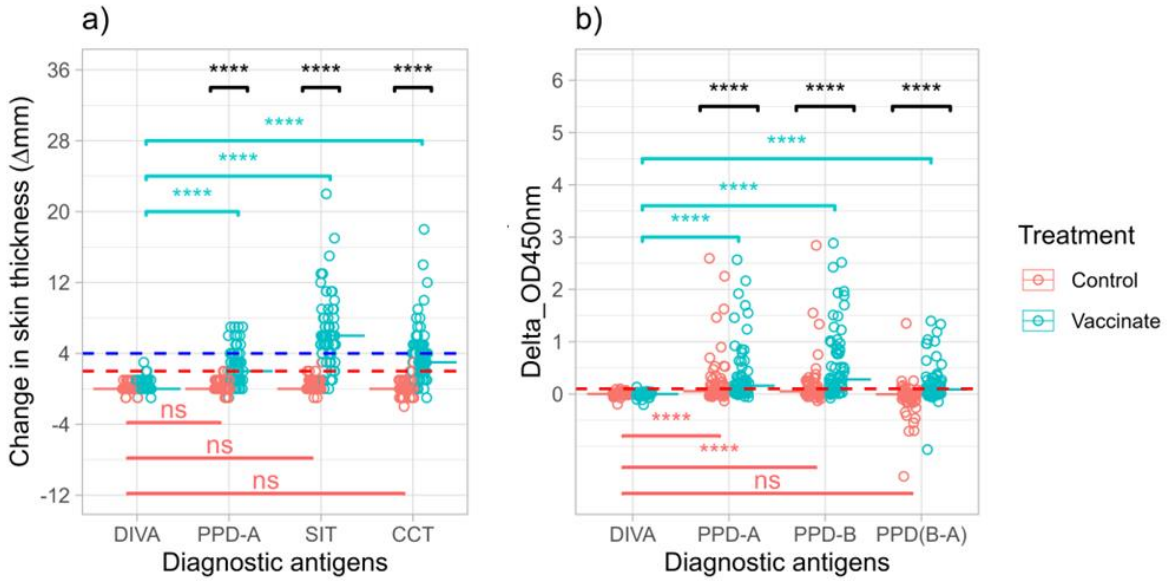


Figure 3.2. Eight-week post-BCG immune response to tuberculin and DIVA antigens. a) changes in skin thickness and b) IFN-γ responses to DIVA and tuberculin in 67 BCG-vaccinated and 67 unvaccinated naïve calves. Skin and IFN-γ test responses to DIVA, avian, and bovine tuberculin were evaluated eight weeks after BCG vaccination. The dotted blue horizontal lines at 4 mm and the dotted red horizontal lines at 2 mm are the cutoffs used for the tuberculin and DIVA skin test antigens, respectively. The dotted red horizontal line at 0.1 is the cutoff values used for all tuberculin and DIVA IFN-γ antigens, and the horizontal lines within the plots of each treatment group represent the median response to all the respective antigens. The statistical significance of differences in skin reaction and IFN-γ production between different antigens within a treatment group were determined using Friedman nonparametric analysis of variance with Bonferroni correction for multiple comparisons. Statistical significance of differences between the control and vaccinated groups were determined using the Wilcoxon rank-sum (Mann–Whitney U) test. (****P<0.0001; ns = not significant (P>0.05)).

Skin and IGRA responses in experimental calves postexposure to infected cattle

Out of 112 animals with visible lesions, 54 (48%) tested positive for DIVA skin, 49 (44%) for DIVA IGRA, and 49 (44%) for CCT. Similar DIVA skin, CCT and DIVA IGRA tests positive rates were observed in *M. bovis* culture PCR-positive animals (Table 3.2). These findings suggest consistent and similar accuracy for DIVA skin, DIVA IGRA, and CCT tests in classifying cattle with confirmed *M. bovis* infection by culture PCR and cattle with lesions suggestive of disease (Table 3.2). On the other hand, bovine minus avian tuberculin IGRA and bovine tuberculin (SIT) skin tests demonstrated higher sensitivity closer to 60% or higher in both confirmed *M. bovis* infection cases and cattle with lesions (Table 3.2).

Table 3.2. Positive Skin and IGRA test responses of experimental calves at the 12th month post-exposure to infected cattle in *M. bovis* PCR, and Gross VL test status at PM.
Note: PCR (Polymerase Chain Reaction), PM (Postmortem), VL (Visible lesion).

PM Tests	Test status	Treatment	N	Tests and Number (%) Positive					
				Skin test			IGRA test		
				DIVA	CCT	SIT	DIVA	PPD (B-A)	PPD-B
M. bovis PCR	Negative	Control	18	7 (39)	5 (28)	10 (56)	3 (17)	9 (50)	17 (94)
		Vaccinate	20	8 (40)	6 (30)	9 (45)	5 (25)	11 (55)	17 (85)
		subtotal	38	**15 (39)**	**11 (29)**	**19 (50)**	**8 (21)**	**20 (53)**	**34 (89)**
	Positive	Control,	47	25 (53)	23 (49)	32 (68)	26 (55)	39 (83)	42 (89)
		Vaccinate	42	16 (38)	17 (40)	30 (71)	16 (38)	29 (69)	38 (90)
		Subtotal	89	**41 (46)**	**40 (45)**	**52 (58)**	**42 (47)**	**68 (76)**	**80 (90)**
Gross VL	Negative	Control	4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)
		Vaccinate	11	2 (18)	2 (18)	5 (45)	1 (9.1)	4 (36)	10 (91)
		Subtotal	15	**2 (13)**	**2 (13)**	**5 (33)**	**1 (13)**	**4 (7)**	**14(93)**
	Positive	Control	61	32 (52)	28 (46)	42 (69)	29 (48)	48 (79)	55 (90)
		Vaccinate	51	22 (43)	21 (41)	34 (67)	20 (39)	36 (71)	45 (88)
		Subtotal	112	**54 (48)**	**49 (44)**	**76 (68)**	**49 (44)**	**84 (75)**	**100 (89)**

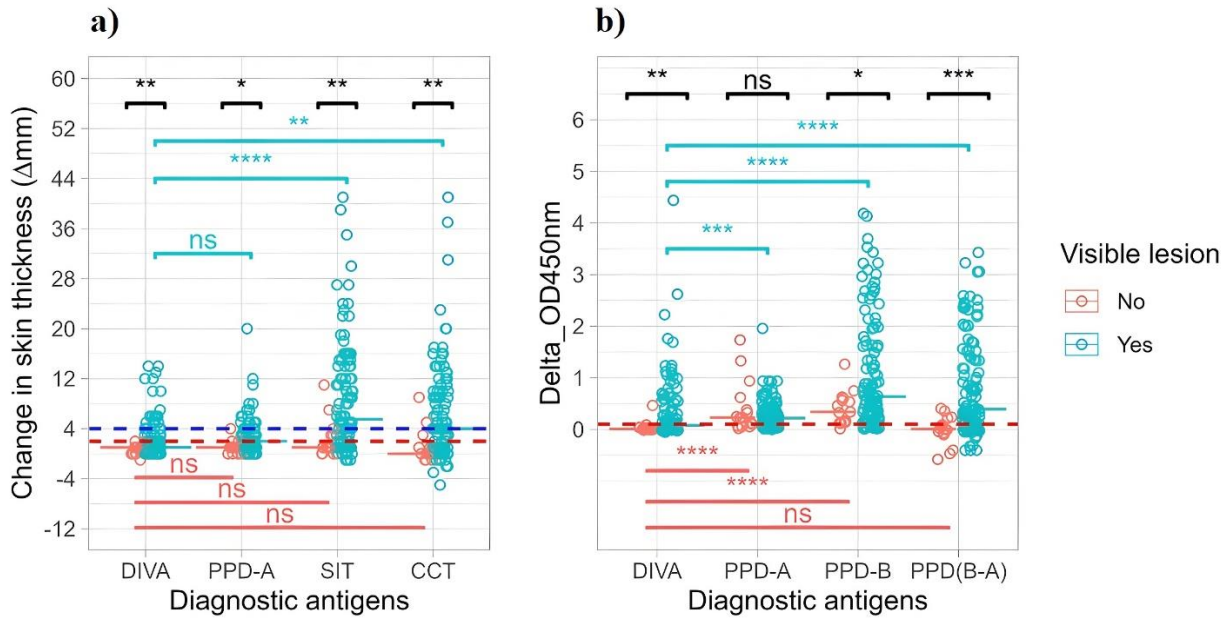


Figure 3.3. Twelve-month postexposure immune response to tuberculin and DIVA antigens. a) change in skin thickness and b) IFN-γ response to DIVA and tuberculin in 65 unvaccinated control and 62 BCG-vaccinated calves exposed to natural infection for a year. Skin and IFN-γ test responses to DIVA and tuberculin were evaluated after 12 months of exposure to infected herds (14 months after BCG vaccination). The dotted blue horizontal lines at 4 mm and the dotted red horizontal lines at 2 mm are the cutoffs used for tuberculin and DIVA skin test antigens, respectively. The dotted red horizontal line at 0.1 is the cutoff used for all tuberculin and DIVA IFN-γ responses. The horizontal line within the plots of animals with and without visible lesions represents the median change in response to all the respective antigens. The statistical significance differences in skin reactions as well as IFN-γ responses between different antigens within the lesioned and non-lesioned groups were determined using Friedman repeated measures nonparametric analysis of variance with Bonferroni correction for multiple comparisons. The statistical significance of differences between the animals with and without visible lesions was determined using the Wilcoxon rank sum (Mann–Whitney U) test. (****P<0.0001; ***P<0.001; **P < 0.01; *P < 0.05; ns = not significant (P>0.05)).

The results of the Mann–Whitney U test indicated that skin and IFN-γ responses to all antigens were significantly greater (P>0.05) in animals with visible lesions, except for the IFN-γ response to avian tuberculin (p = 0.69) (Figure 3.3**a** and 3.3**b**). The rank biserial correlation coefficient (r), which indicates the effect size, showed very large differences for all antigens. Tuberculin antigens elicited significantly greater skin and IGRA responses than DIVA antigens in animals with visible lesions (p < 0.0001) (Figure 3.3**a**). Friedman repeated measures

nonparametric ANOVA, with a Bonferroni correction for multiple comparisons, demonstrated a significantly greater skin test response in the SIT ($p < 0.0001$) and CCT ($p < 0.01$) tests than in the DIVA skin test while the skin test responses to avian tuberculin and DIVA were equivalent ($p>0.05$). However, there were no significant differences in the skin test responses to any of the tuberculin or DIVA antigens ($p = 0.07$) among the animals without visible lesions. In contrast, both avian and bovine tuberculin antigens induced significantly greater IFN-γ responses than DIVA IGRA ($p < 0.0001$) in the animals without visible lesions, while the bovine minus avian tuberculin and DIVA IGRA test responses were comparable ($p>0.05$) (Figure 3.3**b**).

Sensitivity of skin tests and IFN-γ assays compared against *M. bovis* culture PCR test

The performance measures of all skin and IGRA test antigens evaluated at the 12th month postexposure in the BCG-vaccinated and unvaccinated animals were presented in Table 3.3. The findings indicate that the CCT at the standard cutoff (>4 mm), DIVA skin test at cutoff (≥2 mm), and DIVA IGRA tests show comparable levels of accuracy and performance, albeit with an overall relative sensitivity of less than 50%. At the end of the exposure period, out of 47 culture PCR-positive unvaccinated cattle, 25 and 23 had positive skin fold thickness responses to the DIVA and CCT skin tests, respectively, whereas out of 42 culture PCR-positive BCG-vaccinated group, 16 and 17 animals had positive skin reactions to the DIVA and CCT skin tests, respectively (Table 3.3). The results indicate that the DIVA and CCT have overall relative sensitivities to culture PCR status of 46% (95% CI: 36, 56) and 45% (95% CI: 35, 55), respectively. The DIVA IGRA test demonstrated an overall relative sensitivity of 47% (95% CI: 37, 57). On the other hand, both the bovine and bovine minus avian tuberculin IGRA tests with the SIT skin test demonstrate comparable accuracy and performance, with significantly greater overall sensitivity of over 60% compared to CCT, DIVA skin, and DIVA IGRA tests regardless of the treatment group. Although the relative sensitivities of all tests were slightly greater in the unvaccinated control calves than in the BCG-vaccinated calves, these differences were not statistically significant. The sensitivity of all skin tests and IFN-γ assays was similar, with no statistically significant differences, when evaluated against the combined visible lesions or *M. bovis* PCR status reference standard Supplementary Table 3.2) compared to the *M. bovis* culture PCR status reference (Table 3.3).

Table 3.3. Relative sensitivity of IGRA and skin tests with respect to *M. bovis* culture PCR positivity in BCG vaccinated and unvaccinated control animals at the 12th month postexposure

Treatment	Test	Antigen	Cutoff	Status	*M. bovis* Culture PCR		Sensitivity (95%CI)	LR+	LR-	Accuracy
					Positive	Negative				
BCG vaccinated	Skin	DIVA	≥ 2 mm	Positive	16	8	38 (25, 53)	0.95	1.03	0.45
				Negative	26	12				
		CCT	> 4 mm	Positive	17	6	40 (27, 56)	1.35	0.85	0.50
				Negative	25	14				
		SIT	≥ 4 mm	Positive	27	7	64 (49, 77)	1.84	0.55	0.65
				Negative	15	13				
	IGRA	DIVA	≥ 0.1	Positive	16	5	38 (25, 53)	1.52	0.83	0.50
				Negative	26	15				
		PPD (B-A)	≥ 0.1	Positive	29	11	69 (54, 81)	1.26	0.69	0.61
				Negative	13	9				
		PPD-B	≥ 0.1	Positive	38	17	90 (78, 96)	1.06	0.63	0.66
				Negative	4	3				
Unvaccinated controls	Skin	DIVA	≥ 2 mm	Positive	25	7	53 (39, 67)	1.37	0.77	0.55
				Negative	22	11				
		CCT	> 4 mm	Positive	23	5	49 (35, 63)	1.76	0.71	0.55
				Negative	24	13				
		SIT	≥ 4 mm	Positive	31	10	66 (52, 78)	1.19	0.77	0.60
				Negative	16	8				
	IGRA	DIVA	≥ 0.1	Positive	26	3	55 (41, 69)	3.32	0.54	0.63
				Negative	21	15				
		PPD (B-A)	≥ 0.1	Positive	39	9	83 (70, 91)	1.66	0.34	0.74
				Negative	8	9				
		PPD-B	≥ 0.1	Positive	42	17	89 (77, 95)	0.95	1.91	0.66
				Negative	5	1				

Correlations between antemortem diagnostic tests of bTB

The correlation between skin and IFN-γ responses to DIVA and tuberculin antigens were evaluated using the nonparametric Spearman's rank correlation coefficient (ρ). The DIVA skin test response was strongly correlated with the CCT (Spearman's $\rho = 0.65$, $p < 0.0001$) and SIT (Spearman's $\rho = 0.71$, $p < 0.0001$) tuberculin skin tests responses. Similarly, the DIVA IFN-γ test responses were strongly positively correlated with both the bovine minus avian tuberculin (Spearman's $\rho = 0.79$, $p < 0.0001$) and the bovine tuberculin IFN-γ test responses (Spearman's $\rho = 0.82$, $p < 0.0001$). The correlation between DIVA skin and DIVA IFN-γ test responses was

also strong and positive (Spearman's ρ=0.73; 95% CI=0.64–0.81, p<0.0001), as was that between the CCT skin and bovine minus avian tuberculin IFN-γ responses (Spearman's ρ=0.79; 95% CI=0.71–0.85, p<0.0001) (see Supplementary Figure 3.2). All the above correlations were statistically significant and had large effect sizes, suggesting that both DIVA and tuberculin antigens used either in skin or IGRA induce similar immunological responses in bTB-infected animals (Figure 3.4).

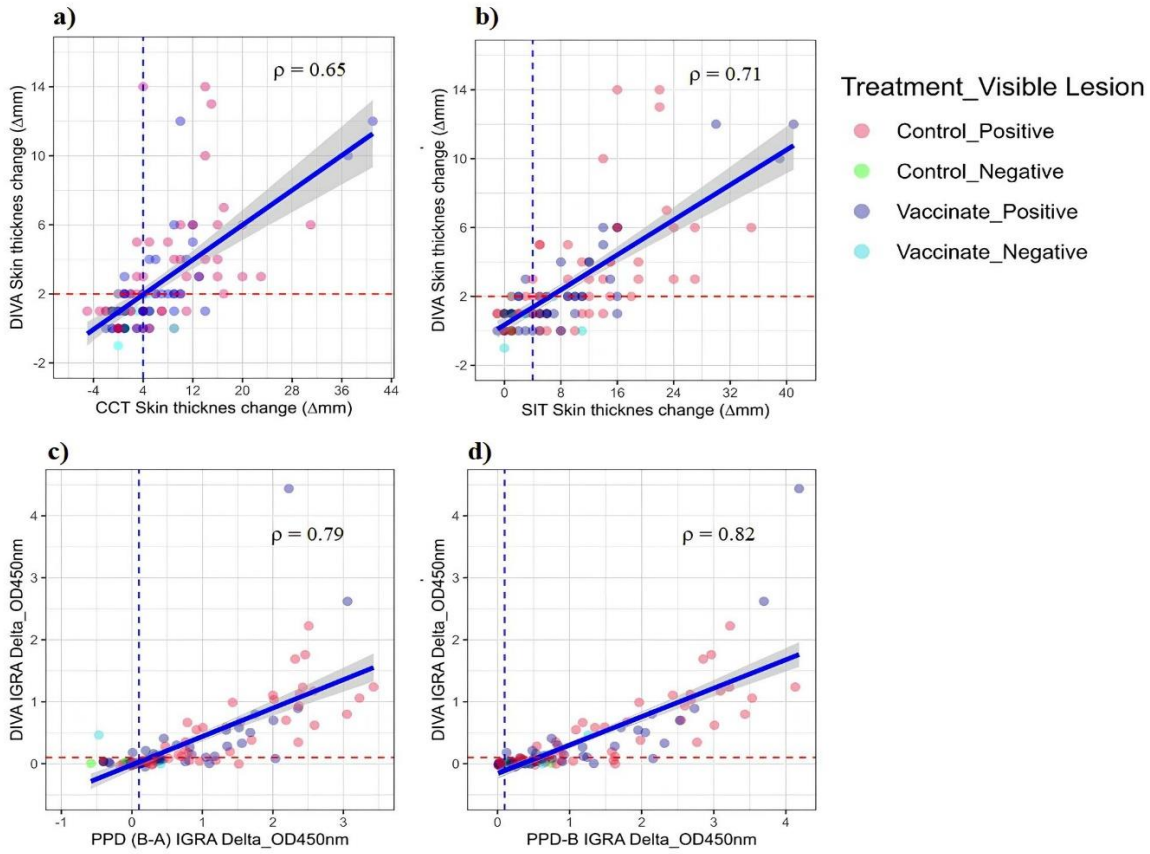


Figure 3.4. Correlation between OD450 nm levels of IFN-γ and skin test responses a) Between the DIVA skin and CCT skin tests. **b**) Between the DIVA skin and SIT skin tests. **c)** Between the DIVA IGRA and bovine minus avian tuberculin IFN-γ tests. **d)** Between the DIVA IGRA and bovine tuberculin IFN-γ tests. The solid-colored circles represent individual animals with treatment and visible lesions status combinations. The dashed vertical and horizontal lines at 0.1 are the cut-offs used for tuberculin and DIVA IGRA IFN-γ responses. The dashed vertical and horizontal lines at 4 and 2 are the cut-offs used for the skin thickness in the SIT/CCT and DIVA skin test responses, respectively.

Overlapping and distinct responses to multiple skin and IGRA test antigens

All the three tests: DIVA IGRA, DIVA skin and CCT tests are concordant on 69% (88/127) of all tested animals at the end of the exposure period (Figure 3.5 and Supplementary Table 3.3). The BCG-vaccinated animals had concordant outcomes for 74% (46/62) of animals using the DIVA IGRA and CCT skin tests. The CCT tuberculin skin and DIVA skin tests showed concordant test responses in 86% (56/65) of controls and 82% (51/62) vaccinated animals. Between the DIVA IGRA and DIVA skin tests, a similar concordance of 86% (56/65) was observed in unvaccinated controls while 79% (49/62) was seen in BCG vaccinated cattle (Figure 3.5 and Supplementary Table 3.3). The bovine minus avian tuberculin IGRA and DIVA IGRA test results are concordant in 71% (46/65) of controls and in 63% (39/62) of vaccinated animals (Supplementary Figure 3.3 and Table 3.4). Diagnostic status based on the DIVA IFN-γ and DIVA skin test formats showed substantial agreement, with observed agreement on 83% of the cases and a kappa value of 0.645 (95% CI=0.51, 0.78), suggesting a level of concordance beyond that expected by chance. The bovine tuberculin and bovine minus avian tuberculin IGRA tests and bovine tuberculin skin test (SIT) are concordant on 70% (89/127) of the animals. In the unvaccinated control group, animals that tested positive for all test antigens except for bovine tuberculin IGRA were positive in postmortem tests (lesions, culture, or PCR). However, the bovine tuberculin IGRA test showed positive results in all postmortem test-negative unvaccinated and BCG-vaccinated animals. In BCG-vaccinated cattle, the DIVA skin test, DIVA IGRA test, and bovine minus avian tuberculin IGRA each tested positive in one postmortem test negative animal. Additionally, the bovine tuberculin skin test was positive in two postmortem test negative cattle.

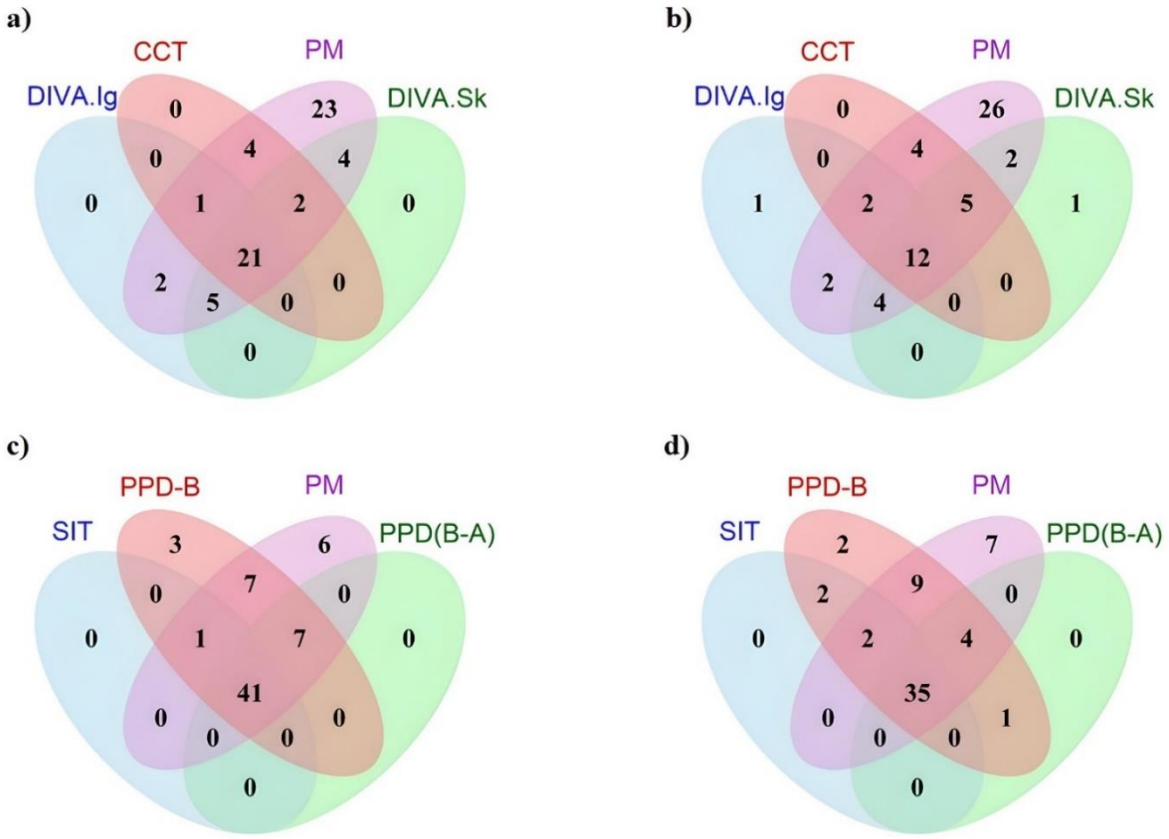


Figure 3.5. Venn diagram comparing the number of cattle positives for postmortem visible lesions, culture or *M. bovis* PCR (PM) with the number of (**a**) unvaccinated control and (**b**) vaccinated cattle tested positive on the DIVA IGRA (DIVA.Ig), CCT or DIVA skin test (DIVA.Sk) (**c**) unvaccinated and (**d**) vaccinated cattle tested positive on the SIT skin test, PPD-B or PPD (B-A) IGRA tests. All numbers within the purple circle represent animals that tested positive in the postmortem test (62 unvaccinated and 57 BCG vaccinated). All numbers outside the purple circle represent animals that tested negative in the postmortem test (3 unvaccinated controls and 5 BCG vaccinated).

3.5 Discussion

Incorporating BCG vaccination into control strategies has the potential to offer a cost-effective and sustainable method of controlling bTB in regions where the "test and cull" strategy might not be feasible or persistent wildlife reservoirs pose challenges. Studies show that BCG vaccination reduces bTB pathology and transmission in cattle [7,8,10]. BCG, the most widely used vaccine against human tuberculosis is approved for some wildlife species such as the Eurasian

badger [1,38]. However, deployment of BCG vaccination to control bTB in cattle requires validation of a diagnostic assay with reliable sensitivity, specificity, and DIVA capability.

This study evaluated immune responses to tuberculin and DIVA peptide cocktails (ESAT-6, CFP10, and Rv3615c) in IFN-γ and skin tests in 67 BCG-vaccinated and 67 unvaccinated controls prior to exposure and in 65 unvaccinated and 62 BCG-vaccinated cattle after one year of exposure to natural infection. Eight weeks post BCG, all DIVA skin and IGRA tests as well as all tuberculin skin tests demonstrated 100% specificity in the unvaccinated control calves. In vaccinated calves, DIVA IGRA and skin tests had specificities of 98.5% and 94.0%, respectively. All DIVA skin and IGRA test positive calves at preexposure were BCG-vaccinated that showed responses close to the marginal cutoff values in their respective tests. However, upon retesting at the 4th and 2nd month post-exposure, respectively, they tested negative in these same tests except one. This suggests that the initial marginally positive test responses before exposure were likely false positives. This aligns with previously reported lower proportions of positive DIVA IFN-γ responses in BCG-vaccinated calves [24] and positive DIVA skin test responses in Gudair-vaccinated calves [39]. However, it is difficult to explain why such erratic positive responses to DVIA antigens in both skin and IGRA tests occur in vaccinated calves.

In contrast to DIVA, tuberculin antigens elicited significantly higher skin and IFN-γ responses eight-weeks post-BCG in vaccinated cattle. This elevated pre-exposure response is as expected given that the DIVA antigens were selected specifically not to elicit an immune response in BCG vaccinates in contrast to tuberculin that contains a broader range of antigens that can cross-react with BCG. Several previous studies have also reported a higher skin thickness response to tuberculin than to DIVA antigens in infected cattle with lesions [12,13,17,39,40] and greater IFN-γ response to tuberculin compared to ESAT-6 in *M. bovis* culture-positive cattle than in *M. bovis* culture-negative animals [39,41]. A positive bovine minus avian as well as bovine tuberculin IFN-γ response was also observed in unvaccinated control calves at eight-weeks post BCG and in vaccinated cattle without visible lesions at the end of exposure. Positive tuberculin IFN-γ response in unvaccinated control at preexposure may have been due to nonspecific immune response to environmental mycobacterial exposure. The IFN-γ responses biased towards avian tuberculin, suggest sensitization to environmental mycobacteria. This further suggests that the tuberculin antigens lack specificity in regions where animals are possibly exposed to environmental mycobacteria, leading to immune sensitization. The observed nonspecific positive IFN-γ test response to tuberculin in unvaccinated and

noninfected control cattle in the current is consistent with previous reports [15,19]. Young cattle are reported to have more NK cells and exhibit nonspecific IFN-γ production in as much as one-third of the calves [42,43]. A strong avian tuberculin-biased response has also been reported in Gudair-vaccinated and noninfected control cattle [39].

The DIVA skin and IFN-γ responses were significantly lower than those of tuberculin; however, DIVA tests performed comparably to the comparative cervical test (CCT). These three tests showed less than 50% sensitivity in cattle that were confirmed positive for *M. bovis* in culture PCR or through the presence of lesions indicative of possible infection. In contrast, the bovine and bovine minus avian tuberculin IGRA tests, as well as the SIT skin test, showed significantly higher sensitivities of about 60% and above in the same groups compared to the CCT, DIVA skin, and DIVA IGRA tests. However, these tests are less specific in unvaccinated controls exposed to environmental mycobacteria and BCG-vaccinated cattle. More than 20% of animals with a positive immune response to all skin and IGRA tests, indicating possible infection, were *M. bovis* culture PCR-negative. This suggests a lack of sensitivity in mycobacterial culture for detecting potentially infected cattle. Three controls and five vaccinates (100%) of claves that tested negative in all postmortem tests (bTB lesions, culture, or PCR) also tested positive in the bovine tuberculin minus nil IGRA test (Figure 3.5). This demonstrates the lack of specificity of the bovine tuberculin in the IGRA test. This lack of specificity could be due to environmental mycobacterial exposure or the effect of BCG, which suggests that using the IGRA bovine tuberculin alone in areas with a high likelihood of exposure to environmental mycobacteria is inappropriate.

Previous research reported DIVA skin and IGRA test sensitivities higher than 63% in experimentally and naturally infected tuberculous cattle [19,39]. Several factors, including regional differences related to herd size, breed, age, stage of disease, repeated tuberculin skin testing, the prevalence of environmental mycobacteria, helminth infections, and pathogens other than mycobacteria causing granulomas, might have contributed to the variations and lower relative sensitivity observed in our study. Repeated tuberculin skin tests have been reported to cause skin desensitization in naturally infected cattle, leading to a lower overall sensitivity [44,45]. We also noticed a decrease in the amplitude of the skin test response in the terminal test conducted for the fourth repeated time (see Supplementary Figure 3.2). Despite regular deworming, 22% of our study animals had an active liver fluke infection with a lower mean total lesion score compared to animals without active fasciolosis postmortem, suggesting

a negative effect of active fasciolosis on lesion severity (p = 0.07). A reduced magnitude of skin response to tuberculin has been reported in cattle co-infected with tuberculosis and liver flukes [46]. In a study conducted in Ireland, animals with fewer than 3.6 lesions at slaughter exhibited no reaction to the tuberculin skin test [47].

Our study found a strong significant correlation between the DIVA peptide cocktail and tuberculin antigen responses in skin fold thickness increase and ΔOD450 nm values of IFN-γ responses. This finding agrees with the previously reported high correlation between DIVA- and tuberculin-induced skinfold thickness and IFN-γ OD450 nm [41,48]. Although the correlation does not indicate accuracy, a significant correlation between the immune responses elicited by DIVA and tuberculin antigens in the skin and IGRA tests suggests that both may measure the same underlying condition. The DIVA skin or DIVA IFN-γ tests used in combination with CCT yielded concordant test outcomes in approximately three-quarters or more of the same individual controls and vaccinated animals. The similar responses to these three tests, regardless of vaccination status, further suggest that the tests are consistent across different treatment groups and have comparable performances in measuring similar underlying disease status.

The limitations of this study include the use of diagnostic data collected to evaluate the efficacy of BCG through a natural transmission experiment [10], that was not specifically designed for diagnostic test evaluation. Although Bayesian Latent Class Analysis is recommended for evaluating measures of diagnostic performance when a gold standard is not available, we used culture PCR test status as a reference standard to calculate the relative sensitivity. However, convergence of the standard Walter-Hui model for latent class analysis of diagnostic tests depends on having a set of at least two diagnostic test results for individuals from two populations with distinct prevalence. This latter requirement is not met for our data, limiting the value and reliability of these methods in this context.

The sensitivities of all the tests are not significantly different when using *M. bovis* culture PCR test status as a reference standard compared to using combinations of visible lesions and *M. bovis* culture PCR status as a reference (Table 3.3, Supplementary Table 3.3 and Table 3.5, Supplementary Figure 3.4**)**. This suggests that the performance of these tests in detecting positive cases remains consistent, regardless of whether the reference standard is culture PCR or a combination of visible lesions and culture PCR test results. However, both tests are

imperfect to be considered as a true gold standard since lesions are nonspecific and culture, though considered the gold standard, has less sensitive. Test sensitivity can vary depending on different epidemiological contexts influenced by breed, immunological status, co-infections, and regional strain differences. Therefore, the relative sensitivity of the tests reported in this study may not accurately reflect the performance of the tests in the field under varying epidemiological conditions.

Overall, our results confirm that DIVA antigens can be used to detect tuberculosis infection in BCG-vaccinated cattle with high specificity and sensitivity comparable to that of CCT. Thus, the DIVA antigens used in both IGRA and skin tests hold a promise for practical implementation alongside BCG vaccination. This approach is particularly relevant in areas where the test-and-slaughter strategy is not economically feasible and there is no alternative implementable bTB control option in resource-poor countries such as Ethiopia.

In general, these findings underscore the low sensitivity of the current antemortem bTB diagnostic tests and the need for more sensitive diagnostic tests, which are crucial for effective bTB control. In a population with a high prevalence of bTB, a test and slaughter strategy using low sensitivity tests is not expected to be effective in controlling the disease. Both the mean, and median bTB prevalence exceed 30%, in large and medium-sized herds in Central Ethiopia[4]. When a high prevalence of bTB is observed within a herd in some high-income countries, all animals in the infected herd are culled. In countries with lower bTB prevalence and more financial resources, total culling may be feasible and effective in eradicating the disease. However, in Ethiopia, the combination of high bTB prevalence, economic limitations, and logistical constraints makes total culling impractical. Culling the entire herd presents several significant challenges including difficulties in obtaining replacement herds, farmers' reluctance to cull high milk-producing cows, and financial constraints associated with compensating for the loss of numerous dairy cattle. Given these challenges, vaccination presents a more viable and economically feasible alternative to total culling. The deployment of BCG vaccination for bTB control requires highly specificic and sensitive DIVA diagnostics.

Therefore, important alternative measures to prevent the transmission and spread of the disease include BCG vaccination of newborn calves and testing replacement livestock using WOAH recommended standard bTB diagnostics before purchasing. Farmers are also more likely to accept and participate in vaccination programs than culling programs.

3.6 References

1. WOAH. Mammalian tuberculosis (infection with *Mycobacterium tuberculosis* complex), In: Manual of diagnostic tests and vaccines for terrestrial animals World Organisation for Animal Health, Paris. (2022).

2. Allen, A. R., Skuce, R. A. & Byrne, A. W. Bovine Tuberculosis in Britain and Ireland – A Perfect Storm? the Confluence of Potential Ecological and Epidemiological Impediments to Controlling a Chronic Infectious Disease. *Front. Vet. Sci.* **5**, 109 (2018).

3. Sibhat, B. *et al.* Bovine tuberculosis in Ethiopia: A systematic review and meta-analysis. *Preventive Veterinary Medicine* **147**, 149–157 (2017).

4. Almaw, G. *et al.* The variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia provides opportunities for targeted intervention. *PLoS ONE* **16**, e0254091 (2021).

5. Glanville, R. J. Australia's colourful path to tuberculosis freedom. *Irish Veterinary Journal* **76**, 15 (2023).

6. Conlan, A. J. K. *et al.* Potential Benefits of Cattle Vaccination as a Supplementary Control for Bovine Tuberculosis. *PLoS Comput Biol* **11**, e1004038 (2015).

7. Ameni, G. *et al.* Vaccination of calves with *Mycobacterium bovis* Bacillus Calmette-Guerin reduces the frequency and severity of lesions of bovine tuberculosis under a natural transmission setting in Ethiopia. *Transbound Emerg Dis* **65**, 96–104 (2018).

8. Ameni, G., Vordermeier, M., Aseffa, A., Young, D. B. & Hewinson, R. G. Field Evaluation of the Efficacy of *Mycobacterium bovis* Bacillus Calmette-Guérin against Bovine Tuberculosis in Neonatal Calves in Ethiopia. *Clin Vaccine Immunol* **17**, 1533–1538 (2010).

9. Conlan, A. J. K., Vordermeier, M., De Jong, M. C. & Wood, J. L. The intractable challenge of evaluating cattle vaccination as a control for bovine Tuberculosis. *eLife* **7**, e27694 (2018).

10. Fromsa, A. *et al.* BCG vaccination reduces bovine tuberculosis transmission, improving prospects for elimination. *Science* **383**, eadl3962 (2024).

11. Bayissa, B. *et al.* Evaluation of the Efficacy of BCG in Protecting Against Contact Challenge with Bovine Tuberculosis in Holstein-Friesian and Zebu Crossbred Calves in Ethiopia. *Front. Vet. Sci.* **8**, 702402 (2021).

12. Whelan, A. O. *et al.* Development of a Skin Test for Bovine Tuberculosis for Differentiating Infected from Vaccinated Animals. *J Clin Microbiol* **48**, 3176–3181 (2010).

13. Srinivasan, S. *et al.* A defined antigen skin test for the diagnosis of bovine tuberculosis. *Sci. Adv.* **5**, eaax4899 (2019).

14. Vordermeier, H. M., Jones, G. J., Buddle, B. M., Hewinson, R. G. & Villarreal-Ramos, B. Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. *Annu. Rev. Anim. Biosci.* **4**, 87–109 (2016).

15. Sidders, B. *et al.* Screening of Highly Expressed Mycobacterial Genes Identifies Rv3615c as a Useful Differential Diagnostic Antigen for the *Mycobacterium tuberculosis* Complex. *Infect Immun* **76**, 3932–3939 (2008).

16. Flores-Villalva, S. *et al.* Specificity of the Tuberculin Skin Test Is Modified by Use of a Protein Cocktail Containing ESAT-6 and CFP-10 in Cattle Naturally Infected with *Mycobacterium bovis*. *Clinical and Vaccine Immunology* **19**, 797–803 (2012).

17. Bayissa, B. *et al.* Field evaluation of specific mycobacterial protein-based skin test for the differentiation of *Mycobacterium bovis*- infected and Bacillus Calmette Guerin-vaccinated crossbred cattle in Ethiopia. *Transbounding Emerging Dis* **69**, (2022).

18. Blanco, F. C. *et al.* Identifying Bacterial and Host Factors Involved in the Interaction of *Mycobacterium bovis* with the Bovine Innate Immune Cells. *Front. Immunol.* **12**, 674643 (2021).

19. Vordermeier, H. *et al.* Use of Synthetic Peptides Derived from the Antigens ESAT-6 and CFP-10 for Differential Diagnosis of Bovine Tuberculosis in Cattle. *Clinical Diagnostic Laboratory Immunology* **8**, 571–578 (2001).

20. Jones, G. J. *et al.* Test performance data demonstrates utility of a cattle DIVA skin test reagent (DST-F) compatible with BCG vaccination. *Sci Rep* **12**, 12052 (2022).

21. Lopez-Valencia, G. *et al.* Field evaluation of the protective efficacy of *Mycobacterium bovis* BCG vaccine against bovine tuberculosis. *Research in Veterinary Science* **88**, 44–49 (2010).

22. Nugent, G., Yockney, I. J., Whitford, J., Aldwell, F. E. & Buddle, B. M. Efficacy of oral BCG vaccination in protecting free-ranging cattle from natural infection by *Mycobacterium bovis*. *Veterinary Microbiology* **208**, 181–189 (2017).

23. Nugent, G., Yockney, I. J., Cross, M. L. & Buddle, B. M. Low-dose BCG vaccination protects free-ranging cattle against naturally-acquired bovine tuberculosis. *Vaccine* **36**, 7338–7344 (2018).

24. Subramanian, S. *et al.* Defined Antigen Skin Test for Bovine Tuberculosis Retains Specificity on Revaccination With Bacillus Calmette–Guérin. *Front. Vet. Sci.* **9**, 814227 (2022).

25. Vordermeier, H. M. *et al.* Correlation of ESAT-6-Specific Gamma Interferon Production with Pathology in Cattle following *Mycobacterium bovis* BCG Vaccination against Experimental Bovine Tuberculosis. *Infect Immun* **70**, 3026–3032 (2002).

26. QIAGEN. DNeasy ® Blood & Tissue Handbook. (2023).

27. Fromsa, A. *et al.* Bovine tuberculosis in Central Ethiopian slaughterhouses and the identification of causative mycobacteria by multiplex real-time PCR. *BMC Microbiology* **24**, 394 (2024).

28. Ministry of Education (MoE). National Research Ethics Review Technical Guideline. (2022).

29. R Core Team. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. https://www.R-project.org URL https://www.R-project.org/. (2023).

30. RStudio Team. RStudio: Integrated Development for R. RStudio. (2020).

31. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. (2016).

32. Patil, I. Visualizations with statistical details: The 'gstatsplot' approach. *Journal of Open Source Software* **6**, 3167 (2021).

33. Ranganathan, P. & Aggarwal, R. Understanding the properties of diagnostic tests – Part 2: Likelihood ratios. *Perspectives in Clinical Research* **9**, 99–102 (2018).

34. Ranganathan, P. & Aggarwal, R. Common pitfalls in statistical analysis: Understanding the properties of diagnostic tests – Part 1. *Perspect Clin Res* **9**, 40–43 (2018).

35. Portney, L. G. *Foundations of Research and Evidence: Applications to Evidence-Based Practice*. (F. A. Davis Company, Philadelphia, PA, USA, 2020).

36. Drewe, J. A., Tomlinson, A. J., Walker, N. J. & Delahay, R. J. Diagnostic Accuracy and Optimal Use of Three Tests for Tuberculosis in Live Badgers. *PLOS ONE* **5**, e11196 (2010).

37. McHugh, M. L. Interrater reliability: the kappa statistic. *Biochemia Medica* (2012) doi:10.11613/bm.2012.031.

38. Robinson, P. A. *et al.* BCG vaccination against tuberculosis in European badgers (Meles meles): A review. *Comparative Immunology, Microbiology and Infectious Diseases* **35**, 277–287 (2012).

39. Middleton, S. *et al.* A molecularly defined skin test reagent for the diagnosis of bovine tuberculosis compatible with vaccination against Johne's Disease. *Sci Rep* **11**, 2929 (2021).

40. Jones, G. J., Whelan, A., Clifford, D., Coad, M. & Vordermeier, H. M. Improved skin test for differential diagnosis of bovine tuberculosis by the addition of Rv3020c-derived peptides. *Clin Vaccine Immunol* **19**, 620–622 (2012).

41. Buddle, B. *et al.* Differentiation between *Mycobacterium bovis* BCG-Vaccinated and *M. bovis*-Infected Cattle by Using Recombinant Mycobacterial Antigens. *Clinical Diagnostic Laboratory Immunology* **6**, 1–5 (1999).

42. Kulberg, S., Boysen, P. & Storset, A. K. Reference values for relative numbers of natural killer cells in cattle blood. *Developmental & Comparative Immunology* **28**, 941–948 (2004).

43. Olsen, I. *et al.* Bovine NK Cells Can Produce Gamma Interferon in Response to the Secreted Mycobacterial Proteins ESAT-6 and MPP14 but Not in Response to MPB70. *Infect Immun* **73**, 5628–5635 (2005).

44. Coad, M. *et al.* Repeat tuberculin skin testing leads to desensitisation in naturally infected tuberculous cattle which is associated with elevated interleukin-10 and decreased interleukin-1 beta responses. *Veterinary Research* **41**, 14–14 (2010).

45. Lakew, M. *et al.* Utility of the Intradermal Skin Test in a Test-and-Cull Approach to Control Bovine Tuberculosis: A Pilot Study in Ethiopia. *Front. Vet. Sci.* **9**, 823365 (2022).

46. Claridge, J. *et al.* Fasciola hepatica is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nat Commun* **3**, 853 (2012).

47. Byrne, A. W. *et al.* Modelling the variation in skin-test tuberculin reactions, post-mortem lesion counts and case pathology in tuberculosis-exposed cattle: Effects of animal characteristics, histories and co-infection. *Transbound Emerg Dis* **65**, 844–858 (2018).

48. Casal, C. *et al.* Evaluation of two cocktails containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the interferon-γ assay for diagnosis of bovine tuberculosis. *Preventive Veterinary Medicine* **105**, 149–154 (2012).

Author contributions:

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CHAPTER FOUR: BOVINE TUBERCULOSIS IN CENTRAL ETHIOPIAN SLAUGHTERHOUSES AND THE IDENTIFICATION OF CAUSATIVE MYCOBACTERIA BY MULTIPLEX REAL-TIME PCR

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4.1 Abstract

Background: Bovine tuberculosis (bTB) is a chronic disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC) that ultimately leads to the development of progressive granulomatous lesions. Although the disease is widespread, especially in crossbred cattle in Ethiopia, routine investigations and surveillance are lacking. Thus, the aim of this study was to determine the prevalence, associated risk factors, and species of mycobacteria causing bTB in slaughtered cattle at four slaughterhouses in Central Ethiopia.

Methods: Postmortem examination of 7,640 cattle was conducted using a cross-sectional slaughterhouse survey. A total of 388 tuberculous-like lesions (TBLs) were collected from 173 animals and cultured. Six target genes were used to differentiate mycobacterial species using multiplex qPCR (m-qPCR). Multivariate logistic regression analyses and related odds ratios (ORs) were used to gauge the strength of the associations between risk factors, TBL incidence and culture growth.

Results: The prevalence of TBL was 2.3% (95% CI = 2.0-2.6). Logistic regression analysis indicated an increased risk of TBL in crossbred cattle (OR = 11.8, 95% CI: 6.4, 21.2, p<0.001). Animals slaughtered at Adama (OR = 3.2, 95% CI: 1.2, 7.3, p= 0.009) or Burayu (OR = 5.8, 95% CI: 3.9, 8.9, p<0.001) had a greater occurrence of TBL than those slaughtered at Sululta. There were significantly more TBL-positive lesions in the lungs and lymph nodes related to the lung (OR = 7.1; 95% CI: 2.7, 24.5, p<0.001) and the head lymph node (OR= 5.6; 95% CI: 1.8, 21.7; p=0.006) compared to gut associated lymph nodes. Among the 173 TBL-positive animals, 36% (95% CI = 28.8, 43.2), and among the 388 TBL-positive tissues, 24.2% (95% CI = 20, 29) were culture and m-qPCR positive. All the culture-generated isolates were positive for *M. bovis* in m-qPCR. Among them, two animals had mixed infections including one zebu cattle tested positive for both *M. caprae* and *M. bovis*, and a crossbred cow tested positive for both *M. tuberculosis* and *M. bovis* in m-qPCR. This suggests persistent transmission within the cattle population, posing a substantial public health threat.

Conclusion: This study revealed an eleven-fold greater risk of bTB-related lesions in crossbred cattle compared to local zebu cattle. This finding highlights the necessity for targeted interventions, continuous vigilance, and thorough carcass inspection to mitigate public health risks.

Keywords: *Bovine tuberculosis; slaughterhouse; tuberculous-like lesions; Mycobacterium tuberculosis complex; cattle*

4.2 Introduction

Bovine tuberculosis (bTB) is a chronic cattle disease that negatively impacts productivity and poses serious threats to public health and the livestock economy. It is caused by members of the *Mycobacterium tuberculosis* complex (MTBC) that contains *M. bovis*, *M. caprae, M. tuberculosis*, *M. africanum, M. bovis* bacillus Calmette-Guérin (BCG), *M. microti, M. suricate, M. pinnipedii, M. orygis, M. mungi, M. canettii* and Dassie Bacillus [1]. Although MTBC member species are host-adapted to certain species, investigations have demonstrated that each MTBC subspecies can be transmitted from one reservoir host to another and cause tuberculosis in humans as well as in multiple domestic and wildlife animal species [2].

Ethiopia is estimated to have a cattle population of 66 million, and families are dependent on cattle for food and income [3]. bTB is a disease of economic significance which is endemic within the cattle population of Ethiopia [4]. The zoonotic potential of the MTBC has been demonstrated by documentation of *M. bovis* infections in humans [5–7] and identification of the human adapted *M. tuberculosis* strains in cattle [8–10], pigs [11], camels [12], sheep and goats [13, 14]. There is no national control strategy for bTB and infected animals are neither culled nor restricted from movement. The occurrence of both *M. tuberculosis* and *M. bovis* infection in humans and livestock populations in Ethiopia highlights the potential threat to both livestock production and human health.

Several investigators have developed and validated multiplex qPCR methodologies targeting variable genomic regions affected by insertions, deletions, and single nucleotide polymorphism (SNP) events within the MTBC, allowing for species-level identification of the group [15–18]. The insertion element IS1081 was shown to be exclusively present in the MTBC in 6 copies and consequently was validated and employed to differentiate MTBC from nontuberculous mycobacteria (NTM) [16, 19]. A region of difference (RD7) present in *M. tuberculosis* but deleted in *M. bovis, M. bovis* BCG, *M. orygis*, and *M. caprae* strains was found to be a useful genetic marker for the collective identification of human-adapted *M. tuberculosis* lineages [1]. The deletion of the Rv1510 gene internal to RD4 is absent from all *M. bovis* and *M. bovis* BCG strains but is present in other MTBC strains and was demonstrated to be a specific genotypic marker of *M. bovis* and *M. bovis* BCG strains [1, 19]. A single nucleotide polymorphism (SNP) with a characteristic C-to-T replacement at the 690 bp position of the lepA gene is *M. caprae* specific and was validated as a specific target for *M. caprae* detection [18, 20]. A specific single

nucleotide polymorphism (SNP) with a G to C substitution at position 698 in the Rv0444c gene was also identified and validated as a specific diagnostic target for *M. orygis* [15, 21].

Detection of tuberculous lesions at slaughter is a cost-effective method of passive surveillance for bTB enabling monitoring of progress towards elimination or reduction of zoonotic TB. As well as enabling epidemiological surveillance, slaughterhouse inspection of animals destined for consumption is critical for removing animals with bTB from the food supply chain and reducing the risk of zoonotic transmission. Although there have been several slaughterhouse surveys of bTB in Ethiopia, most studies conducted in central Ethiopia are over a decade old and the sample sizes were small often involving a single slaughterhouse (Supplementary Table 4.1). The diversity of the MTBC species associated with bTB is not also well understood. Thus, our research addresses these important gaps in the current literature and provides much-needed current data on bTB prevalence, which is crucial given potential changes in cattle management practices and disease control efforts over time. The current study is distinguished by its comprehensive sampling strategy, examining a large sample size of 7,640 cattle across four slaughterhouses in central Ethiopia. This broad approach offers a more representative picture of the current bTB situation in the region. Furthermore, unlike many previous studies that relied solely on gross pathology, our research combines detailed meat inspection with culture and multiplex qPCR for species identification, providing more accurate and detailed information on the causative agents of bTB in central Ethiopia. Our study explored bTB prevalence in slaughtered crossbred cattle, which are increasingly important in Ethiopia's developing dairy sector but potentially more susceptible to bTB, thereby providing important insights into the risks associated with the ongoing intensification of dairy production in the country.

4.3 Materials and Methods

4.3.1 Study areas

This was a cross-sectional study conducted at four slaughterhouses—Adama, Burayu, Bishoftu (Elfora), and Sululta—from May 2018 to March 2020. The slaughterhouses were selected purposefully based on ease of access and willingness to participate in the study. Although the slaughterhouses in our study were purposefully selected, they are similar to the broader slaughterhouses in central Ethiopia in key aspects. Specifically, the slaughterhouses in our study primarily handle old Zebu oxen culled from draught power, just like most central Ethiopian slaughterhouses. Furthermore, most animals processed in central Ethiopian

slaughterhouses come from similar livestock supply markets [22]. This close resemblance in the type of animals and sources implies representativeness to other slaughterhouses in central Ethiopia. Sululta and Burayu slaughterhouses are two of the five domestic slaughterhouses that supply cattle meat to Addis Ababa. Sululta slaughterhouses has an annual slaughter capacity of 34,000 heads of cattle, whilst Burayu slaughterhouses slaughters up to 13,000 cattle each year accounting for 13% of beef supply to Addis Ababa [22]. The Adama slaughterhouse processes an average of 200 to 300 heads of cattle per day, totaling around 55,000 to 80,000 cattle annually [23]. The locations of the slaughterhouses are displayed in Figure 4.1. Adama is a zonal town of Eastern Shoa in the Regional State of Oromia, Central Ethiopia, situated 100 km southeast of Addis Ababa along the road that connects the capital with Dire Dawa and the seaports of Djibouti. Bishoftu is a town of Ada'a district in the East Shoa Zone of the Oromia Region State in central Ethiopia, which is located 48 kilometers from Addis Ababa in the southeast direction. Burayu is a district town in the Oromia Region located 15 km west and directly adjacent to the capital Addis Ababa. Sululta is a district town located 26 km north of Addis Ababa.

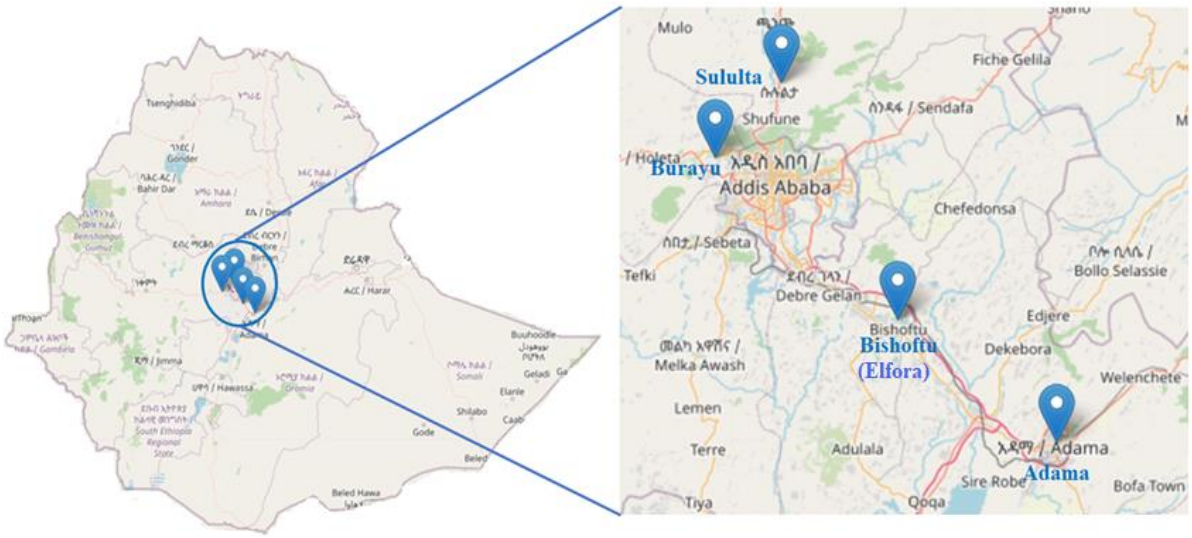


Figure 4.1: Locations of the study slaughterhouses produced and marked using the leaflet package in R: Left) Study locations are indicated by blue ballons within the circle. Right) Study areas zoomed with expanded view.

4.3.2 Study animals

The study animals were cattle that were slaughtered at the four slaughterhouses and consequently subjected to routine meat inspection. Animals slaughtered at Adama, Burayu, and

Bishoftu (Elfora) were all male Zebu cattle, whereas at Sululta crossbred and zebu cattle as well as males and females were included. Sululta is located in the North Shoa zone, which has the highest population of *B. indicus* and *B. taurus* crossbred dairy cows desired for increased milk production and reared under intensive farming systems supplemented with commercial concentrated feed [24]. Zebu cattle are indeed primarily raised under extensive farming systems across most parts of Ethiopia, especially in lowland and mid-altitude areas, where they graze on natural pastures primarily relying on available grazing lands with minimal inputs. However, some Zebu cattle, particularly those destined for meat markets in and around urban population centers, may undergo 3-4 months of intensive fattening before slaughter. This extensive management of Zebu cattle contrasts with the more intensive systems used for crossbred cattle more typically associated with dairy production as also noted above. Cattle slaughtered for meat consumption at all slaughterhouses included in the current study, came from various parts of the country including Sheger, Wollo, Menize, Gonder, Jimma, Assela, Adama, Jiru, Woliata, Borena, Wollega, Sidama, and Harar [22, 25]. The source of the cattle cannot be traced back since routine documentation is uncommon, and animals are sold at multiple markets before slaughter.

4.3.3 Postmortem examination (PM) and sample collection

Postmortem examinations were conducted at the Adama, Burayu, Bishoftu (Elfora), and Sululta slaughterhouses by trained and experienced meat inspectors. Before beginning sample collection, the meat inspectors were also given additional refresher training from an experienced researcher on the diagnosis and detection of bTB-suggestive lesions. In each animal, all lung lobes and the mandibular, parotid, retropharyngeal, mediastinal, bronchial, hepatic, and mesenteric lymph nodes were thoroughly inspected and evaluated for the presence of one or more distinct spots of typical "tuberculous" granulomas characterized by abscess, caseous, and calcified lesions (Figure 4.2). The examination also included other relevant organs, such as the spleen, liver, kidneys, and related lymph nodes. Each lobe of the lung was compressed and palpated for granulomatous tuberculosis lesions deep within the parenchyma. To help identify the status of bTB, lymph nodes of the head, the lung, the GIT and others such as prescapular, prefemoral and mammary lymph nodes were cut into 1–2 cm thick slices and carefully examined. Then, all the lymph nodes or tissues with visible tuberculosis lesions (TBLs) were sampled from each TBL-positive animal and were collected in 50 mL sterile Falcon tubes containing 10 mL of 0.9% saline solution. The samples were transported to the

Microbiology Laboratory of the Aklilu Lemma Institute of Pathobiology of Addis Ababa University (ALPB-AAU) while the cold chain was kept using icebox at 4ºCand in the laboratory, stored at -20°C until processing. Bacterial culture and isolation were performed at the same laboratory.

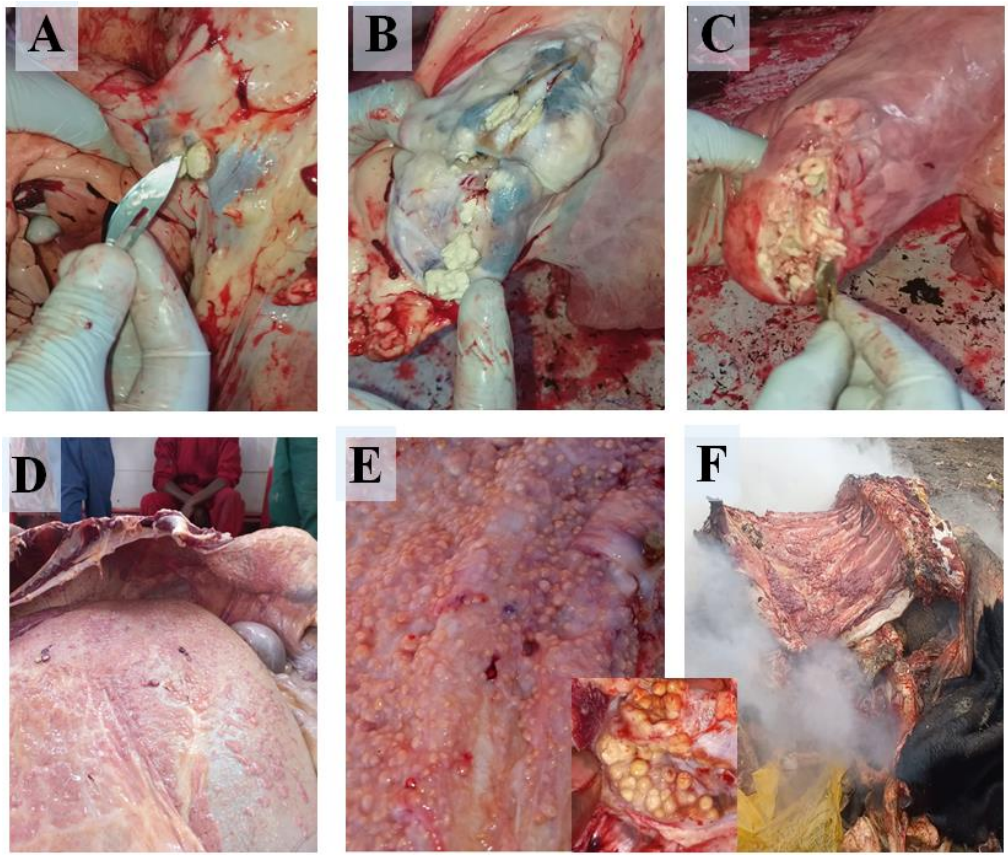


Figure 4.2: Different types of bTB lesions observed. A) A light yellow, dry caseous lesion in the left bronchial lymph node; B) A white thick creamy caseous lesion within enlarged caudal mediastinal lymph node; C) Cavitary tuberculous lesions with white creamy caseous abscess in the lung lobe D) Generalized case of miliary bTB numerous circumscribed raised lesions covering the whole peritoneum diaphragm and spleen, E) The same miliary bTB case with numerous yellowish small tuberculous lesions on parietal pleural surface of the rib cage. Similar several small yellowish lesions embedded within the sub-vertebral and rib cage muscles (insert). F) Complete disposal of the whole carcass of the same case by burning.

4.3.4 Mycobacterial culture from tissues

For mycobacterial isolation, the collected lymph node, lung, or other tissue specimens were cut into thin slices with sterile blades and homogenized with a sterile mortar and pestle and/or a stomacher. Then, an equal volume of 4% NaOH (sodium hydroxide) was added to

decontaminate the homogenate and continuously agitated with slow horizontal and vertical motions for 15 minutes to ensure thorough mixing and decontamination. Next, PBS is added to fill the tubes to 15 ml capacity and centrifuged at 1,106 g for 15 min. After discarding the supernatant, a drop of phenol red indicator was added to the sediment until it became red. Then, 2N HCL (hydrogen chloride) was added dropwise to neutralize the sediment. The change in the color of the solution from purple to yellow confirmed neutralization. Thereafter, 0.1 ml of the suspension from each sample was spread onto replicate slants of Lowenstein–Jensen (LJ) media supplemented with sodium pyruvate or glycerol (Sigma Aldrich, Co, St. Louis, USA). Cultures were then incubated at 37°C under aerobic conditions for up to 8 weeks. The culture tubes were observed weekly for growth of mycobacterial colonies. Cultures that did not exhibit growth on the slants after 8th week were considered to be negative. Culture-positive isolates were stained with Ziehl-Neelsen (ZN). The presence of acid-fast bacilli (AFB) in the colony smears confirmed culture-positive cases.

All culturing work was conducted in a biosafety level 3 (BSL-3) bacteriology laboratory of the Aklilu Lemma Institute of Pathobiology at Addis Ababa University (ALPB-AAU). The laboratory is specifically designed to ensure the safety of laboratory personnel and prevent potential exposure to pathogenic mycobacteria. It is equipped with specialized safety features, including ventilation systems that maintain negative pressure and HEPA filters that filter the air to prevent the release of airborne pathogens. To minimize the risk of inhalation or contact with infectious mycobacteria, all personnel working in the laboratory wear personal protective equipment (PPE) including gowns, N95 respirators, and gloves. Tissue homogenization, culturing, and the opening of culture slant tubes are performed within biosafety level 3 safety cabinets, which provide a sterile and contained environment. Rigorous decontamination protocols are followed for all equipment and surfaces using chemical disinfectants, and waste materials are autoclaved to ensure complete sterilization.

4.3.5 DNA extraction

Using a sterile loop, a loopful of cells was transferred from acid-fast bacilli (AFB)-positive LJ medium to Eppendorf tubes containing 300 μl of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) and killed by incubation at 85°C for 60 minutes on a heating block. After cell inactivation, the suspension was washed with PBS, and

DNA was extracted using the CTAB-chloroform technique as described in a previous study [26].

4.3.6 Detection and speciation of MTBC by mRT–PCR

The target genes used in the current multiplex qPCR assay were tested and validated in previous studies [15, 16, 18, 21]. The DNA was amplified by two-step real-time multiplex qPCR at AHI (Animal Health Institute), Sebeta, Ethiopia. In the first step of the multiplex qPCR cycle, three primer-probe sets were used.1) A 100 bp IS1081 insertion sequence found in all MTBC species was used to identify all MTBCs. 2) Deletion of yrbE3A was used to collectively identify animal-adapted MTBC species, including *M. caprae*, *M. bovis,* and *M. orygis*. 3) The 'RD7' deletion in the mce3D gene was used to collectively identify human-adapted *M. tuberculosis* lineages (1–6). In the second step of the multiplex qPCR cycle*,* another three primer sets were used. 1) The intergenic RD4 gene deletion was used to detect *M. bovis* specifically; 2) a single nucleotide polymorphism (SNP) in the lepA (Rv2404c) gene was used to specifically identify *M. caprae;* and 3) a single nucleotide polymorphism (SNP) in the rskA gene was used to specifically detect *M. orygis*. The details of all primer and probe sets used in the first and second RT–PCR steps are presented in Supplementary Table 4.2.

DNA amplification was performed in a final volume of 20 μL of reaction mixture. The reaction mixture consisted of 10 μL of master mix for primer gene expression with ROX dye, 0.5 microliter (μL) of each primer, 5 μL of DNA template, and 3.5 μL of nuclease-free water. Amplification was performed on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The thermocycler protocol included one cycle of 3 minutes at 95°C for activation of the polymerase, 40 cycles of 15 sec at 95°C for denaturation, and 40 cycles of 60 sec each at 63°C for annealing/extension. The sample was classified as PCR positive if the machine returned a Ct value less than 40 for all primer–probe combinations.

4.3.7 Positive case definition for MTBC infection

In our study, an animal or sample was considered "positive" for MTBC infection when it tested positive by both culture and m-qPCR.

4.3.8 Statistical analysis

At the slaughterhouses, identifiers of each examined animal, such as identification number, date of examination, breed, sex, organ with lesion, and others, were recorded on a hardcopy form. The data were then transcribed to Microsoft Excel 2010. The data in Excel were imported to R software version 4.3.2 [27]. We developed three logistic regression models to assess the probability of animals demonstrating visible TB lesions (TBL) (Table 4.1) and the probability of *M. bovis* being cultured per animal (Table 4.3) and per tissue sample (Table 4.4). To this end we built three multivariate logistic regression models corresponding to these response variables, fitted using the glm function from the base stats package (with the default log link function) and the same model building strategy. First, we carried out a univariable screen on the collected risk factors (slaughterhouse, breed, sex and for the final model body region of the sampled tissue) with a generous inclusion threshold. All variables below this threshold ($p < 0.1$) were considered for inclusion in multivariate models. We then carried out a stepwise variable selection (forwards and backwards) based on the Akaike Information Criterion (AIC). As only one lesioned animal was collected from the Bishoftu (Elfora) slaughterhouse (that was culture negative) samples from this animal were excluded from the two models for the probability of culture (Table 4.3, Table 4.4) to avoid overfitting. Summary measures were calculated and tabulated using the 'gtsummary' package version 1.7.2 [28]. The odds ratio (OR) of the reference groups (Ref.) in our logistic regression analysis is 1, the default used as a baseline against which an increase or decrease in the odds of bTB occurring is measured relative to the other group/s. The OR greater than 1 indicates increased odds, an OR less than 1 indicates decreased odds, and an OR of 1 indicates no difference (or change) in odds of the bTB occurring relative to the reference group. A map indicating the locations of slaughterhouse towns was generated and annotated using the leaflet R package version 2.2.2 [29]. Values of $P< 0.05$ and 95% CIs were considered statistically significant.

4.3.9 Ethical approval

Ethical approval was obtained from the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Institutional Research Ethics and Review Committee, Minute's Reference No.: ALIPB IRB/001/2017/2018, The experiment was conducted according to the Technical Guideline for National Research Ethics Review, ensuring compliance with the guidelines [30]. Additionally, the study adhered to and reported following the ARRIVE guidelines 2.0.

4.4 Results

Prevalence of tuberculous lesions and associated risk factors

A total of 7,640 animals were evaluated, and characteristic tuberculous-like lesions were observed in 173 animals, with an overall lesion prevalence of 2.3% (95% CI: 2.0, 2.6). The prevalence of TB lesions varied by breed, sex, and slaughterhouse. The prevalence of lesions was significantly greater in crossbred cattle compared to local zebu cattle and in Burayu and Adama slaughterhouses than in Sululta (Table 4.1). Multivariable logistic regression revealed that females had a lower risk of TBL lesions, with an odds ratio of 0.42 (95% CI: 0.24 to 0.76), while crosses had a greater risk, with an odds ratio of 11.8 (95% CI: 6.4, 21.2). When compared to cattle slaughtered at the Sululta slaughterhouse used as reference, those slaughtered at the Adama and Burayu slaughterhouses showed significantly higher probabilities of having TBL lesions, with odds ratios of 3.2 (95% CI, 1.2-7.3) and 5.8 (95% CI, 3.9-8.9), respectively (Table 4.1).

Table 4.1: Estimated risk factors from univariate and multivariate logistic regression models for the risk of TBL in cattle examined at four slaughterhouses in central Ethiopia: Sululta, Burayu, Adama, and Bishoftu (Elfora).

Variables	N (%) Examined Animals	N (%) Lesioned Animals	95% CI2	Coefficient estimate	95%CI	Univariate Models			Multivariate Model		
						OR	95% CI	p value	OR	95% CI	p value
Intercept				**-4.8**	**-5.2, -4.5**				**-**	**-**	**< 0.001**
				Sex							
Male	6052	125 (2.1)	[1.7, 2.5]	Ref	Ref	Ref.	Ref.		Ref.	Ref.	
Female	1588	48 (3.0)	[2.3, 4.0]	-0.83	-1.4, -0.3	1.5	1.1, 2.1	**0.023**	0.4	0.2, 0.8	**0.003**
Breed											
Zebu	6287	112(1.8)	[1.5, 2.1]	Ref	Ref	Ref.	Ref.		Ref.	Ref.	
Cross	1353	61(4.5)	[3.5, 5.8]	2.4	1.8, 3.0	2.6	1.9, 3.6	**<0.001**	11.8	6.4, 21.2	**<0.001**
Slaughterhouses											
Sululta	5723	95 (1.7)	[1.4, 2.0]	Ref	Ref	Ref.	Ref.		Ref.	Ref.	
Adama	230	6 (2.6)	[1.1, 5.7]	1.1	0.2, 2.0	1.6	0.6, 3.4	0.3	3.2	1.2, 7.3	**0.009**
Burayu	1539	71 (4.6)	[3.7, 5.8]	1.7	1.3, 2.1	2.9	2.1, 3.9	**<0.001**	5.8	3.9, 8.9	**<0.001**
Bishoftu (Elfora)	148	1 (0.7)	[0.01, 4.]	-0.2	-1.9, 1.5	0.4	0.0, 1.8	0.4	0.8	0.1, 3.9	0.8
Total	**7640**	**173 (2.3)**	**[2.0, 2.6]**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

N (%) = number (percent)

OR = odds ratio, CI = confidence interval

*The OR for the reference groups (Ref.) is 1, the baseline against which an increase or decrease relative risk compared to the other groups is measured

Distribution of bTB lesions in the carcass

The distribution of lesions suggestive of bTB in the examined organs and lymph nodes is shown in Table 4.2. Tuberculous lesions were most common in the lungs and lung lymph nodes, representing 61% (236/388) of all lesions, followed by 18% (71/388) in the head lymph nodes and 17% (66/388) in the gastrointestinal tract (GIT) lymph nodes, whereas in the other superficial lymph nodes, including the prescapular, prefemoral and mammary lymph nodes, TBL was observed in only 4% (15/388) of the total collection of lesions.

Table 4.2: Sampled lymph nodes and tissues with tuberculous-like lesions (TBLs) from the thoracic cavity, head, GIT, and other superficial body regions of cattle

Body region	LNs or tissue TBL Positive	Number TBL positive	Number (%) Culture* & m-qPCR Positive	95% CI
Lung associated	Lung tissue	34	13 (38)	23, 56
	Tracheobronchial LNs	78	25(32)	22, 44
	Mediastinal LNs	106	35 (33)	24, 43
	Diaphragm	12	3 (25)	7, 57
	Thoracic wall	6	1(17)	1, 64
	Subtotal	**236**	**77 (33)**	**27, 39**
Head associated	Retropharyngeal LNs	29	9 (31)	16, 51
	Mandibular LNs	36	4 (11)	4, 27
	Parotid LNs	6	0 (0)	0, 48
	Subtotal	**71**	**13 (18)**	**10, 30**
GIT associated	Hepatic LNs	13	2 (15)	3, 46
	Mesenteric LNs	48	2 (4)	1, 15
	Liver tissue	1	0 (0)	0, 95
	Spleen tissue	3	0 (0)	0, 69
	Pelvic cavity LNs	1	0 (0)	0, 95
	Subtotal	**66**	**4 (6)**	**2, 16**
Others superficial	Prefemoral LNs	2	0 (0)	0, 80
	Prescapular LNs	12	0 (0	0, 30
	Mammary LNs	1	0 (0	0, 95
	Subtotal	**15**	**0 (0)**	0, 24
	Total	**388**	**94 (24.2)**	**20, 29**

TBL = Tuberculous-like lesions, GIT = Gastrointestinal tract, LNs = Lymph nodes
Confidence intervals were computed using ‘tbl_summary()’ function of ‘gtsummary’ package by the default, "Wilson" method. *Culture Positive: refers to observed growth of mycobacterial colonies confirmed by presence of acid-fast bacilli (AFB) in colony smears stained with ZN

Culture-positive mycobacterial growth and influencing factors

From the 173 animals, a total of 388 TB lesions were collected. Of the 173 animals with lesions, 63 (36%), and of the 388 lesions sampled, 94 (24.2) were culture and m-qPCR-positive. On a step-wise forwards and backwards variable selection procedure based on the Akaike Information Criterion (AIC), slaughterhouse was dropped out of the final selected regression models for no significant difference among slaughterhouses on culture and m-qPCR confirmation at both the animal (Table 4.3) and tissue (Table 4.4) levels. A multivariate logistic regression revealed significantly higher culture and m-qPCR positivity in cross breed cattle OR of 17.2 (95% CI: 5.1, 79.3, p <0.001), but no significant difference between sexes (Table 4.3). Tuberculous-like lesions from male and crossbred cattle produced a significantly greater positive mycobacterial culture and m-qPCR positive test, with an OR of 2.5 (95% CI: 1.1, 5.7, p=0.031) and 6.2 (95% CI: 2.7, 14.7, p <0.001), than TB-like lesions collected from female and zebu cattle, respectively. There were significantly more culture and m-qPCR positive tuberculous lesions in the lungs and lymph nodes of the lungs (OR =7.1; 95% CI: 2.7, 24.5, <0.001) and head lymph nodes (OR= 5.6; 95% CI: 1.8, 21.7; p=0.006) compared to lymph nodes of GIT (Table 4.4).

Table 4.3: Estimated risk factors from univariate and multivariate logistic regression models for the probability of successful culture from lesions stratified by sex, breed, slaughterhouse and anatomical site of lesion.

Variable	N culture & m-qPCR/TBL positive animals	% (95% CI) culture & m-qPCR positive lesioned animals	Coefficient estimate	95%CI	Univariate Models			Multivariate Model		
					OR	95% CI	p value	OR	95% CI	p value
Intercept			2.3	-4.0, -1.0	-	-	**0.001**	-	-	**0.001**
Sex										
Female	29/48	60 (45, 74)			Ref	Ref		Ref	Ref	
Male	34/125	27 (20, 36)	0.9	-0.3, 2.5	0.2	0.1, 0.5	**<0.001**	2.6	0.7, 12.2	0.2
Breed										
Zebu	22/112	20 (13, 28)			Ref.	Ref.		Ref	Ref	
Cross	41/61	67 (54, 78)	2.9	1.7, 4.4	8.4	4.2, 17.4	**<0.001**	17.2	5.1, 79.3	**<0.001**

N = Number

OR = odds ratio, CI = confidence interval

TBL = Tuberculous-like lesions, GIT = Gastrointestinal tract, LN = Lymph node

*The OR for the reference groups (Ref.) is 1, the baseline against which an increase or decrease relative risk compared to the other groups is measured

Table 4.4: Estimated risk factors from multivariate logistic regression models for the probability of successful culture from all TB lesions stratified by sex, breed, and anatomical site of lesion.

Variables	N culture and m-qPCR /TBL positive tissues	% (95% CI) culture and m-qPCR positive lesioned tissues	Coefficient estimate	95%CI	Univariate Models			Multivariate Model		
					OR	95% CI	p value	OR	95% CI	p value
Intercept	-	-	-4.2	-5.8, -2.9	-	-	**<0.001**	-	-	**<0.001**
Sex										
Female	44/132	33 (26, 42)	Ref	Ref	Ref	Ref		Ref	Ref	
Male	50/240	21 (16, 26)	0.9	0.1, 1.8	0.50	0.3, 0.8	**0.005**	2.5	1.1, 5.7	**0.031**
Breed										
Zebu	34/215	16 (12, 21)	Ref	Ref	Ref	Ref		Ref	Ref	
Cross	60/157	38 (31, 46)	1.8	1.0, 2.7	3.35	2.1, 5.9	**< 0.001**	6.2	2.7, 14.7	**<0.001**
Body region										
GIT LNs and tissues	4/66	6 (2, 16)	Ref	Ref	Ref	Ref		Ref	Ref	
Lung LNs and tissues	77/236	33 (27, 39)	2.0	1.0, 3.2	7.5	3.0, 25.4	**<0.001**	7.1	2.7, 24.5	**<0.001**
Head LNs	13/70	19 (11, 29)	1.7	0.6, 3.1	3.5	1.2, 13.1	**0.035**	5.6	1.8, 21.7	**0.006**
Other	0/15	0 (0,25)	-14	-247, -42	0.0	0.0, 1,581	> 0.9	0.0	0.0, 0.0	>0.9
Total	94/388	24.2 (20, 29)								

N = number, % = percent,
OR = odds ratio, CI = confidence interval
TBL = Tuberculous-like lesions, GIT = Gastrointestinal tract, LN = Lymph node
*The OR for the reference groups (Ref.) is 1, the baseline against which an increase or decrease relative risk compared to the other groups is measured

Multiplex qPCR

Amplification of IS1081 was detected in all 94 isolates (100%), confirming their identity as MTBC species. Of the 94 MTBCs confirmed by IS1081, 92 (98%) tested positive for the animal-adapted MTBC species. Among the four samples that tested positive for *M. tuberculosis*, three showed late amplification (Ct >37), which may be a false positive. On the other hand, one culture isolate (1%) from mediastinal lymph node of a crossbred cow at the Sululta slaughterhouse showed early amplification (Ct = 25.7) for *M. tuberculosis*, suggesting a potential true positive case. This isolate also showed positive amplification for animal MTBC-specific primer-probe sets (Ct = 17.2) and *M. bovis*-specific primer-probe sets (Ct = 18.0) (Figure 4.3). In the second stage of the PCR cycle, all 94 samples (100%) tested positive for *M. bovis* according to the specific probe set. One culture isolate (1%) from tracheobronchial lymph node of a local male zebu tested positive for both *M. caprae* (Ct = 19.4) and *M. bovis* (Ct = 31.8) (Table 4.5 and Figure 4.3). However, none (0%) of the isolates tested positive for the *M. orygis*-specific PCR primer-probe sets.

Table 4.5: Number of positive amplifications of the target species genes in DNA samples extracted by the CTAB-chloroform method

Target species	Number detected	Number undetected	Ct values		
			Median	Mean	Range
IS1081 (All MTBC)	94	0	19.6	22.0	14.7, 35.7
Animal adapted MTBC	92	2	19.3	21.4	14.0, 34.6
M. tuberculosis	4*	90	38.6	35.7	25.7, 39.9
M. bovis	94	0	20.4	23.0	15.0, 37.0
M. caprae	1	93	19.4	19.4	-
M. orygis	0	94	-	-	-

*Only one had Ct value of 25.7, the other three had Ct values >37, likely to be false positives

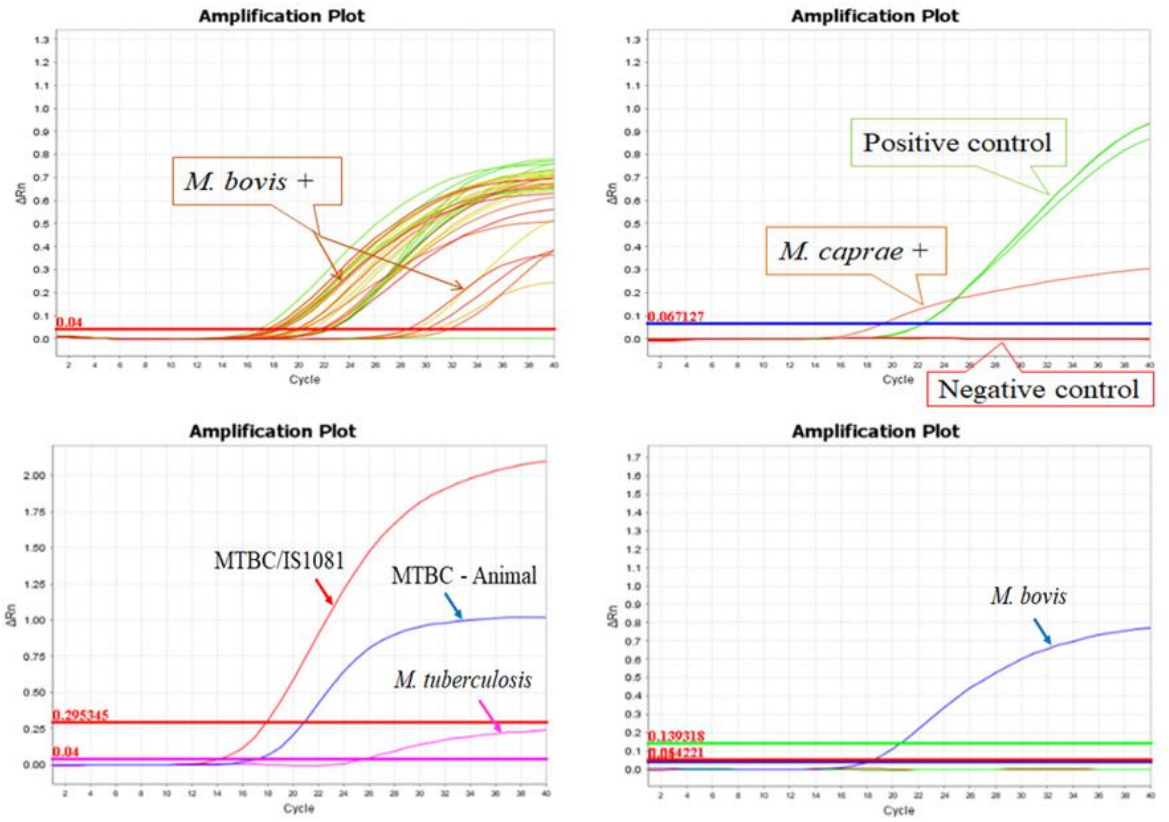


Figure 4.3: Amplification plot showing PCR-positive *M. bovis*, *M. tuberculosis and M. caprae* samples. Upper panel) Amplification plot showing partially selected PCR-positive *M. bovis* and single PCR-positive *M. caprae* samples. Lower panel) A sample with mixed *M. bovis* and *M. tuberculosis,* the plots of first step PCR are shown in the left and second step PCR in the right. The most abundant target IS1081 and MTC Ani already reached maximum plateau when the *M. tuberculosis* target started to rise and cross the cycle threshold probably leading to suppressed amplification due to early consumption of the nucleotides in the reaction mixture

4.5 Discussion

Bovine tuberculosis remains a significant concern for animal health and public health, particularly in developing countries with growing livestock industries. In Ethiopia, where crossbreeding programs are expanding and raw animal product consumption is common, understanding the prevalence, risk factors, and causative agents of bTB is crucial for developing effective control strategies. This study aimed to investigate these aspects in cattle slaughtered at four central

Ethiopian abattoirs, providing insights into the current status of bTB and its potential implications for both animal and human health in the region.

This study revealed an overall prevalence of 2.3% (95% CI = 2.0-2.6) for TBL in cattle carcasses across the four studied slaughterhouses in central Ethiopia. This prevalence aligns with previous reports from different parts of Ethiopia, including 2.8% from Bahirdar [31], 1.1% from Hawassa [32], and 1.5% from Addis Ababa [33]. Our findings are also consistent with prevalence ranges reported in other African countries, such as 0.78 to 1.90% reported in Nigeria [34, 35], 1.8% in Mali [36], and 0.18 to 0.82% in Cameroon [37], 0.9% (148/16753) in Rwanda [38], and 2.7 in in Burkina Faso [39]. However, it's important to note that other studies from Ethiopia and sub-Saharan African countries have reported higher prevalences ranging from 4 - 11% based on lesion detection [9, 40–49]. This variability in reported prevalence highlights the complexity of bTB surveillance and the influence of various factors on prevalence estimates.

The prevalence of bTB lesions observed in our study may be influenced by several factors and is likely a lower bound given the general lack of sensitivity in detecting lesions during routine meat inspections may lead to underestimation of true prevalence. A meta-analysis indicated that the sensitivity of TBL detection by necropsy at meat inspection was 46% [50]. Additionally, practical constraints in abattoir settings, such as uncooperative attitudes of butchers and time constraints on meat inspectors, may hinder thorough inspections, potentially leading to missed lesions. The predominance of zebu cattle, which may have lower risks of exposure and susceptibility to bTB compared to intensively reared exotic breeds, may also contribute to the apparently lower observed prevalence in settings where zebu cattle predominate [4]. Furthermore, postmortem examination at slaughterhouses typically reveals only more advanced forms of bTB, likely underestimating the true prevalence of infection in the cattle population [33, 34, 37, 47].

Hence, to more accurately assess the actual prevalence of bTB, a comprehensive approach would be beneficial. This could involve conducting ante-mortem skin tests and/or IGRA, followed by post-mortem examination of test-positive animals. Additionally, direct detection of causative organisms through PCR, irrespective of the presence or absence of visible lesions, could provide a more sensitive measure of bTB prevalence. Nonetheless, the 2.3% TBL prevalence we observed

underscores the persistent presence of bTB in the Ethiopian cattle population and indicates a continued risk for both animal health and potential zoonotic transmission, particularly given the common practices of raw meat and milk consumption in the region.

Our study revealed that crossbred cattle had a substantially higher risk of TBL compared to local zebu cattle (OR = 11.8, 95% CI: 6.4, 21.2, p<0.001). This aligns with previous studies reporting greater bTB prevalence in Holstein-Friesian and crossbred cattle compared to zebu breeds in Ethiopia and Uganda [4, 44, 51]. The increased susceptibility of crossbred cattle likely stems from a combination of genetic factors and intensive management practices associated with dairy production systems.

In this study, we observed variation in bTB risk across slaughterhouse locations, with animals slaughtered at the Adama (OR = 3.2, 95% CI: 1.2, 7.3, p= 0.009) and Burayu (OR = 5.8, 95% CI: 3.9, 8.9, p<0.001) having a significantly greater risk of bTB lesions than those at Sululta. This regional variation is consistent with previous studies [41, 42, 44, 52] and can be attributed to differences in farming system, breed, age, and contact with wildlife. A meta-analysis of 21 studies revealed a higher pooled prevalence of bTB lesion (5.1% nationally and 5.0% in central Ethiopian slaughterhouses) than our findings of 2.3% [4]. The wide range (0.9 to 13.2%) of the reported prevalences [4] underscores the variability in bTB prevalence and detection sensitivity across studies. These findings highlight the complex interplay of factors influencing bTB prevalence and emphasize the need for targeted interventions, particularly in intensive dairy systems.

Tuberculous lesions were predominantly observed in the lungs and lung-associated lymph nodes, suggesting that the respiratory tract is the primary route of bTB infection for these study animals. This is consistent with previous studies in Ethiopia and other African countries [32–34, 37, 40, 41, 47, 53], although some studies have reported different patterns of organ involvement [36, 43].

Culture and m-qPCR positivity rates varied across anatomical sites: 33% (77/236) for thoracic cavity lesions, 18% (13/71) for head region lesions, 6% (4/67) for the gastrointestinal lesions. No lesions from superficial lymph nodes were culture and m-qPCR positive. The higher culture and

m-qPCR positivity rate in thoracic lesions suggests that TBLs in this region may have a higher specificity for bTB, aligning with the understanding that the respiratory route is the primary mode of infection. Conversely, lower rates in other sites indicate that TBLs in these areas may have lower specificity for bTB and could be caused by other granuloma-inducing pathogens. These findings underscore the importance of combining gross pathological examination with bacteriological culture and/or molecular techniques for definitive bTB diagnosis. They also suggest that prioritizing thoracic cavity inspection during meat examination could increase bTB detection efficiency. However, lesions in other anatomical sites shouldn't be disregarded, as they could represent early or disseminated bTB infections. The predominance of respiratory lesions also has implications for bTB control strategies, suggesting that measures aimed at reducing close animal contact, improving ventilation, and implementing appropriate biosecurity measures could be particularly effective in reducing transmission, especially in intensive farming systems.

Of the 388 TBLs collected, 94 (24.2%) were culture and m-qPCR positive, consistent with some previous studies [48, 56, 57]. All culture-positive isolates were identified as *M. bovis* by m-qPCR, confirming its predominant role in bTB in this region. Based on culture and m-qPCR confirmed infection, the prevalence of bTB was 0.8% (95% CI: 0.6-1.0) in the current study. We detected two cases of mixed infections: one zebu cattle with *M. caprae* and *M. bovis*, and a crossbred cow with *M. tuberculosis* and *M. bovis*. The detection of *M. tuberculosis* in a bovine host suggests potential human-to-animal transmission, consistent with previous reports [8–10, 48].

It is important to note that our study employed m-qPCR on DNA from culture isolates, a method that offers significant advantages over traditional culture techniques for detecting MTBC lineages in animal tuberculosis. This approach is important given the expanding understanding of MTBC, which includes *M. bovis, M. orygis, M. caprae*, and *M. tuberculosis* sensu stricto as causative agents of mammalian tuberculosis [54–56]. Unlike time-consuming traditional methods, m-qPCR provides rapid (within hours), highly specific and sensitive detection, even with small sample volumes. It simultaneously identifies multiple MTBC species/lineages, overcoming limitations of traditional culture methods in distinguishing between MTBC members. The potential adaptation of m-qPCR for direct testing on clinical specimens could significantly reduce diagnosis time. Moreover, this technique offers a more comprehensive view of zoonotic tuberculosis (zTB)

epidemiology, addressing the recent call for more precise identification of zTB causative agents [54].

The detection of *M. bovis* in all culture-positive samples using m-qPCR confirms the ongoing presence of bTB in cattle, presenting a persistent zoonotic risk. This risk is amplified by the common practice of consuming raw meat and unpasteurized milk in Ethiopia. The identification of *M. tuberculosis* in a bovine host highlights the complex, bidirectional nature of tuberculosis transmission in settings with close human-animal contact. The higher prevalence of bTB in crossbred cattle is particularly concerning as Ethiopia's dairy industry grows, potentially increasing human exposure to bTB. The predominance of respiratory tract lesions indicates that aerosol transmission is the main route of bTB spread, emphasizing the risk for individuals in close contact with infected animals.

These findings underscore the necessity for a One Health approach to tuberculosis control in Ethiopia, involving integrated human and animal health surveillance, improved food safety measures, and public education about the risks of consuming raw animal products. Enhanced surveillance, improved diagnostics, and coordinated control efforts across human and animal health sectors are crucial to effectively mitigate the public health risks associated with bTB bovine tuberculosis.

One of the limitations of our study is related to the culturing procedure. Our study's culture recovery rate of 24.2% from TBL-positive specimens might have been influenced by several methodological factors. These include the potentially harsh decontamination method (4% NaOH for 15 minutes), lower-than-optimal centrifugation speed (1,106 g), lack of sediment resuspension, shorter incubation period (8 weeks versus 12-16 weeks in some studies), and sole reliance on Löwenstein-Jensen medium. These factors may have contributed to lower mycobacterial recovery rates compared to some other studies [38, 39, 48]. To address these issues, future studies should consider modifying the current culture procedure to optimize mycobacterial recovery. However, the use of m-qPCR for species identification provided rapid and specific results, offering advantages over traditional culture methods in detecting multiple MTBC lineages and identifying mixed infections.

Additional study limitations include our inability to trace cattle origin, small sample sizes from some slaughterhouses, and lack of age data for study animals. These factors limit the generalizability of our findings to the broader cattle population in the study area and highlight the challenges in bTB surveillance and underscore the need for standardized, optimized protocols in mycobacterial detection and identification. Such standardization would enhance the accuracy and comparability of bTB prevalence data across studies and regions. Despite these limitations, our study provides valuable insights into bTB prevalence and characteristics in central Ethiopian cattle, contributing to the broader understanding of this important zoonotic disease in the region.

Conclusions and recommendations

Our study reveals the ongoing challenge of bTB in Central Ethiopia, with a 2.3% prevalence of tuberculous-like lesions and a higher risk in crossbred cattle. The identification of *M. bovis* as the primary causative agent, alongside rare cases of *M. tuberculosis* and *M. caprae*, underscores the complex nature of bTB and its zoonotic potential. These findings highlight the need for enhanced surveillance and control strategies, particularly in the growing dairy sector. We recommend implementing more sensitive diagnostic techniques, focusing control efforts on intensive dairy farms, strengthening public health measures, and adopting a One Health approach that integrates animal and human TB surveillance and control programs. Future research should explore transmission dynamics between humans and cattle, evaluate control strategies across different production systems, and establish long-term, nationwide surveillance to comprehensively address bTB and risk for zoonotic TB in Ethiopia.

Animal Ethics declaration:

Not applicable. The study was conducted on cattle slaughtered to supply meat for the human population by the Municipalities.

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4.6 References

1. Huard RC, de Oliveira Lazzarini LC, Butler WR, van Soolingen D, Ho JL. PCR-Based Method to Differentiate the Subspecies of the *Mycobacterium tuberculosis* Complex on the Basis of Genomic Deletions. J Clin Microbiol. 2003;41:1637–50.

2. Langer AJ, LoBue PA. Public health significance of zoonotic tuberculosis caused by the *Mycobacterium tuberculosis* complex. In: Zoonotic Tuberculosis: *Mycobacterium bovis* and other pathogenic mycobacteria. Third Edition. Iowa, USA: John Wiley & Sons, Inc.; 2014.

3. ETHIOPIAN STATISTICAL SERVICES. REPORT ON LIVESTOCK AND LIVESTOCK CHARACTERISTICS (PRIVATE PEASANT HOLDINGS). Annual Agricultural Sample Survey. Addis Ababa, Ethiopia: FEDERAL DEMOCRATIC REPUBLIC OF ETHIOPIA ETHIOPIAN STATISTICS SERVICE AGRICULTURAL SAMPLE SURVEY; 2022.

4. Sibhat B, Asmare K, Demissie K, Ayelet G, Mamo G, Ameni G. Bovine tuberculosis in Ethiopia: A systematic review and meta-analysis. Preventive Veterinary Medicine. 2017;147:149–57.

5. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, *et al.* Mycobacterial Lineages Causing Pulmonary and Extrapulmonary Tuberculosis, Ethiopia. Emerg Infect Dis. 2013;19:460–3.

6. Nuru A, Mamo G, Zewude A, Mulat Y, Yitayew G, Admasu A, *et al.* Preliminary investigation of the transmission of tuberculosis between farmers and their cattle in smallholder farms in northwestern Ethiopia: a cross-sectional study. BMC Research Notes. 2017;10:31–31.

7. Kidane D, Olobo JO, Habte A, Negesse Y, Aseffa A, Abate G, *et al.* Identification of the Causative Organism of Tuberculous Lymphadenitis in Ethiopia by PCR. J Clin Microbiol. 2002;40:4230–4.

8. Ameni G, Vordermeier HM, Firdessa R, Aseffa A, Hewinson RG, Gordon SV, *et al.* *Mycobacterium tuberculosis* infection in grazing cattle in central Ethiopia. Veterinary Journal. 2011;188:359–61.

9. Ameni G, Desta F, Firdessa R. Molecular typing of *Mycobacterium bovis* isolated from tuberculosis lesions of cattle in north eastern Ethiopia. Veterinary Record. 2010;167:138–41.

10. Berg S, Firdessa R, Habtamu M, Gadisa E, Mengistu A, Yamuah L, *et al.* The Burden of Mycobacterial Disease in Ethiopian Cattle: Implications for Public Health. PLoS ONE. 2009;4:e5068.

11. Arega S, Conraths F, Ameni G. Prevalence of tuberculosis in pigs slaughtered at two abattoirs in Ethiopia and molecular characterization of *Mycobacterium tuberculosis* isolated from tuberculous-like lesions in pigs. BMC Vet Res. 2013;9:97.

12. Gumi B, Schelling E, Berg S, Firdessa R, Erenso G, Mekonnen W, *et al.* Zoonotic Transmission of Tuberculosis Between Pastoralists and Their Livestock in South-East Ethiopia. EcoHealth. 2012;9:139–49.

13. Deresa B, Conraths FJ, Ameni G. Abattoir-based study on the epidemiology of caprine tuberculosis in Ethiopia using conventional and molecular tools. Acta Vet Scand. 2013;55:15.

14. Mamo G, Abebe F, Worku Y, Legesse M, Medhin G, Bjune G, *et al.* Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of *Mycobacterium tuberculosis* from Goat. Veterinary Medicine International. 2012;2012:1–8.

15. Duffy SC, Srinivasan S, Schilling MA, Stuber T, Danchuk SN, Michael JS, *et al.* Reconsidering *Mycobacterium bovis* as a proxy for zoonotic tuberculosis: a molecular epidemiological surveillance study. Lancet Microbe. 2020;1:e66–73.

16. Dykema PE, Stokes KD, Beckwith NR, Mungin JW, Xu L, Vickers DJ, *et al.* Development and validation of a direct real-time PCR assay for *Mycobacterium bovis* and implementation into the United States national surveillance program. preprint. PeerJ PrePrints; 2016.

17. Reddington K, O'Grady J, Dorai-Raj S, Maher M, Van Soolingen D, Barry T. Novel Multiplex Real-Time PCR Diagnostic Assay for Identification and Differentiation of *Mycobacterium tuberculosis*, *Mycobacterium canettii*, and *Mycobacterium tuberculosis* Complex Strains. J Clin Microbiol. 2011;49:651–7.

18. Reddington K, O'Grady J, Dorai-Raj S, Niemann S, Van Soolingen D, Barry T. A Novel Multiplex Real-Time PCR for the Identification of Mycobacteria Associated with Zoonotic Tuberculosis. PLoS ONE. 2011;6:e23481.

19. Taylor GM, Worth DR, Palmer S, Jahans K, Hewinson RG. Rapid detection of *Mycobacterium bovis* DNA in cattle lymph nodes with visible lesions using PCR. BMC Vet Res. 2007;3:12.

20. Domogalla J, Prodinger WM, Blum H, Krebs S, Gellert S, Müller M, *et al.* Region of Difference 4 in Alpine *Mycobacterium caprae* Isolates Indicates Three Variants. J Clin Microbiol. 2013;51:1381–8.

21. Saïd-Salim B, Mostowy S, Kristof AS, Behr MA. Mutations in *Mycobacterium tuberculosis Rv0444c* , the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in *Mycobacterium bovis*. Molecular Microbiology. 2006;62:1251–63.

22. Abera D, Mego L, Amenu K, Cook ElizabethAJ, Alonso S. A Closer Look at Livestock Markets and Meat Supply Chain in Ethiopia: Implications for Sustainable Urban Food System. 2023. https://www.ilri.org/knowledge/publications/closer-look-livestock-markets-and-meat-supply-chain-ethiopia-implications. Accessed 29 Jun 2024.

23. Birmaduma G, Yesihak Y, Mohammad Y K. Evaluation of physical Facilities, Operation and Management Practice in Selective Public Abattoirs in Eastern Oromia, Ethiopia. Int J Agric Sc Food Technol. 2019;5:043–9.

24. Brandsma W, Mengistu D, Kassa B, Yohannes M, Lee J van der. The major Ethiopian milksheds : an assessment of development potential. 2013.

25. Kumsa B. Cystic echinococcosis in slaughtered cattle at Addis Ababa Abattoir enterprise, Ethiopia. Veterinary and Animal Science. 2019;7:100050.

26. Amaro A, Duarte E, Amado A, Ferronha H, Botelho A. Comparison of three DNA extraction methods for *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. avium. Lett Appl Microbiol. 2008;47:8–11.

27. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria; 2023.

28. Sjoberg D, Whiting K, Curry M, Lavery J, Larmarange J. Reproducible Summary Tables with the gtsummary Package. The R Journal. 2021;13:570–80.

29. Cheng J, Schloerke B, Karambelkar B, Xie Y. leaflet: Create Interactive Web Maps with the JavaScript "Leaflet" Library. 2024.

30. Ministry of Education (MoE). National Research Ethics Review Technical Guideline. 2022.

31. Nuru A, Zewude A, Mohammed T, Wondale B, Teshome L, Getahun M, *et al.* Nontuberculosis mycobacteria are the major causes of tuberculosis like lesions in cattle slaughtered at Bahir Dar Abattoir, northwestern Ethiopia. BMC Vet Res. 2017;13:237.

32. Regassa A, Tassew A, Amenu K, Megersa B, Abunna F, Mekibib B, *et al.* A cross-sectional study on bovine tuberculosis in Hawassa town and its surroundings, Southern Ethiopia. Trop Anim Health Prod. 2010;42:915–20.

33. Asseged B, Woldesenbet Z, Yimer E, Lemma E. Evaluation of Abattoir Inspection for the Diagnosis of *Mycobacterium bovis* Infection in Cattle at Addis Ababa Abattoir. Tropical Animal Health and Production. 2004;36:537–46.

34. Ejeh EF, Raji MA, Bello M, Lawan FA, Francis MI, Kudi AC, *et al.* Prevalence and Direct Economic Losses from Bovine Tuberculosis in Makurdi, Nigeria. Veterinary Medicine International. 2014;2014:e904861.

35. Sa'idu AS, Mohammed S, Ashafa M, Gashua MM, Mahre MB, Maigado AI. Retrospective study of bovine tuberculosis in Gombe Township Abattoir, Northeastern Nigeria. Int J Vet Sci Med. 2017;5:65–9.

36. Müller B, Steiner B, Bonfoh B, Fané A, Smith NH, Zinsstag J. Molecular characterisation of *Mycobacterium bovis* isolated from cattle slaughtered at the Bamako abattoir in Mali. BMC Vet Res. 2008;4:26.

37. Ndukum JA, Kudi AC, Bradley G, Ane-Anyangwe IN, Fon-Tebug S, Tchoumboue J. Prevalence of bovine tuberculosis in abattoirs of the littoral and Western highland regions of cameroon: a cause for public health concern. Vet Med Int. 2010;2010:495015.

38. Habarugira G, Rukelibuga J, Nanyingi MO, Mushonga B. Bovine tuberculosis in Rwanda: Prevalence and economic impact evaluation by meat inspection at Société des Abattoirs de Nyabugogo-Nyabugogo Abattoir, Kigali. J S Afr Vet Assoc. 2014;85:5 pages.

39. Kanyala E, Shuaib YA, Schwarz NG, Andres S, Richter E, Sawadogo B, *et al.* Prevalence and Molecular Characterization of *Mycobacterium bovis* in Slaughtered Cattle Carcasses in Burkina Faso; West Africa. Microorganisms. 2022;10:1378.

40. Abbate JM, Arfuso F, Iaria C, Arestia G, Lanteri G. Prevalence of Bovine Tuberculosis in Slaughtered Cattle in Sicily, Southern Italy. Animals (Basel). 2020;10:1473.

41. Damene H, Tahir D, Diels M, Berber A, Sahraoui N, Rigouts L. Broad diversity of *Mycobacterium tuberculosis* complex strains isolated from humans and cattle in Northern Algeria suggests a zoonotic transmission cycle. PLoS Negl Trop Dis. 2020;14:e0008894.

42. Egbe NF, Muwonge A, Ndip L, Kelly RF, Sander M, Tanya V, *et al.* Abattoir-based estimates of mycobacterial infections in Cameroon. Sci Rep. 2016;6:24320.

43. Müller B, Vounatsou P, Ngandolo BNR, Diguimbaye-Djaïbe C, Schiller I, Marg-Haufe B, *et al.* Bayesian receiver operating characteristic estimation of multiple tests for diagnosis of bovine tuberculosis in Chadian cattle. PLoS One. 2009;4:e8215.

44. Nalapa DP, Muwonge A, Kankya C, Olea-Popelka F. Prevalence of tuberculous lesion in cattle slaughtered in Mubende district, Uganda. BMC Veterinary Research. 2017;13:73.

45. Okeke LA, Fawole O, Muhammad M, Okeke IO, Nguku P, Wasswa P, *et al.* Bovine tuberculosis: a retrospective study at Jos abattoir, Plateau State, Nigeria. Pan Afr Med J. 2016;25.

46. Sahraoui N, Müller B, Guetarni D, Boulahbal F, Yala D, Ouzrout R, *et al.* Molecular characterization of *Mycobacterium bovis* strains isolated from cattle slaughtered at two abattoirs in Algeria. BMC Veterinary Research. 2009;5:4.

47. Teklu A, Asseged B, Yimer E, Gebeyehu M, Woldesenbet Z. Tuberculous lesions not detected by routine abattoir inspection : the experience of the Hossana municipal abattoir, Southern Ethiopia: -EN- -FR- -ES-. Rev Sci Tech OIE. 2004;23:957–64.

48. Borham M, Oreiby A, El-Gedawy A, Hegazy Y, Hemedan A, Al-Gaabary M. Abattoir survey of bovine tuberculosis in tanta, centre of the Nile delta, with in silico analysis of gene mutations and protein–protein interactions of the involved mycobacteria. Transbounding Emerging Dis. 2022;69:434–50.

49. Ejo M, Haile B, Tsegaye Tariku, Nigatu S, Kebede E, Bitew AB, *et al.* Bacteriological and molecular characterization of *Mycobacterium bovis* isolates from tuberculous lesions collected among slaughtered cattle, Northwest Ethiopia. BMC Microbiology. 2021;21:286.

50. Nuñez-Garcia J, Downs SH, Parry JE, Abernethy DA, Broughan J, Cameron A, *et al.* Edinburgh Research Explorer Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. Preventive veterinary medicine. 2017;153:94–107.

51. Tekle Y, Mamo G, Ameni G, Ftiwi M. Assessment of Bovine Tuberculosis like Lesions and Its Risk Factors in Cattle Slaughtered at Hawassa University and Municipal Abattoirs, Southern Ethiopia. J Vet Sci Med. 2017;5:01–9.

52. Katale BZ, Mbugi EV, Kendal S, Fyumagwa RD, Kibiki GS, Godfrey-Faussett P, *et al.* Bovine tuberculosis at the human-livestock-wildlife interface: is it a public health problem in Tanzania? A review. Onderstepoort J Vet Res. 2012;79:463.

53. Menin Á, Fleith R, Reck C, Marlow M, Fernandes P, Pilati C, *et al.* Asymptomatic Cattle Naturally Infected with *Mycobacterium bovis* Present Exacerbated Tissue Pathology and Bacterial Dissemination. PLoS ONE. 2013;8:e53884.

54. Duffy SC, Marais B, Kapur V, Behr MA. Zoonotic tuberculosis in the 21st century. The Lancet Infectious Diseases. 2024;24:339–41.

55. Zwyer M, Çavusoglu C, Ghielmetti G, Pacciarini ML, Scaltriti E, Van Soolingen D, *et al.* A new nomenclature for the livestock-associated *Mycobacterium tuberculosis* complex based on phylogenomics. Open Res Europe. 2021;1:100.

56. WOAH. Mammalian tuberculosis (infection with *Mycobacterium tuberculosis* complex), In: Manual of diagnostic tests and vaccines for terrestrial animals World Organisation for Animal Health, Paris. 2022.

CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Several studies have demonstrated a substantially higher prevalence of bTB in exotic and crossbred cattle raised under intensive dairy farming systems around urban and peri-urban regions of Ethiopia. In central Ethiopia, the animal level prevalence ranges from 25% to 39% while the herd prevalence exceeds 50% in the intensive dairy herds (Firdessa *et al.* 2012; Almaw *et al.* 2021; Tulu *et al.* 2021). Nevertheless, the disease remains uncontrolled due to economic constraints and lack of feasible control strategies. The extensive crossbreeding to expand intensive dairy farming to meet growing milk demand will further increase the incidence of bTB, signifying an urgent need for a cost-effective and feasible control strategy. Currently, tuberculin test-and-slaughter alongside movement restriction are the primary methods employed for bTB control. While these strategies have led to the elimination or very low prevalence of bTB in many high-income countries, they are economically infeasible in LMIC, leaving these regions with limited options for disease control. In addition, wildlife reservoirs made eradication extremely difficult in some regions, despite the test-and-slaughter approach was introduced and applied for decades. Moreover, WOAH has discouraged the antimycobacterial treatment of TB in livestock (WOAH, 2024) due to economic impracticality of long-term treatment with multiple drugs and the risk of resistance to anti-mycobacterial medications used for human tuberculosis, precluding the treatment and cure of bTB in livestock and leaving control as the only strategy to deal with bTB. These impeding limitations call for an alternative control methods implementable in LMICs. The utilization of the BCG vaccine may constitute a cost-effective approach to bTB control LMICs and regions with wildlife reservoirs.

Conventional vaccine efficacy evaluations have shown that BCG confers partial protection by reducing the severity and frequency of pathology (Vordermeier *et al.*, 2009; Ameni *et al.*, 2010b; Lopez-Valencia *et al.*, 2010; Ameni *et al.*, 2018; Bayissa *et al.*, 2021), though its overall direct protective effect against bTB infection is 25% (Srinivasan *et al.*, 2021). The partial protective effect of BCG is anticipated to decrease the infectiousness of vaccinated animals. However, the traditional vaccine efficacy evaluations, relied on fixed endpoint outcomes and failed to measure the indirect effect of the vaccine on disease transmission. As a result, our current knowledge limited to the variable and low direct effect of BCG along with sensitization to tuberculin, has

hindered the adoption of the vaccine for bTB control strategy. Understanding this association and the performance of DIVA diagnostic tests in differentiating infected among vaccinated animals can have significant implications for reconsidering the use of BCG for bTB control, particularly in resource-limited settings where test-and-slaughter approaches are economically unfeasible. However, this has never been evaluated and it remains unclear whether the reduction in disease severity translates into decreased infectiousness of vaccinated animals.

To address this knowledge gap, we evaluated the impact of BCG vaccination on bTB transmission using a crossover design in a natural transmission experiment, assessed the long-term impact of vaccination on bTB transmission among cattle herds using a mechanistic transmission model, compared tuberculin with defined peptide antigen cocktails (ESAT-6, CFP-10, and Rv3615c) in skin and IGRA tests.

Direct and indirect efficacies of BCG vaccine

The results of this study revealed that BCG vaccination significantly reduces the severity of lesions in vaccinated animals compared to unvaccinated controls. This is reflected on higher indirect effect of 74% reduction in bTB transmission. This indirect effect exceeded the direct efficacy of 58% against infections, resulting in an overall efficacy of 89%. This confirms the assumption that vaccinated animals with less severe lesions are likely to be less infectious (Ameni *et al.*, 2018), This result provides definitive evidence for partial protection as the mechanism contributing to the high indirect effects of BCG vaccine. A recent study conducted in Holstein Friesian x Swedish Red crossbred dairy herd in central Chile reported similar direct BCG vaccine efficacy of 58.5% at 18th month post vaccination using the same antigens (ESAT6/CFP-10 and Rv3615c) in the IGRA test (Contreras *et al.*, 2023).

The basic reproduction rate (Ro) and the effective reproduction number (R) are concepts used to understand the transmission of infectious diseases and control strategies in a population. The basic R is defined as the average number of secondary cases produced by a single infectious individual introduced into a completely susceptible population. The effective R refers to the average number of secondary infections caused by a single infected individual in a population where control measures are implemented. Effective control measures should reduce R below one. In this study,

the estimated baseline R_0 is 3.1, highlighting the high transmission potential of bTB in a susceptible population in the absence of control strategies. Previous investigations reported 1.34 before control measures were applied in France (Bouchez-Zacria *et al.*, 2023) and 1.1 (Cox *et al.*, 2005) in the UK where the long-standing costly test-and- slaughter program is implemented, indicating the challenges of bTB control in regions where wildlife reservoirs of infection exist.

Our analysis of the pair-wise reproduction number shows that neither direct nor indirect protection alone is sufficient to prevent transmission, as the posterior densities for R_0 overlap with the critical threshold value of 1. The R_0 value falls significantly below 1 only when both sentinel and seeder animals are vaccinated. This underscores the importance of combined direct and indirect effects of BCG vaccination for bTB elimination. These results indicate that BCG is more effective at reducing onward transmission than preventing infection, suggesting its important role in herd immunity. This indicates that BCG could complement or replace expensive test-and-slaughter control programs, potentially enabling a cost-effective bTB control strategy, especially in resource-limited regions where test-and-slaughter approaches are impractical due to economic and sociocultural constraints. For regions where long-established bTB control through test-and-slaughter programs is implemented, but wildlife reservoirs make eradication challenging, the high indirect effect of BCG mitigates concerns about its use due to limited direct protection.

The efficacies in this study were recorded under the highest infectious pressure, where 100% of infected seeders were housed with susceptible sentinels in a one-to-one ratio. This scenario is unlikely in many field situations, indicating the possibility of an equal or better efficacy under field conditions. This assumption is supported by the BCG efficacy reported from New Zealand, where subcutaneous and oral vaccination with BCG against natural exposure in low-prevalence environments showed efficacy of 85.7% and 67.4% against detectable TB lesions at the slaughterhouse and infection by *M. bovis*, respectively (Nugent *et al.*, 2017, 2018).

Prospects for elimination of bTB by BCG vaccination

In the absence of systematic testing and legislative restriction on trading infected cattle, movement can facilitate long-distance transmission of bTB. To guide disease control and prevention policies, stochastic metapopulation model is commonly used to simulate the transmission of infectious

diseases in heterogeneous cattle populations with interconnected spatial networks to evaluate the efficacy of interventions. In this research, we developed a stochastic metapopulation transmission model calibrated using bTB test data from Mekele, Gondar, and Hawassa to project the potential expansion of bTB in emerging dairy markets over the next 50 years. We compared baseline scenarios without vaccination with scenarios where animals are protected only by a direct effect or with the combined direct and indirect effects. The model indicates that baseline scenarios without vaccination can lead to an endemic animal-level prevalence of approximately 50% in all the three regions, demonstrating the magnitude of the potential risk posed by unregulated cattle trading on bTB spread. This slow rate of progression taking the full 50-year span due to the relatively infrequent cattle trades offers a window of opportunity for vaccination or other interventions targeted on the cattle movement networks. In contrast to the baseline scenarios, routine vaccination of all neonatal calves with direct efficacy alone reduces the predicted spread, but doesn't bring R below 1, whereas the total vaccine efficacy of 89% prevents the spread and reduces the population average R to below 1 within a decade. This means bTB elimination can only be achieved as a result of the combined direct and indirect effects of BCG vaccination in common with our experimental system, implying that relying on BCG vaccination alone, elimination. may take decades to achieve requiring a long-term plan and vaccinating multiple generations of neonatal calves.

Implications for the deployment of BCG in the field

The estimated within herd reproduction ratios (R_0) shows large variability among Ethiopian dairy herds ranging from 1.3 to 17.3 with a population average of 3.26 (95% CrI:2.7-4.0%). This means that the reproduction number may not fall below 1 in all settings, indicating the need for integrated intervention strategies for effective control and elimination in large herds with high prevalence and Ro above 4.0 (Figure 2.3C). Nevertheless, vaccination could limit the spread and reduce prevalence in endemic settings, making more rapid measures, such as test-and-slaughter or test-and-segregate strategies economically viable. The reproduction number of bTB is affected by the effectiveness of control measures, herd size, animal movement and contact rates between infected and susceptible animals. Larger herd sizes create more opportunities for disease transmission. A previous study showed a basic reproduction ratio of 1.5 in a herd of 30 cattle, which rises to 4.9 in

herds of 400 (Conlan *et al.*, 2012). In large herds with high prevalence, integrating vaccination with test-and-cull, test-and-segregation, early calf segregation, movement restriction of infected cattle, and biosecurity may help accelerate bTB control and elimination (Srinivasan *et al.*, 2021; Lakew *et al.*, 2022, 2024).

Performance of DIVA antigens compared to tuberculin

BCG is commonly used to prevent *M. tuberculosis* infection in humans, but it is not authorized for use in livestock. The primary barriers to BCG vaccine use in livestock are incomplete direct protection and interference with the specificity of tuberculin-based tests (Conlan *et al.*, 2015). A study indicated that up to 80% of vaccinated but uninfected cattle tested positive six months after vaccination, though this proportion drop to 10% nine months postvaccination (Jones *et al.*, 2017). Although BCG interferes with the tuberculin test, its relevance for LMICs is disputed, especially in regions where routine tuberculin testing and bTB control programs are not implemented despite high endemic prevalence. In such settings, BCG vaccination may remain beneficial. To alleviate this concern, progress has also been made in DIVA diagnostic assays, enabling to differentiate infected from vaccinated animals essential for integration with BCG vaccination for bTB control without compromising disease surveillance (Conlan *et al.*, 2015; Vordermeier *et al.*, 2016).

In this study, we evaluated defined peptide cocktail antigens (ESAT-6, CFP10, Rv3615c) along with PPDs in the INF-γ and skin test formats to determine how well they distinguish vaccinated from infected animals (DIVA). The performance of defined antigens was evaluated on a total of 134 Zebu and Holstein–Friesian crossbred calves, divided equally into 67 vaccinated and 67 unvaccinated control groups. These same antigens were tested in 65 unvaccinated control and 62 BCG-vaccinated animals that survived a year of exposure to infection through reactor animals under natural transmission setting. Then, the responses of the same group of animals to PPD antigens in the INF-γ and skin test were compared with the outcomes of the defined antigens INF-γ and skin test responses.

Pre-exposure to infected herds, DIVA skin and IGRA tests exhibited 94.0% and 98.5% specificity in vaccinated cattle, respectively, while both test formats exhibited 100% in unvaccinated cattle. These findings underline the significance of these tests in minimizing false positives in vaccinated

cattle and highlight their potential for integration with BCG vaccination in bTB control programs. At the end of the postexposure period, the DIVA and CCT skin tests demonstrated relative sensitivities of 46% and 45%, respectively, whereas the DIVA IGRA test showed a sensitivity of 47% indicating comparable performance of the three tests against *M. bovis* infection confirmed by culture PCR. The low sensitivity of these diagnostic tests can limit the detection of truly infected animals. In resource limited regions, where the effectiveness of vaccination programs can be monitored through slaughterhouse detection of lesions to complement the low sensitivity of the DIVA tests, vaccination remains beneficial. However, the test-and-cull or test-and segregate control strategies depending on such low-sensitivity tests may not be effective, highlighting the need to develop and validate improved diagnostic tests that can accurately identify bTB in both vaccinated and unvaccinated cattle to allow effective control measures and safeguard both livestock health and public safety. Including additional mycobacterial peptides unique to pathogenic mycobacteria that exhibit no cross-reactivity with BCG and environmental mycobacterial strains in the current DIVA cocktails may enhance sensitivity while maintaining specificity, thereby addressing existing limitations.

Prevalence of bTB at slaughterhouses

Integrating slaughterhouse surveys into the bTB control strategy is vital for the bTB control and eradication. It plays a key role to detect infected animals to safeguard public health and provide essential data to understand the prevalence, the associated risk factors, and the possible spread of disease among different cattle populations to inform surveillance strategy and targeted interventions. In high income countries, slaughterhouse surveillance has been critical to achieving and maintaining OTF status, which is crucial for unhindered access to international markets. In contrast, inadequate surveillance and lack of control measures have contributed to a high prevalence in developing countries. The findings of our slaughterhouse survey indicated that crossbred cattle are at a significantly higher risk of developing bTB lesions, with an odds ratio of 11.8 compared to local Zebu cattle. This is likely due to a combination of genetic factors and intensive management practices, suggesting the need for targeted bTB control measures prioritizing intensive dairy production systems that predominantly keep crossbred cattle. The primary causative agent of bTB identified in this study was *M. bovis* along with rare cases of *M.*

tuberculosis and *M. caprae*, indicating the multi-species causes of the disease. These findings have implications for animal health, food safety, and public health. This study revealed an overall prevalence of 2.3% for TBL in cattle carcasses across the four studied slaughterhouses in central Ethiopia, indicating the persistent occurrence of bTB in the Ethiopian cattle population and a continued risk for both animal health and potential zoonotic transmission. Culture and m-qPCR positive tuberculous lesions were predominantly observed in the lungs and lung-associated lymph nodes. The predominance of respiratory lesions has implications for bTB control strategies, suggesting the importance of measures aimed at reducing close animal contact, improving ventilation, and implementing appropriate biosecurity measures to reduce transmission, especially in intensive farming systems. Given the zoonotic potential of the identified MTBC species in this study and the common practice of raw meat and unpasteurized milk consumption in Ethiopia, public education on the risks of transmission, sources of infection and food safety practices, including boiling or pasteurization of milk and cooking meat before consumption is recommended.

Conclusions

This is the first study to reveal the previously unrecognized superior indirect effects of BCG vaccination in reducing the transmission of bTB, exceeding its direct protective effect. This aspect was often overlooked in the previous traditional vaccine efficacy evaluations that exclusively focused on the direct effect of the vaccine against infection. Our experimental study and mechanistic transmission model analyses show that combined direct and indirect effects of BCG reduce the reproduction number below one, indicating the possibility of eliminating bTB. The findings suggest the effectiveness of BCG in the field is likely to be greater and provide key missing evidence of the potential benefits of a BCG-vaccination to accelerate control of bTB in LMIC regions, where the disease remains endemic and uncontrolled. This provides a strong support for the adoption of BCG vaccine as an effective control strategy and potential elimination of bTB. The DIVA antigens used in both the skin and IGRA DIVA tests show high specificity in differentiating infected from vaccinated animals, with minimal cross-reactivity to BCG-vaccine. However, their diagnostic sensitivity is low, similar to that of the CCT, underlining the need for development of more sensitive diagnostic tests. Although not a finding, the cross-over design used

in this study can be applied to investigate other vaccines and interventions for infectious diseases in animals and humans, particularly where indirect effects are crucial to the outcome.

In summary, our findings strongly support the need for a policy to integrate BCG vaccination into the bTB control strategy, prioritizing and targeting high-risk intensive dairy cattle production systems. However, the influence of disease prevalence, cattle breed, farming system, revaccination, and cross-species transmission on BCG vaccine efficacy, is still unknown. Additionally, the impacts and magnitude of the bTB burden on animal production, productivity, and public health are poorly understood, making it difficult to establish a strong business case for implementing a national bTB control program.

Recommendations

Based on the above conclusions, the following recommendations are forwarded:

1. The estimates of direct and indirect vaccine effects in this study come from a two-year challenge and the long-term protective effect of BCG vaccination or revaccination beyond two years is not well-known. Therefore, further research on the duration of BCG immunity and the potential to extend protection for the lifetime of a dairy cow through revaccination with BCG, DNA, protein, or viral subunit vaccines is required.
2. The natural transmission experiment of the current trial was conducted in a closed cattle herd without contact with other domestic or wild animal species and does not account for cross-species transmission. Therefore, further research is needed to assess the potential benefits of cattle vaccination in reducing the risk of cross-species transmission from other sympatric hosts kept on the same premises as cattle, such as pigs, goats, sheep, or free-living wildlife. The involvement of wild animals either as maintenance or spillover hosts for *M, bovis* has not been assessed and confirmed in Ethiopia. Thus, further research is needed to clarify their role in bTB transmission.
3. Develop and execute a pilot BCG vaccination trial in intensive dairy farms with varying herd prevalence and evaluate vaccine efficacy alone or combined with other control measures such as test-and-segregate strategy and/or early-age calf separation.
4. Conduct further studies to refine both tuberculin and DIVA tests for improved prediction of the risk of disease transmission.

5. The magnitude of the bTB burden on animal production, productivity, and public health, remain largely uncharacterized. Thus, future research should conduct comprehensive investigation into the impact of bTB on animal production and explore the transmission dynamics between humans and cattle using a One Health approach. This is essential to perform economic cost-benefit analyses and establish a better business case for vaccine-based bTB control strategy. Thus, the economic feasibility of BCG vaccination need to be studied.
6. Policy recommendations

   Several studies indicated a high prevalence of bTB in intensive dairy farms located in central Ethiopia. However, the disease has remained largely uncontrolled due to limited resources to implement the costly test-and-slaughter strategy. In the absence of a national bTB control strategy, the consistent presence of uncontrolled bTB causes economic loss to livestock farmers, threatens food security and poses substantial public health risks. In this study, BCG has shown an indirect efficiency 74%, higher than the direct protection of 58%, resulting in a total efficacy of 89%. Our experimental study and modelling analyses revealed combined direct and indirect efficacies capable of reducing the reproduction number below one, which is crucial for elimination of the disease. In support of our findings, the new WOAH guidelines (WOAH, 2024) recognize BCG vaccination as bTB controlling tool in resource-limited settings where herd prevalence is higher than 5% with no routine surveillance, and vaccination does not impact trading. Policymakers should therefore develop guidelines to adopt BCG vaccination for integration into national bTB control programs, especially in the high prevalence intensive dairy sector, aligned with the recommendation by WOAH. The economic justification for vaccination of local Zebu cattle is likely weak, because vaccinating large cattle herds in the extensive farming systems is challenging and costly. In addition, the prevalence is lower in Zebu cattle, estimated at 4.1%, and similar immune responses to BCG and *M. bovis* can create a barrier to livestock export and international trade. Thus, prioritizing high risk dairy farms optimizes the limited resource allocation and utilization.

   Implementation could start with a pilot trial focusing on neonatal calf vaccination in the intensive dairy farms in central Ethiopia. However, before planning any intervention, it

may be necessary to raise awareness about the potential benefits of vaccination and assess the acceptability of the vaccine among dairy farmers, as well as public acceptance of products from vaccinated animals. Overall, the deployment of BCG vaccination can offer a cost-effective and sustainable bTB control strategy to reduce the burden of bTB, preserve productive livestock and improve productivity. Since bTB is a zoonotic disease, reducing transmission in cattle could subsequently lower the risk of human infection and improve public health. Though it remains to be proven, reduction in infectiousness of vaccinated cattle could lower the likelihood of spillover transmission to wildlife, while the direct protective effect of the vaccine could prevent cattle from being infected by wildlife. Several wild animal species are expected to come in contact with livestock in Ethiopia, but their involvement either as maintenance or spillover host is unknown. Nonetheless, based on wildlife maintenance hosts in other parts of the globe, the closely related species such as warthog (*Phacochoerus africanus*), bushpig (*Potamochoerus larvatus*), and honey badger (*Mellivora capensis*) that are known to interact with livestock in Ethiopia can serve as potential reservoirs of infection. It has been proposed to vaccinate cattle herds and/or wild animals that share their grazing land to prevent bTB. Badger vaccination authorized in the UK has been proven to protect badgers (Robinson *et al.*, 2012) with potential to contribute to bTB control in cattle.

A pilot trial in volunteer commercial dairy farms will offer an opportunity to gather additional data related to the impact of vaccination on bTB prevalence and productivity. The challenges that may be encountered during pilot vaccination trial will enable to refine and tailor future vaccination strategies to specific herd and husbandry practices. To implement, sustain and achieve the long-term success of vaccination approach for bTB control, collaboration among all stakeholders, including dairy producers, veterinary and public health authorities and decision makers, vaccine and diagnostic agent manufacturers, and international regulatory bodies should be established. To ensure availability of the vaccine and fit for purpose DIVA test, production and marketing approval, as well as compliance with guidelines, is essential.

REFERENCES

Abalos, P., Valdivieso, N., Pérez de Val, B., Vordermeier, M., Benavides, M., Alegria, R., Saadi, K., Wistuba, M., Ortega, C., Sánchez, N., Retamal, P., 2022. Vaccination of Calves with the *Mycobacterium bovis* BCG Strain Induces Protection against Bovine Tuberculosis in Dairy Herds under a Natural Transmission Setting. Anim. Open Access J. MDPI 12. doi:10.3390/ani12091083

Abe, C., Hirano, K., Wada, M., Kazumi, Y., Takahashi, M., Fukasawa, Y., Yoshimura, T., Miyagi, C., Goto, S., 1993. Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified *Mycobacterium Tuberculosis* Direct Test. J Clin Microbiol 31, 3270–3274. doi:10.1128/jcm.31.12.3270-3274.1993

Ahmed, M., Tezera, L. B., Elkington, P. T., & Leslie, A. J. (2022). The paradox of immune checkpoint inhibition re-activating tuberculosis. European Respiratory Journal, *60*(5). https://doi.org/10.1183/13993003.02512-2021

Ali, M.Z., 2013. BOVINE TUBERCULOSIS. doi:10.13140/RG.2.2.10600.55045

Allen, A.R., Skuce, R.A., Byrne, A.W., 2018. Bovine Tuberculosis in Britain and Ireland – A Perfect Storm? the Confluence of Potential Ecological and Epidemiological Impediments to Controlling a Chronic Infectious Disease. Front. Vet. Sci. 5, 109. doi:10.3389/fvets.2018.00109

Almaw, G., Conlan, A.J.K., Ameni, G., Gumi, B., Alemu, A., Guta, S., Gebre, S., Olani, A., Garoma, A., Shegu, D., Yimesgen, L., Nigussie, D., Wood, J.L.N., Abebe, T., Mihret, A., Berg, S., the ETHICOBOTS consortium, 2021. The variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia provides opportunities for targeted intervention. PLOS ONE 16, e0254091. doi:10.1371/journal.pone.0254091

Ameni, G., Aseffa, A., Hewinson, G., Vordermeier, M., 2010a. Comparison of different testing schemes to increase the detection *Mycobacterium bovis* infection in Ethiopian cattle. Trop. Anim. Health Prod. 42, 375–383. doi:10.1007/s11250-009-9429-1

Ameni, G., Desta, F., Firdessa, R., 2010. Molecular typing of *Mycobacterium bovis* isolated from tuberculosis lesions of cattle in north eastern Ethiopia. Vet. Rec. 167, 138–141. doi:10.1136/vr.b4881

Ameni, G., Tafess, K., Zewde, A., Eguale, T., Tilahun, M., Hailu, T., Sirak, A., Salguero, F.J., Berg, S., Aseffa, A., Hewinson, R.G., Vordermeier, H.M., 2018. Vaccination of calves with *Mycobacterium bovis* Bacillus Calmette–Guerin reduces the frequency and severity of lesions of bovine tuberculosis under a natural transmission setting in Ethiopia. Transbound. Emerg. Dis. 65, 96–104. doi:10.1111/tbed.12618

Ameni, G., Vordermeier, H.M., Firdessa, R., Aseffa, A., Hewinson, R.G., Gordon, S.V., Berg, S., 2011. *Mycobacterium tuberculosis* infection in grazing cattle in central Ethiopia. Vet. J. 188, 359–361. doi:10.1016/j.tvjl.2010.05.005

Ameni, G., Vordermeier, M., Aseffa, A., Young, D.B., Hewinson, R.G., 2010b. Field Evaluation of the Efficacy of *Mycobacterium bovis* Bacillus Calmette-Guérin against Bovine Tuberculosis in Neonatal Calves in Ethiopia. Clin. Vaccine Immunol. 17, 1533–1538. doi:10.1128/CVI.00222-10

Araújo, C.P., Osório, A.L.A.R., Jorge, K.S.G., Ramos, C.A.N., Souza Filho, A.F., Vidal, C.E.S., Vargas, A.P.C., Roxo, E., Rocha, A.S., Suffys, P.N., Fonseca Júnior, A.A., Silva, M.R., Barbosa Neto, J.D., Cerqueira, V.D., Araújo, F.R., 2014. Direct detection of *Mycobacterium tuberculosis* complex in bovine and bubaline tissues through nested-PCR. Braz. J. Microbiol. 45, 633–640. doi:10.1590/S1517-83822014000200035

Arega, S., Conraths, F., Ameni, G., 2013. Prevalence of tuberculosis in pigs slaughtered at two abattoirs in Ethiopia and molecular characterization of *Mycobacterium tuberculosis* isolated from tuberculous-like lesions in pigs. BMC Vet. Res. 9, 97. doi:10.1186/1746-6148-9-97

Ashford, D.A., Whitney, E., Raghunathan, P., Cosivi, O., 2001. Epidemiology of selected mycobacteria that infect humans and other animals: -EN- -FR- -ES-. Rev. Sci. Tech. OIE 20, 325–337. doi:10.20506/rst.20.1.1266

Asimov, I., 2015. Oxidative stress and redox regulation: adaptation, damage, repair, denescence and death, in: Free Radicals in Biology and Medicine. Oxford University Press, Great Clarendon Street, Oxford, OX2 6DP, United Kingdom, pp. 200–283.

Asmare, K., Sibhat, B., Demissie, K., Mamo, G., Skjerve, E., Ameni, G., 2020. Tuberculosis in small ruminants and dromedary camels in Ethiopia: A systematic review and meta-analysis. Prev. Vet. Med. 185, 105181. doi:10.1016/j.prevetmed.2020.105181

Averbakh, M.M., Ergeshow, A., 2018. Interaction between *Mycobacterium tuberculosis* and Human Host: Role of Cytokines in Pathogenesis and Treatment Monitoring, in: Kayembe, J.-M.N. (Ed.), Tuberculosis. InTech. doi:10.5772/intechopen.76543

Bayissa, B., Sirak, A., Worku, A., Zewude, A., Zeleke, Y., Chanyalew, M., Gumi, B., Berg, S., Conlan, A., Hewinson, R.G., The ETHICOBOTS Consortium, Wood, J.L.N., Vordermeier, H.M., Ameni, G., 2021. Evaluation of the Efficacy of BCG in Protecting Against Contact Challenge with Bovine Tuberculosis in Holstein-Friesian and Zebu Crossbred Calves in Ethiopia. Front. Vet. Sci. 8, 702402. doi:10.3389/fvets.2021.702402

Bayissa, B., Sirak, A., Zewude, A., Worku, A., Gumi, B., Berg, S., Hewinson, R.G., Wood, J.L.N., Jones, G.J., the ETHICOBOTS consortium, Vordermeier, H.M., Ameni, G., 2022. Field evaluation of specific mycobacterial protein-based skin test for the differentiation of *Mycobacterium bovis*- infected and Bacillus Calmette Guerin-vaccinated crossbred cattle in Ethiopia. Transbound. Emerg. Dis. 69. doi:10.1111/tbed.14252

Bengis, R.G., Keet, D.F., Michel, A.L., Kriek, N.P., 2001. Tuberculosis, caused by *Mycobacterium bovis*, in a kudu (Tragelaphus strepsiceros) from a commercial game

farm in the Malelane area of the Mpumalanga Province, South Africa. Onderstepoort J. Vet. Res. 68, 239–241.

Bernitz, N., Kerr, T.J., Goosen, W.J., Chileshe, J., Higgitt, R.L., Roos, E.O., Meiring, C., Gumbo, R., de Waal, C.R., Clarke, C., Smith, K., Goldswain, S., Sylvester, T.T., Kleynhans, L., Dippenaar, A., Buss, P., Cooper, D., Lyashchenko, K.P., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2021. Review of Diagnostic Tests for Detection of *Mycobacterium bovis* Infection in South African Wildlife. Frontiers in Veterinary Science 8, 588697–588697. doi:10.3389/fvets.2021.588697

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D.V., Warren, R.M., Van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (Syncerus caffer). Preventive Veterinary Medicine 169, 104700. doi:10.1016/j.prevetmed.2019.104700

Blanco, F.C., Soria, M., Bianco, M.V., Bigi, F., 2012. Transcriptional Response of Peripheral Blood Mononuclear Cells from Cattle Infected with *Mycobacterium bovis*. PLoS ONE 7, e41066. doi:10.1371/journal.pone.0041066

Bottai, D., Frigui, W., Sayes, F., Di Luca, M., Spadoni, D., Pawlik, A., Zoppo, M., Orgeur, M., Khanna, V., Hardy, D., Mangenot, S., Barbe, V., Medigue, C., Ma, L., Bouchier, C., Tavanti, A., Larrouy-Maumus, G., Brosch, R., 2020. TbD1 deletion as a driver of the evolutionary success of modern epidemic *Mycobacterium tuberculosis* lineages. Nat Commun 11, 684. doi:10.1038/s41467-020-14508-5

Bouchez-Zacria, M., Ruette, S., Richomme, C., Lesellier, S., Payne, A., Boschiroli, M.-L., Courcoul, A., Durand, B., 2023. Analysis of a multi-type resurgence of *Mycobacterium bovis* in cattle and badgers in Southwest France, 2007-2019. Vet. Res. 54, 41. doi:10.1186/s13567-023-01168-8

Bretou, M., Kumari, A., Malbec, O., Moreau, H.D., Obino, D., Pierobon, P., Randrian, V., Saez, P.J., Lennon-Dumenil, A.-M., 2016. Dynamics of the membranecytoskeleton interface in MHC class II-restricted antigen presentation. Immunol. Rev. 272, 39–51.

Brites, D., Gagneux, S., 2017. The Nature and Evolution of Genomic Diversity in the *Mycobacterium tuberculosis* Complex, in: Gagneux, S. (Ed.), Strain Variation in the *Mycobacterium tuberculosis* Complex: Its Role in Biology, Epidemiology and Control, Advances in Experimental Medicine and Biology. Springer International Publishing, Cham, pp. 1–26. doi:10.1007/978-3-319-64371-7_1

Brites, D., Loiseau, C., Menardo, F., Borrell, S., Boniotti, M.B., Warren, R., Dippenaar, A., Parsons, S.D.C., Beisel, C., Behr, M.A., Fyfe, J.A., Coscolla, M., Gagneux, S., 2018. A New Phylogenetic Framework for the Animal-Adapted *Mycobacterium tuberculosis* Complex. Front. Microbiol. 9, 2820. doi:10.3389/fmicb.2018.02820

Brodin, P., Majlessi, L., Brosch, R., Smith, D., Bancroft, G., Clark, S., Williams, A., Leclerc, C., Cole, S.T., 2004. Enhanced Protection against Tuberculosis by Vaccination with Recombinant *Mycobacterium microti* Vaccine That Induces T Cell Immunity against Region of Difference 1 Antigens. J. Infect. Dis. 190, 115–122. doi:10.1086/421468

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Gutierrez, Cristina, Hewinson, G., Kremer, K., Linda M. Parsons, Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D., van Soolingen, D., Cole, S.T., 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. U. S. A. 99, 3684–3689. doi:10.1073/pnas.052548299

Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., Wedlock, D.N., 2013. Subcutaneous administration of a 10-fold-lower dose of a commercial human tuberculosis vaccine, *Mycobacterium bovis* bacillus Calmette-Guerin Danish, induced levels of protection against bovine tuberculosis and responses in the tuberculin intradermal test similar to those induced by a standard cattle dose. Clin. Vaccine Immunol. CVI 20, 1559–1562. doi:10.1128/CVI.00435-13

Buddle, B.M., Vordermeier, H.M., Chambers, M.A., de Klerk-Lorist, L.-M., 2018. Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife. Front. Vet. Sci. 5, 259. doi:10.3389/fvets.2018.00259

Casal, C., Bezos, J., Díez-Guerrier, A., Álvarez, J., Romero, B., De Juan, L., Rodriguez-Campos, S., Vordermeier, M., Whelan, A., Hewinson, R.G., Mateos, A., Domínguez, L., Aranaz, A., 2012. Evaluation of two cocktails containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the interferon-γ assay for diagnosis of bovine tuberculosis. Prev. Vet. Med. 105, 149–154. doi:10.1016/j.prevetmed.2012.02.007

Chai, Q., Wang, L., Liu, C.H., Ge, B., 2020. New insights into the evasion of host innate immunity by *Mycobacterium tuberculosis*. Cell. Mol. Immunol. 17, 901–913. doi:10.1038/s41423-020-0502-z

Chancellor, A., Gadola, S.D., Mansour, S., 2018. The versatility of the CD 1 lipid antigen presentation pathway. Immunology 154, 196–203. doi:10.1111/imm.12912

Chang, Y., Widgren, S., de Jong, M.C.M., Tratalos, J.A., More, S.J., Hartemink, N., 2024. Evaluating the effectiveness of badger vaccination combined with cattle test-and-removal in managing Bovine Tuberculosis: Insights from a two-host and multi-route transmission model. Prev. Vet. Med. 106386. doi:10.1016/j.prevetmed.2024.106386

Chileshe, J., Goosen, W.J., Buss, P.E., Van Helden, P.D., Warren, R., Parsons, S.D.C., Miller, M.A., 2019a. A commercial ELISA for detection of interferon gamma in white rhinoceros. J VET Diagn Invest 31, 531–536. doi:10.1177/1040638719843955

Chileshe, J., Roos, E.O., Goosen, W.J., Buss, P., Hausler, G., Rossouw, L., Manemela, T., Van Helden, P., Warren, R., Parsons, S.D., Miller, M., 2019b. An interferon-gamma release assay for the diagnosis of the *Mycobacterium bovis* infection in white rhinoceros

(Ceratotherium simum). Veterinary Immunology and Immunopathology 217, 109931. doi:10.1016/j.vetimm.2019.109931

Chugh, S., Bahal, R.K., Dhiman, R., Singh, R., 2024. Antigen identification strategies and preclinical evaluation models for advancing tuberculosis vaccine development. npj Vaccines 9, 57. doi:10.1038/s41541-024-00834-y

Conlan, A.J.K., Brooks Pollock, E., McKinley, T.J., Mitchell, A.P., Jones, G.J., Vordermeier, M., Wood, J.L.N., 2015. Potential Benefits of Cattle Vaccination as a Supplementary Control for Bovine Tuberculosis. PLOS Comput. Biol. 11, e1004038. doi:10.1371/journal.pcbi.1004038

Conlan, A.J.K., McKinley, T.J., Karolemeas, K., Pollock, E.B., Goodchild, A.V., Mitchell, A.P., Birch, C.P.D., Clifton-Hadley, R.S., Wood, J.L.N., 2012. Estimating the Hidden Burden of Bovine Tuberculosis in Great Britain. PLoS Comput. Biol. 8, e1002730. doi:10.1371/journal.pcbi.1002730

Conlan, A.J.K., Vordermeier, M., De Jong, M.C., Wood, J.L., 2018. The intractable challenge of evaluating cattle vaccination as a control for bovine Tuberculosis. eLife 7, e27694. doi:10.7554/eLife.27694

Constable, P.D., Hinchcliff, K.W., Done, S.H., Grünberg, W., 2017. Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats, 11th ed. Elsevier Ltd, St. Louis, Missouri, USA.

Contreras, C., Alegría-Moran, R., Duchens, M., Ábalos, P., López, R., Retamal, P., 2023. Specific and non-specific effects of *Mycobacterium bovis* BCG vaccination in dairy calves. Front. Vet. Sci. 10, 1278329. doi:10.3389/fvets.2023.1278329

Correia-Neves, M., Fröberg, G., Korshun, L., Viegas, S., Vaz, P., Ramanlal, N., Bruchfeld, J., Hamasur, B., Brennan, P., Källenius, G., 2019a. Biomarkers for tuberculosis: the case for lipoarabinomannan. ERJ Open Res 5, 00115–02018. doi:10.1183/23120541.00115-2018

Correia-Neves, M., Sundling, C., Cooper, A., Källenius, G., 2019b. Lipoarabinomannan in Active and Passive Protection Against Tuberculosis. Front. Immunol. 10, 1968. doi:10.3389/fimmu.2019.01968

Costa, P., Botelho, A., Couto, I., Viveiros, M., InÃ¡cio, J., 2014. Standing of nucleic acid testing strategies in veterinary diagnosis laboratories to uncover *Mycobacterium tuberculosis* complex members. Front. Mol. Biosci. 1. doi:10.3389/fmolb.2014.00016

Courcoul, A., Moyen, J.-L., Brugère, L., Faye, S., Hénault, S., Gares, H., Boschiroli, M.-L., 2014. Estimation of Sensitivity and Specificity of Bacteriology, Histopathology and PCR for the Confirmatory Diagnosis of Bovine Tuberculosis Using Latent Class Analysis. PLOS ONE 9, e90334. doi:10.1371/journal.pone.0090334

Cousins, D.V., Dawson, D.J., 1999. Tuberculosis due to *Mycobacterium bovis* in the Australian population: cases recorded during 1970-1994. Int. J. Tuberc. Lung Dis. Off. J. Int. Union Tuberc. Lung Dis. 3, 715–721.

Cox, D.R., Donnelly, C.A., Bourne, F.J., Gettinby, G., McInerney, J.P., Morrison, W.I., Woodroffe, R., 2005. Simple model for tuberculosis in cattle and badgers. Proc. Natl. Acad. Sci. U. S. A. 102, 17588–17593. doi:10.1073/pnas.0509003102

Crawshaw, T.R., Chanter, J.I., McGoldrick, A., Line, K., 2014. A proof of concept study to assess the potential of PCR testing to detect natural *Mycobacterium bovis* infection in South American camelids. Ir. Vet. J. 67, 5. doi:10.1186/2046-0481-67-5

Curtsinger, J.M., Mescher, M.F., 2010. Inflammatory cytokines as a third signal for T cell activation. Curr. Opin. Immunol. 22, 333–340. doi:10.1016/j.coi.2010.02.013

De Garine-Wichatitsky, M., Caron, A., Kock, R., Tschopp, R., Munyeme, M., Hofmeyr, M., Michel, A., 2013. A review of bovine tuberculosis at the wildlife–livestock–human interface in sub-Saharan Africa. Epidemiol. Infect. 141, 1342–1356. doi:10.1017/S0950268813000708

De Jong, M.C.M., Kimman, T.G., 1994. Experimental quantification of vaccine-induced reduction in virus transmission. Vaccine 12, 761–766. doi:10.1016/0264-410X(94)90229-1

de la Rua-Domenech, R., de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res. Vet. Sci. 81, 190–210. doi:10.1016/j.rvsc.2005.11.005

De Libero, G., Singhal, A., Lepore, M., Mori, L., 2014. Nonclassical T Cells and Their Antigens in Tuberculosis. Cold Spring Harbor Perspectives in Medicine 4, a018473–a018473. doi:10.1101/cshperspect.a018473

de Lisle, G.W., Green, R.S., Buddle, B.M., 2017. Factors affecting the gamma interferon test in the detection of bovine tuberculosis in cattle. J. Vet. Diagn. Invest. 29, 198–202.

De Martino, M., Lodi, L., Galli, L., Chiappini, E., 2019. Immune Response to *Mycobacterium tuberculosis*: A Narrative Review. Front. Pediatr. 7, 350. doi:10.3389/fped.2019.00350

Deresa, B., Conraths, F.J., Ameni, G., 2013. Abattoir-based study on the epidemiology of caprine tuberculosis in Ethiopia using conventional and molecular tools. Acta Vet. Scand. 55, 15. doi:10.1186/1751-0147-55-15

Domogalla, J., Prodinger, W.M., Blum, H., Krebs, S., Gellert, S., Müller, M., Neuendorf, E., Sedlmaier, F., Büttner, M., 2013. Region of Difference 4 in Alpine *Mycobacterium caprae* Isolates Indicates Three Variants. J. Clin. Microbiol. 51, 1381–1388. doi:10.1128/JCM.02966-12

Dorfleutner, A., Chu, L., Stehlik, C., 2015. Inhibiting the inflammasome: one domain at a time. Immunol. Rev. 265, 205–216.

Duffy, S.C., Venkatesan, M., Chothe, S., Poojary, I., Verghese, V.P., Kapur, V., Behr, M.A., Michael, J.S., 2021. Development of a Multiplex Real-Time PCR Assay for *Mycobacterium bovis* BCG and Validation in a Clinical Laboratory. Microbiol. Spectr. 9, e01098-21. doi:10.1128/Spectrum.01098-21

Dykema, P.E., Stokes, K.D., Beckwith, N.R., Mungin, J.W., Xu, L., Vickers, D.J., Reising, M.M., Bravo, D.M., Thomsen, B.V., Robbe-Austerman, S., 2016. Development and validation of a direct real-time PCR assay for *Mycobacterium bovis* and implementation into the United States national surveillance program (preprint). PeerJ PrePrints. doi:10.7287/peerj.preprints.1703v1

Egbe, N.F., Muwonge, A., Ndip, L., Kelly, R.F., Sander, M., Tanya, V., Ngwa, V.N., Handel, I.G., Novak, A., Ngandalo, R., Mazeri, S., Morgan, K.L., Asuquo, A., Bronsvoort, B.M.D.C., 2016. Abattoir-based estimates of mycobacterial infections in Cameroon. Sci. Rep. 6, 24320. doi:10.1038/srep24320

Elnaggar, M.M., Abdellrazeq, G.S., Elsisy, A., Mahmoud, A.H., Shyboub, A., Sester, M., Khaliel, S.A., Singh, M., Torky, H.A., Davis, W.C., 2017. Evaluation of antigen specific interleukin-1β as a biomarker to detect cattle infected with *Mycobacterium bovis*. Tuberculosis 105, 53–59. doi:10.1016/j.tube.2017.04.009

European Commission, 2013. Summary Report on the Outcome of EU Co-Financed Animal Disease Eradication and Monitoring Programmes (2007–2011) (No. SANCO/2013/10941). Brussels: European Commission.

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2023. The European Union One Health 2022 Zoonoses Report. EFSA J. 21. doi:10.2903/j.efsa.2023.8442

Fang, L., Lin, W., Jia, H., Gao, X., Sui, X., Guo, X., Hou, S., Jiang, Y., Zhu, L., Zhu, H., Ding, J., Jiang, L., Xin, T., 2020. Potential Diagnostic Value of the Peripheral Blood Mononuclear Cell Transcriptome From Cattle With Bovine Tuberculosis. Front. Vet. Sci. 7, 295. doi:10.3389/fvets.2020.00295

FDA-NIH Biomarker Working Group, 2016. BEST (Biomarkers, EndpointS, and other Tools) Resource. Food and Drug Administration (US), Silver Spring (MD).

Fellag, M., Loukil, A., Drancourt, M., 2020. The puzzle of the evolutionary natural history of tuberculosis. New Microbes New Infect. 41, 100712. doi:10.1016/j.nmni.2020.100712

Figueiredo, E.E.D.S., Carvalho, R.C.T., Silvestre, F.G., Lilenbaum, W., Fonseca, L.S., Silva, J.T., Paschoalin, V.M.F., 2010. Detection of *Mycobacterium bovis* DNA in nasal swabs from tuberculous cattle by a multiplex PCR. Braz. J. Microbiol. 41, 386–390. doi:10.1590/S1517-83822010000200020

Firdessa, R., Berg, S., Hailu, E., Schelling, E., Gumi, B., Erenso, G., Gadisa, E., Kiros, T., Habtamu, M., Hussein, J., Zinsstag, J., Robertson, B.D., Ameni, G., Lohan, A.J., Loftus, B., Comas, I., Gagneux, S., Tschopp, R., Yamuah, L., Hewinson, G., Gordon, S.V., Young, D.B., Aseffa, A., 2013. Mycobacterial Lineages Causing Pulmonary and Extrapulmonary Tuberculosis, Ethiopia. Emerg. Infect. Dis. 19, 460–463. doi:10.3201/eid1903.120256

Firdessa, R., Tschopp, R., Wubete, A., Sombo, M., Hailu, E., Erenso, G., Kiros, T., Yamuah, L., Vordermeier, M., Hewinson, R.G., Young, D., Gordon, S.V., Sahile, M., Aseffa, A., Berg, S., 2012. High Prevalence of Bovine Tuberculosis in Dairy Cattle in Central Ethiopia: Implications for the Dairy Industry and Public Health. PLoS ONE 7, e52851. doi:10.1371/journal.pone.0052851

Gagneux, S., Small, P.M., 2007. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. Lancet Infect. Dis. 7, 328–337. doi:10.1016/s1473-3099(07)70108-1

Gao, X., Guo, X., Li, M., Jia, H., Lin, W., Fang, L., Jiang, Y., Zhu, H., Zhang, Z., Ding, J., Xin, T., 2019. Interleukin 8 and Pentaxin (C-Reactive Protein) as Potential New Biomarkers of Bovine Tuberculosis. J Clin Microbiol 57, e00274-19. doi:10.1128/JCM.00274-19

Garcia, A.A., Borja, E., Reid, A., Samy, V., Singh, S., Whittington, R.J., Toribio, J.-A.L.M.L., 2022. Bovine tuberculosis control in Fiji: Retrospective study findings for 2015 to 2020. Front. Vet. Sci. 9, 972120. doi:10.3389/fvets.2022.972120

Gaudino, S.J., Kumar, P., 2019. Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. Front. Immunol. 10, 360. doi:10.3389/fimmu.2019.00360

Goletti, D., Lee, M.R., Wang, J.Y., Walter, N., Ottenhoff, T.H.M., 2018. Update on tuberculosis biomarkers: From correlates of risk, to correlates of active disease and of cure from disease. Respirology 23, 455–466. doi:10.1111/resp.13272

Gong, Y., Wang, J., Li, F., Zhu, B., 2023. Polysaccharides and glycolipids of *Mycobacterium tuberculosis* and their induced immune responses. Scand J Immunol 97, e13261. doi:10.1111/sji.13261

Goodchild, A.V., Clifton-Hadley, R.S., 2001. Cattle-to-cattle transmission of *Mycobacterium bovis*. Tuberculosis 81, 23–41. doi:10.1054/tube.2000.0256

Goodchild, A.V., Downs, S.H., Upton, P., Wood, J.L.N., de la Rua-Domenech, R., 2015. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Vet. Rec. 177, 258–258. doi:10.1136/vr.102961

Goosen, W.J., Cooper, D., Miller, M.A., Van Helden, P.D., Parsons, S.D.C., 2015. IP-10 Is a Sensitive Biomarker of Antigen Recognition in Whole-Blood Stimulation Assays Used for the Diagnosis of *Mycobacterium bovis* Infection in African Buffaloes (Syncerus caffer). Clin Vaccine Immunol 22, 974–978. doi:10.1128/CVI.00324-15

Gumi, B., Schelling, E., Berg, S., Firdessa, R., Erenso, G., Mekonnen, W., Hailu, E., Melese, E., Hussein, J., Aseffa, A., Zinsstag, J., 2012. Zoonotic Transmission of Tuberculosis Between Pastoralists and Their Livestock in South-East Ethiopia. EcoHealth 9, 139–149. doi:10.1007/s10393-012-0754-x

Halloran, M.E., Haber, M., Longini, I.M., Struchiner, C.J., 1991. Direct and Indirect Effects in Vaccine Efficacy and Effectiveness. Am. J. Epidemiol. 133, 323–331. doi:10.1093/oxfordjournals.aje.a115884

Hance, A.J., Grandchamp, B., Lévy-Frébault, V., Lecossier, D., Rauzier, J., Bocart, D., Gicquel, B., 1989. Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol. Microbiol. 3, 843–849. doi:10.1111/j.1365-2958.1989.tb00233.x

Haydon, D.T., Cleaveland, S., Taylor, L.H., Laurenson, M.K., 2002. Identifying reservoirs of infection: a conceptual and practical challenge. Emerg. Infect. Dis. 8, 1468–1473. doi:10.3201/eid0812.010317

Hemati, Z., Derakhshandeh, A., Haghkhah, M., Chaubey, K.K., Gupta, S., Singh, M., Singh, S.V., Dhama, K., 2019. Mammalian cell entry operons; novel and major subset candidates for diagnostics with special reference to *Mycobacterium avium* subspecies *paratuberculosis* infection. Vet. Q. 39, 65–75. doi:10.1080/01652176.2019.1641764

Hope, J.C., Khalid, H., Thom, M.L., Howard, C.J., Shaw, D.J., 2023. Protective Efficacy of BCG Vaccination in Calves Vaccinated at Different Ages. Pathogens 12, 789. doi:10.3390/pathogens12060789

Hope, J.C., Thom, M.L., Villarreal-Ramos, B., Vordermeier, H.M., Hewinson, R.G., Howard, C.J., 2005. Vaccination of neonatal calves with *Mycobacterium bovis* BCG induces protection against intranasal challenge with virulent *M. bovis*. Clin. Exp. Immunol. 139, 48–56. doi:10.1111/j.1365-2249.2005.02668.x

Huard, R.C., de Oliveira Lazzarini, L.C., Butler, W.R., van Soolingen, D., Ho, J.L., 2003. PCR-Based Method To Differentiate the Subspecies of the *Mycobacterium tuberculosis* Complex on the Basis of Genomic Deletions. J. Clin. Microbiol. 41, 1637–1650. doi:10.1128/JCM.41.4.1637-1650.2003

Irvine, E.B., O'Neil, A., Darrah, P.A., Shin, S., Choudhary, A., Li, W., Honnen, W., Mehra, S., Kaushal, D., Gideon, H.P., Flynn, J.L., Roederer, M., Seder, R.A., Pinter, A., Fortune, S., Alter, G., 2021. Robust IgM responses following intravenous vaccination with Bacille Calmette–Guérin associate with prevention of *Mycobacterium tuberculosis* infection in macaques. Nat. Immunol. 22, 1515–1523. doi:10.1038/s41590-021-01066-1

Jia, X., Yang, L., Dong, M., Chen, S., Lv, L., Cao, D., Fu, J., Yang, T., Zhang, J., Zhang, X., Shang, Y., Wang, G., Sheng, Y., Huang, H., Chen, F., 2017. The Bioinformatics Analysis of Comparative Genomics of *Mycobacterium tuberculosis* Complex (MTBC) Provides Insight into Dissimilarities between Intraspecific Groups Differing in Host Association,

Virulence, and Epitope Diversity. Front. Cell. Infect. Microbiol. 7, 88. doi:10.3389/fcimb.2017.00088

Jones, G.J., Coad, M., Khatri, B., Bezos, J., Parlane, N.A., Buddle, B.M., Villarreal-Ramos, B., Hewinson, R.G., Vordermeier, H.M., 2017. Tuberculin Skin Testing Boosts Interferon Gamma Responses to DIVA Reagents in *Mycobacterium bovis*-Infected Cattle. Clin. Vaccine Immunol. CVI 24. doi:10.1128/CVI.00551-16

Jones, G.J., Pirson, C., Hewinson, R.G., Vordermeier, H.M., 2010. Simultaneous Measurement of Antigen-Stimulated Interleukin-1β and Gamma Interferon Production Enhances Test Sensitivity for the Detection of *Mycobacterium bovis* Infection in Cattle. Clin Vaccine Immunol 17, 1946–1951. doi:10.1128/CVI.00377-10

Kasir, D., Osman, N., Awik, A., El Ratel, I., Rafei, R., Al Kassaa, I., El Safadi, D., Salma, R., El Omari, K., Cummings, K.J., Kassem, I.I., Osman, M., 2023. Zoonotic Tuberculosis: A Neglected Disease in the Middle East and North Africa (MENA) Region. Dis. Basel Switz. 11, 39. doi:10.3390/diseases11010039

Klepp, L.I., Eirin, M.E., Garbaccio, S., Soria, M., Bigi, F., Blanco, F.C., 2019. Identification of bovine tuberculosis biomarkers to detect tuberculin skin test and IFNγ release assay false negative cattle. Research in Veterinary Science 122, 7–14. doi:10.1016/j.rvsc.2018.10.016

Korma, W., Mihret, A., Chang, Y., Tarekegn, A., Tegegn, M., Tuha, A., Hwang, D., Asefa, M., Hasen, M.O., Kim, S., Tessema, T.S., Lee, H., 2020. Antigen-Specific Cytokine and Chemokine Gene Expression for Diagnosing Latent and Active Tuberculosis. Diagnostics 10, 716. doi:10.3390/diagnostics10090716

Lakew, M., Srinivasan, S., Mesele, B., Olani, A., Koran, T., Tadesse, B., Mekonnen, G.A., Almaw, G., Sahlu, T., Seyoum, B., Beyecha, K., Gumi, B., Ameni, G., Ashenafi, H., Bakker, D., Kapur, V., Gebre, S., 2022. Utility of the Intradermal Skin Test in a Test-and-Cull Approach to Control Bovine Tuberculosis: A Pilot Study in Ethiopia. Front. Vet. Sci. 9, 823365. doi:10.3389/fvets.2022.823365

Lakew, M., Tadesse, B., Srinivasan, S., Aschalew, M., Andarge, B., Kebede, D., Etifu, A., Alemu, T., Yalew, B., Benti, T., Olani, A., Abera, S., Bedada, W., Fromsa, A., Mekonnen, G.A., Almaw, G., Ameni, G., Ashenafi, H., Gumi, B., Bakker, D., Kapur, V., 2024. Assessing the feasibility of test-and-cull and test-and-segregation approaches for the control of high-prevalence bovine tuberculosis in Ethiopian intensive dairy farms. Sci. Rep. 14, 14298. doi:10.1038/s41598-024-64884-x

Lai, R., Ogunsola, A.F., Rakib, T., Behar, S.M., 2023. Key advances in vaccine development for tuberculosis—success and challenges. npj Vaccines 8, 158. doi:10.1038/s41541-023-00750-7

Lu, Z., Gröhn, Y.T., Smith, R.L., Karns, J.S., Hovingh, E., Schukken, Y.H., 2014. Stochastic Modeling of Imperfect Salmonella Vaccines in an Adult Dairy Herd. Bull Math Biol 76, 541–565. doi:10.1007/s11538-013-9931-5

Lam, A., Prabhu, R., Gross, C.M., Riesenberg, L.A., Singh, V., Aggarwal, S., 2017. Role of apoptosis and autophagy in tuberculosis. Am. J. Physiol. - Lung Cell. Mol. Physiol. 313, L218–L229. doi:10.1152/ajplung.00162.2017

Lamont, E.A., Janagama, H.K., Ribeiro-Lima, J., Vulchanova, L., Seth, M., Yang, M., Kurmi, K., Waters, W.R., Thacker, T., Sreevatsan, S., 2014a. Circulating *Mycobacterium bovis* Peptides and Host Response Proteins as Biomarkers for Unambiguous Detection of Subclinical Infection. J Clin Microbiol 52, 536–543. doi:10.1128/JCM.02433-13

Lamont, E.A., Ribeiro-Lima, J., Waters, W.R., Thacker, T., Sreevatsan, S., 2014b. Mannosylated lipoarabinomannan in serum as a biomarker candidate for subclinical bovine tuberculosis. BMC Res Notes 7, 559. doi:10.1186/1756-0500-7-559

Lange, C., Aaby, P., Behr, M.A., Donald, P.R., Kaufmann, S.H.E., Netea, M.G., Mandalakas, A.M., 2022. 100 years of *Mycobacterium bovis* bacille Calmette-Guérin. Lancet Infect. Dis. 22, e2–e12. doi:10.1016/S1473-3099(21)00403-5

Li, M., Wang, M., Wen, Y., Zhang, H., Zhao, G., Gao, Q., 2023. Signaling pathways in macrophages: molecular mechanisms and therapeutic targets. MedComm 4, e349. doi:10.1002/mco2.349

Liebana, E., Johnson, L., Gough, J., Durr, P., Jahans, K., Clifton-Hadley, R., Spencer, Y., Hewinson, R.G., Downs, S.H., 2008. Pathology of naturally occurring bovine tuberculosis in England and Wales. The Veterinary Journal 176, 354–360. doi:10.1016/j.tvjl.2007.07.001

Liu, Y., Larrouy-Maumus, G., 2022. Lipids and glycolipids as biomarkers of mycobacterial infections, in: Biology of Mycobacterial Lipids. Elsevier, pp. 83–104. doi:10.1016/B978-0-323-91948-7.00014-2

Lopez-Valencia, G., Renteria-Evangelista, T., Williams, J.D.J., Licea-Navarro, A., Mora-Valle, A.D.L., Medina-Basulto, G., 2010. Field evaluation of the protective efficacy of *Mycobacterium bovis* BCG vaccine against bovine tuberculosis. Res. Vet. Sci. 88, 44–49. doi:10.1016/j.rvsc.2009.05.022

Malone, K.M., Gordon, S.V., 2017. *Mycobacterium tuberculosis* Complex Members Adapted to Wild and Domestic Animals, in: Gagneux, S. (Ed.), Strain Variation in the *Mycobacterium tuberculosis* Complex: Its Role in Biology, Epidemiology and Control, Advances in Experimental Medicine and Biology. Springer International Publishing, Cham, pp. 135–154. doi:10.1007/978-3-319-64371-7_7

Mamo, G., Abebe, F., Worku, Y., Legesse, M., Medhin, G., Bjune, G., Ameni, G., 2012. Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of

Mycobacterium tuberculosis from Goat. Veterinary Medicine International 2012, 1–8. doi:10.1155/2012/869146

McLoughlin, K.E., Nalpas, N.C., Rue-Albrecht, K., Browne, J.A., Magee, D.A., Killick, K.E., Park, S.D.E., Hokamp, K., Meade, K.G., O'Farrelly, C., Gormley, E., Gordon, S.V., MacHugh, D.E., 2014. RNA-seq Transcriptional Profiling of Peripheral Blood Leukocytes from Cattle Infected with *Mycobacterium bovis*. Front Immunol 5, 396. doi:10.3389/fimmu.2014.00396

Medeiros, L.S., Marassi, C.D., Figueiredo, E.E.S., Leite, J., Ferreira, A.M.R., Lilenbaum, W., 2012. Assessing the histopathology to depict the different stages of bovine tuberculosis infection in a naturally infected herd. Pesquisa Veterinária Brasileira 32, 135–139.

Meisinger, G., 1970. [Economic effects of the elimination of bovine tuberculosis on the productivity of cattle herds. 2. Effect on meat production]. Monatsh. Veterinarmed. 25, 7–13.

Menin, Á., Fleith, R., Reck, C., Marlow, M., Fernandes, P., Pilati, C., Báfica, A., 2013. Asymptomatic Cattle Naturally Infected with *Mycobacterium bovis* Present Exacerbated Tissue Pathology and Bacterial Dissemination. PLoS ONE 8, e53884. doi:10.1371/journal.pone.0053884

Merker, M., Kohl, T.A., Niemann, S., Supply, P., 2017. The Evolution of Strain Typing in the *Mycobacterium tuberculosis* Complex, in: Gagneux, S. (Ed.), Strain Variation in the *Mycobacterium Tuberculosis* Complex: Its Role in Biology, Epidemiology and Control. Springer International Publishing, Cham, pp. 43–78. doi:10.1007/978-3-319-64371-7_3

Mihret, A., 2012. The role of dendritic cells in *Mycobacterium tuberculosis* infection. Virulence 3, 654–659. doi:10.4161/viru.22586

Mishra, M., Kapoor, S., 2022. Multifaceted roles of mycobacterium cell envelope glycolipids during host cell membrane interactions, in: Biology of Mycobacterial Lipids. Elsevier, pp. 105–131. doi:10.1016/B978-0-323-91948-7.00004-X

Morgun, E., Zhu, J., Almunif, S., Bobbala, S., Aguilar, M.S., Wang, J., Conner, K., Cui, Y., Cao, L., Seshadri, C., Scott, E.A., Wang, C.-R., 2023. Vaccination with mycobacterial lipid loaded nanoparticle leads to lipid antigen persistence and memory differentiation of antigen-specific T cells. eLife 12, RP87431. doi:10.7554/eLife.87431

Müller, B., Dürr, S., Alonso, S., Hattendorf, J., Laisse, C.J.M., Parsons, S.D.C., Van Helden, P.D., Zinsstag, J., 2013. Zoonotic *Mycobacterium bovis* –induced Tuberculosis in Humans. Emerg. Infect. Dis. 19, 899–908. doi:10.3201/eid1906.120543

Mwebaza, I., Shaw, R., Li, Q., Fletcher, S., Achkar, J.M., Harding, C.V., Carpenter, S.M., Boom, W.H., 2023. Impact of *Mycobacterium tuberculosis* Glycolipids on the CD4+ T Cell–Macrophage Immunological Synapse. The Journal of Immunology 211, 1385–1396. doi:10.4049/jimmunol.2300107

Nadeem, A., Javed, M., 2022. Omics for Animal Sciences: Principles and Approaches. Nova Science Publishers. doi:10.52305/PCIP7813

Neto, P.S.B., Medeiros, G.B., Morais, D.A., Limeira, C.H., Higino, S.S.S., Araújo, F.R., Azevedo, S.S., Alves, C.J., 2024. Prevalence of bovine tuberculosis: A systematic review and meta-analysis. Pesqui. Veterinária Bras. 44, e07390. doi:10.1590/1678-5150-pvb-7390

Noordhoek, G.T., Kolk, A.H., Bjune, G., Catty, D., Dale, J.W., Fine, P.E., Godfrey-Faussett, P., Cho, S.N., Shinnick, T., Svenson, S.B., 1994. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J. Clin. Microbiol. 32, 277–284. doi:10.1128/jcm.32.2.277-284.1994

Nugent, G., Yockney, I.J., Cross, M.L., Buddle, B.M., 2018. Low-dose BCG vaccination protects free-ranging cattle against naturally-acquired bovine tuberculosis. Vaccine 36, 7338–7344. doi:10.1016/j.vaccine.2018.10.025

Nugent, G., Yockney, I.J., Whitford, J., Aldwell, F.E., Buddle, B.M., 2017. Efficacy of oral BCG vaccination in protecting free-ranging cattle from natural infection by *Mycobacterium bovis*. Vet. Microbiol. 208, 181–189. doi:10.1016/j.vetmic.2017.07.029

Nuñez-Garcia, J., Downs, S.H., Parry, J.E., Abernethy, D.A., Broughan, J., Cameron, A., Cook, A.J., Rua-Domenech, R. de la, Goodchild, A., Gunn, J., More, S.J., Rhodes, S., Rolfe, S., Sharp, M., Upton, P.A., Vordermeier, H., Watson, E., Welsh, M., Whelan, A., Woolliams, J.A., Clifton-Hadley, R., Greiner, M., 2017. Edinburgh Research Explorer Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. Prev. Vet. Med. 153, 94–107. doi:10.1016/j.prevetmed.2017.02.017

Nuru, A., Mamo, G., Zewude, A., Mulat, Y., Yitayew, G., Admasu, A., Medhin, G., Pieper, R., Ameni, G., 2017. Preliminary investigation of the transmission of tuberculosis between farmers and their cattle in smallholder farms in northwestern Ethiopia: a cross-sectional study. BMC Res. Notes 10, 31–31. doi:10.1186/s13104-016-2349-z

O'Brien, D.J., Thacker, T.C., Salvador, L.C.M., Duffiney, A.G., Robbe-Austerman, S., Camacho, M.S., Lombard, J.E., Palmer, M.V., 2023. The devil you know and the devil you don't: current status and challenges of bovine tuberculosis eradication in the United States. Ir. Vet. J. 76, 16. doi:10.1186/s13620-023-00247-8

Ogawa, S., Abe, R., 2019. Signal Transduction Via Co-stimulatory and Co-inhibitory Receptors. Adv. Exp. Med. Biol. 1189, 85–133. doi:10.1007/978-981-32-9717-3_4

Orsel, K., De Jong, M.C.M., Bouma, A., Stegeman, J.A., Dekker, A., 2007. Foot and mouth disease virus transmission among vaccinated pigs after exposure to virus shedding pigs. Vaccine 25, 6381–6391. doi:10.1016/j.vaccine.2007.06.010

Palmer, M.V., 2013. *Mycobacterium bovis*: Characteristics of Wildlife Reservoir Hosts. Transbound. Emerg. Dis. 60, 1–13. doi:10.1111/tbed.12115

Palmer, M.V., Kanipe, C., Boggiatto, P.M., 2022. The Bovine Tuberculoid Granuloma. Pathogens 11, 61. doi:10.3390/pathogens11010061

Parham, P., 2015. The Immune System, Fourth. ed. Garland Science, Taylor & Francis Group, LLC.

Park, H., Jang, H., Kim, C., Chung, B., Chang, C.L., Park, S.K., Song, S., 2000. Detection and Identification of Mycobacteria by Amplification of the Internal Transcribed Spacer Regions with Genus-and Species-Specific PCR Primers. J. Clin. Microbiol. 39, 4080–4085. doi:10.1128/JCM.39.2.828-828.2001

Parlane, N.A., Buddle, B.M., 2015. Immunity and Vaccination against Tuberculosis in Cattle. Curr. Clin. Microbiol. Rep. 2, 44–53. doi:10.1007/s40588-014-0009-4

Parsons, L.M., Brosch, R., Cole, S.T., Somoskövi, A., Loder, A., Bretzel, G., Van Soolingen, D., Hale, Y.M., Salfinger, M., 2002. Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. J Clin Microbiol 40, 2339–2345. doi:10.1128/JCM.40.7.2339-2345.2002

Parsons, S.D.C., McGill, K., Doyle, M.B., Goosen, W.J., Van Helden, P.D., Gormley, E., 2016. Antigen-Specific IP-10 Release Is a Sensitive Biomarker of *Mycobacterium bovis* Infection in Cattle. PLoS ONE 11, e0155440. doi:10.1371/journal.pone.0155440

Pedersen, J.L., Bokil, N.J., Saunders, B.M., 2019. Developing new TB biomarkers, are miRNA the answer? Tuberculosis 118, 101860. doi:10.1016/j.tube.2019.101860

Pollock, J.M., Neill, S.D., 2002. *Mycobacterium bovis* Infection and Tuberculosis in Cattle. Vet. J. 163, 115–127. doi:10.1053/tvjl.2001.0655

Pym, A.S., Brodin, P., Brosch, R., Huerre, M., Cole, S.T., 2002. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*: Role of RD1 in virulence of tubercle bacilli. Mol. Microbiol. 46, 709–717. doi:10.1046/j.1365-2958.2002.03237.x

Ramos, D.F., Silva, P.E.A., Dellagostin, O.A., 2015. Diagnosis of bovine tuberculosis: review of main techniques. Braz. J. Biol. 75, 830–837. doi:10.1590/1519-6984.23613

Ravesloot-Chávez, M.M., Van Dis, E., Stanley, S.A., 2021. The Innate Immune Response to *Mycobacterium tuberculosis* Infection. Annu. Rev. Immunol. 39, 611–637. doi:10.1146/annurev-immunol-093019-010426

Reddington, K., O'Grady, J., Dorai-Raj, S., Maher, M., Van Soolingen, D., Barry, T., 2011a. Novel Multiplex Real-Time PCR Diagnostic Assay for Identification and Differentiation of *Mycobacterium tuberculosis, Mycobacterium canettii,* and *Mycobacterium tuberculosis* Complex Strains. J. Clin. Microbiol. 49, 651–657. doi:10.1128/JCM.01426-10

Reddington, K., O'Grady, J., Dorai-Raj, S., Niemann, S., Van Soolingen, D., Barry, T., 2011b. A Novel Multiplex Real-Time PCR for the Identification of Mycobacteria Associated with Zoonotic Tuberculosis. PLoS ONE 6, e23481. doi:10.1371/journal.pone.0023481

Reis, A.C., Ramos, B., Pereira, A.C., Cunha, M.V., 2021a. Global trends of epidemiological research in livestock tuberculosis for the last four decades. Transbound. Emerg. Dis. 68, 333–346. doi:10.1111/tbed.13763

Reis, A.C., Ramos, B., Pereira, A.C., Cunha, M.V., 2021b. The hard numbers of tuberculosis epidemiology in wildlife: A meta-regression and systematic review. Transbound. Emerg. Dis. 68, 3257–3276. doi:10.1111/tbed.13948

Rhodes, S.G., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2000. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology 99, 195–202. doi:10.1046/j.1365-2567.2000.00944.x

Riojas, M.A., McGough, K.J., Rider-Riojas, C.J., Rastogi, N., Hazbón, M.H., 2018. Phylogenomic analysis of the species of the *Mycobacterium tuberculosis* complex demonstrates that *Mycobacterium africanum, Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti* and *Mycobacterium pinnipedii* are later heterotypic synonyms of *Mycobacterium tuberculosis*. International Journal of Systematic and Evolutionary Microbiology 68, 324–332. doi:10.1099/ijsem.0.002507

Rizzi, C., Bianco, M.V., Blanco, F.C., Soria, M., Gravisaco, M.J., Montenegro, V., Vagnoni, L., Buddle, B., Garbaccio, S., Delgado, F., Leal, K.S., Cataldi, A.A., Dellagostin, O.A., Bigi, F., 2012. Vaccination with a BCG strain overexpressing Ag85B protects cattle against *Mycobacterium bovis* challenge. PloS One 7, e51396. doi:10.1371/journal.pone.0051396

Robinson, P.A., Corner, L.A.L., Courcier, E.A., McNair, J., Artois, M., Menzies, F.D., Abernethy, D.A., 2012. BCG vaccination against tuberculosis in European badgers (Meles meles): A review. Comp. Immunol. Microbiol. Infect. Dis. 35, 277–287. doi:10.1016/j.cimid.2012.01.009

Romero-Adrian, J.L.-M.T.B., Ndez, A.V., 2015. Role of cytokines and other factors involved in the *Mycobacterium tuberculosis* infection. World J. Immunol. 5, 16–50. doi:10.5411/wji.v5.i1.16

Roos, E.O., Olea-Popelka, F., Buss, P., De Klerk-Lorist, L.-M., Cooper, D., Warren, R.M., Van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. IP-10: A potential biomarker for detection of *Mycobacterium bovis* infection in warthogs (Phacochoerus africanus). Veterinary Immunology and Immunopathology 201, 43–48. doi:10.1016/j.vetimm.2018.05.007

Roos, E.O., Scott, L.A., Ndou, S., Olea-Popelka, F., Buss, P.E., de Klerk-Lorist, L.-M., Warren, R.M., van Helden, P.D., Sylvester, T.T., Miller, M.A., Parsons, S.D.C., 2019. Cytokine gene expression assay as a diagnostic tool for detection of *Mycobacterium bovis* infection

in warthogs (Phacochoerus africanus). Sci Rep 9, 16525. doi:10.1038/s41598-019-53045-0

Roy, R.D., Gupta, S.D., 2022. A Comparative Study of Cartridge-based Nucleic Acid Amplification Test and ZiehlÂ-Neelsen Stain with Culture on LowensteinÂ-Jensen Media as Gold Standard for the Diagnosis of Pulmonary Tuberculosis. Indian Journal of Respiratory Care 11, 39–42. doi:10.4103/ijrc.ijrc_123_21

Saïd-Salim, B., Mostowy, S., Kristof, A.S., Behr, M.A., 2006. Mutations in *Mycobacterium tuberculosis Rv0444c* , the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in *Mycobacterium bovis*. Mol. Microbiol. 62, 1251–1263. doi:10.1111/j.1365-2958.2006.05455.x

Scherrer, S., Landolt, P., Friedel, U., Stephan, R., 2019. Distribution and expression of *esat-6* and *cfp-10* in non-tuberculous mycobacteria isolated from lymph nodes of slaughtered cattle in Switzerland. J VET Diagn Invest 31, 217–221. doi:10.1177/1040638718824074

Schiller, I., Oesch, B., Vordermeier, H.M., Palmer, M.V., Harris, B.N., Orloski, K.A., Buddle, B.M., Thacker, T.C., Lyashchenko, K.P., Waters, W.R., 2010. Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication: Review of Bovine Tuberculosis Diagnostics. Transbound. Emerg. Dis. no-no. doi:10.1111/j.1865-1682.2010.01148.x

Schorer, M., Kuchroo, V.K., Joller, N., 2019. Role of Co-stimulatory Molecules in T Helper Cell Differentiation, in: Azuma, M., Yagita, H. (Eds.), Co-Signal Molecules in T Cell Activation, Advances in Experimental Medicine and Biology. Springer Singapore, Singapore, pp. 153–177. doi:10.1007/978-981-32-9717-3_6

Seth, M., Lamont, E.A., Janagama, H.K., Widdel, A., Vulchanova, L., Stabel, J.R., Waters, W.R., Palmer, M.V., Sreevatsan, S., 2009. Biomarker Discovery in Subclinical Mycobacterial Infections of Cattle. PLoS ONE 4, e5478. doi:10.1371/journal.pone.0005478

Sharpe, A.H., Freeman, G.J., 2002. The B7–CD28 superfamily. Nat. Rev. Immunol. 2, 116–126. doi:10.1038/nri727

Shin, S.J., Lee, B.S., Koh, W.-J., Manning, E.J.B., Anklam, K., Sreevatsan, S., Lambrecht, R.S., Collins, M.T., 2010. Efficient Differentiation of *Mycobacterium avium* Complex Species and Subspecies by Use of Five-Target Multiplex PCR. J. Clin. Microbiol. 48, 4057–4062. doi:10.1128/JCM.00904-10

Smith, K., Kleynhans, L., Snyders, C., Bernitz, N., Cooper, D., Van Helden, P., Warren, R.M., Miller, M.A., Goosen, W.J., 2021a. Use of the MILLIPLEX® bovine cytokine/chemokine multiplex assay to identify *Mycobacterium bovis*-infection biomarkers in African buffaloes (Syncerus caffer). Veterinary Immunology and Immunopathology 231, 110152. doi:10.1016/j.vetimm.2020.110152

Smith, K., Kleynhans, L., Warren, R.M., Goosen, W.J., Miller, M.A., 2021. Cell-Mediated Immunological Biomarkers and Their Diagnostic Application in Livestock and Wildlife Infected with *Mycobacterium bovis*. Front. Immunol. 12, 639605. doi:10.3389/fimmu.2021.639605

Srinivasan, S., Conlan, A.J.K., Easterling, L.A., Herrera, C., Dandapat, P., Veerasami, M., Ameni, G., Jindal, N., Raj, G.D., Wood, J., Juleff, N., Bakker, D., Vordermeier, M., Kapur, V., 2021. A Meta-Analysis of the Effect of Bacillus Calmette-Guérin Vaccination Against Bovine Tuberculosis: Is Perfect the Enemy of Good? Front. Vet. Sci. 8, 637580. doi:10.3389/fvets.2021.637580

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. doi:10.1126/sciadv.aax4899

Srinivasan, S., Subramanian, S., Shankar Balakrishnan, S., Ramaiyan Selvaraju, K., Manomohan, V., Selladurai, S., Jothivelu, M., Kandasamy, S., Gopal, D.R., Kathaperumal, K., Conlan, A.J.K., Veerasami, M., Bakker, D., Vordermeier, M., Kapur, V., 2020. A Defined Antigen Skin Test That Enables Implementation of BCG Vaccination for Control of Bovine Tuberculosis: Proof of Concept. Front. Vet. Sci. 7, 391. doi:10.3389/fvets.2020.00391

Stamm, C.E., Collins, A.C., Shiloh, M.U., 2015. Sensing of *Mycobacterium tuberculosis* and consequences to both host and bacillus. Immunol. Rev. 264, 204–219. doi:10.1111/imr.12263

Storek, K.M., Monack, D.M., 2015. Bacterial recognition pathways that lead to inflammasome activation. Immunol. Rev. 265, 112–129.

Subramanian, S., Srinivasan, S., Ramaiyan Selvaraju, K., Vinoli, P.M., Selladurai, S., Ramasamy, B., Kumaragurubaran, K., Bakker, D., Vordermeier, M., Kapur, V., Gopal, D.R., 2022. Defined Antigen Skin Test for Bovine Tuberculosis Retains Specificity on Revaccination With Bacillus Calmette–Guérin. Front. Vet. Sci. 9, 814227. doi:10.3389/fvets.2022.814227

Taylor, G.M., Worth, D.R., Palmer, S., Jahans, K., Hewinson, R.G., 2007. Rapid detection of *Mycobacterium bovis* DNA in cattle lymph nodes with visible lesions using PCR. BMC Vet. Res. 3, 12. doi:10.1186/1746-6148-3-12

Thom, M.L., McAulay, M., Vordermeier, H.M., Clifford, D., Hewinson, R.G., Villarreal-Ramos, B., Hope, J.C., 2012. Duration of Immunity against *Mycobacterium bovis* following Neonatal Vaccination with Bacillus Calmette-Guérin Danish: Significant Protection against Infection at 12, but Not 24, Months. Clin. Vaccine Immunol. 19, 1254–1260. doi:10.1128/CVI.00301-12

Tran, V., Ahn, S.K., Ng, M., Li, M., Liu, J., 2016. Loss of Lipid Virulence Factors Reduces the Efficacy of the BCG Vaccine. Sci. Rep. 6, 29076. doi:10.1038/srep29076

Tschopp, R., Berg, S., Argaw, K., Gadisa, E., Habtamu, M., Schelling, E., Young, D., Young, D.B., Aseffa, A., Zinsstag, J., 2010. BOVINE TUBERCULOSIS IN ETHIOPIAN WILDLIFE. J. Wildl. Dis. 46, 753–762. doi:10.7589/0090-3558-46.3.753

Tschopp, R., Zinsstag, J., Conlan, A., Gemechu, G., Wood, J., 2022. Productivity loss and cost of bovine tuberculosis for the dairy livestock sector in Ethiopia. Prev. Vet. Med. 202, 105616. doi:10.1016/j.prevetmed.2022.105616

Tulu, B., Zewede, A., Belay, M., Zeleke, M., Girma, M., Tegegn, M., Ibrahim, F., Jolliffe, D.A., Abebe, M., Balcha, T.T., Gumi, B., Martineau, H.M., Martineau, A.R., Ameni, G., 2021. Epidemiology of Bovine Tuberculosis and Its Zoonotic Implication in Addis Ababa Milkshed, Central Ethiopia. Front. Vet. Sci. 8, 595511–595511. doi:10.3389/fvets.2021.595511

Van Ingen, J., De Zwaan, R., Dekhuijzen, R., Boeree, M., Van Soolingen, D., 2009. Region of Difference 1 in Nontuberculous *Mycobacterium Species* Adds a Phylogenetic and Taxonomical Character. J Bacteriol 191, 5865–5867. doi:10.1128/JB.00683-09

Van Rhijn, I., Koets, A.P., Im, J.S., Piebes, D., Reddington, F., Besra, G.S., Porcelli, S.A., Van Eden, W., Rutten, V.P.M.G., 2006. The Bovine CD1 Family Contains Group 1 CD1 Proteins, but No Functional CD1d. The Journal of Immunology 176, 4888–4893. doi:10.4049/jimmunol.176.8.4888

Van Rhijn, I., Nguyen, T.K.A., Michel, A., Cooper, D., Govaerts, M., Cheng, T., Van Eden, W., Moody, D.B., Coetzer, J.A.W., Rutten, V., Koets, A.P., 2009. Low cross-reactivity of T-cell responses against lipids from *Mycobacterium bovis* and M. avium paratuberculosis during natural infection. Eur J Immunol 39, 3031–3041. doi:10.1002/eji.200939619

van Soolingen, D., de Haas, P.E., Haagsma, J., Eger, T., Hermans, P.W., Ritacco, V., Alito, A., van Embden, J.D., 1994. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. J. Clin. Microbiol. 32, 2425–2433. doi:10.1128/jcm.32.10.2425-2433.1994

Vitale, F., Capra, G., Maxia, L., Reale, S., Vesco, G., Caracappa, S., 1998. Detection of *Mycobacterium tuberculosis* Complex in Cattle by PCR Using Milk, Lymph Node Aspirates, and Nasal Swabs. J. Clin. Microbiol. 36, 1050–1055. doi:10.1128/JCM.36.4.1050-1055.1998

Vlasova, Saif, 2021. Bovine Immunology: Implications for Dairy Cattle. Front Immunol 12, 12:643206. doi:10.3389/fimmu.2021.643206

Vordermeier, H.M., Brown, J., Cockle, P.J., Franken, W.P.J., Arend, S.M., Ottenhoff, T.H.M., Jahans, K., Hewinson, R.G., 2007. Assessment of Cross-Reactivity between

Mycobacterium bovis and *M. kansasii* ESAT-6 and CFP-10 at the T-Cell Epitope Level. Clin Vaccine Immunol 14, 1203–1209. doi:10.1128/CVI.00116-07

Vordermeier, H.M., Chambers, M.A., Cockle, P.J., Whelan, A.O., Simmons, J., Hewinson, R.G., 2002. Correlation of ESAT-6-Specific Gamma Interferon Production with Pathology in Cattle following *Mycobacterium bovis* BCG Vaccination against Experimental Bovine Tuberculosis. Infect. Immun. 70, 3026–3032. doi:10.1128/IAI.70.6.3026-3032.2002

Vordermeier, H.M., Jones, G.J., Buddle, B.M., Hewinson, R.G., Villarreal-Ramos, B., 2016. Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. Annu. Rev. Anim. Biosci. 4, 87–109. doi:10.1146/annurev-animal-021815-111311

Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J., McAulay, M., Rhodes, S.G., Thacker, T., Gilbert, S.C., McShane, H., Hill, A.V.S., Xing, Z., Hewinson, R.G., 2009. Viral Booster Vaccines Improve *Mycobacterium bovis* BCG-Induced Protection against Bovine Tuberculosis. Infect. Immun. 77, 3364–3373. doi:10.1128/IAI.00287-09

Vordermeier, M., 2014. DEVELOPMENT OF CATTLE TB VACCINES, in: The Art and Science of Tuberculosis Vaccine Development. Oxford University Press, Malaysia, pp. 853–883.

Waters, R., Vordermeier, M., 2018. Immunological diagnosis., in: Bovine Tuberculosis, CABI Books. pp. 173–190. doi:10.1079/9781786391520.0173

Wanzala, S.I., Palmer, M.V., Waters, W.R., Thacker, T.C., Carstensen, M., Travis, D.A., Sreevatsan, S., 2017. Evaluation of pathogen-specific biomarkers for the diagnosis of tuberculosis in white-tailed deer (Odocoileus virginianus). American Journal of Veterinary Research 78, 729–734.

Ward, S.K., Abomoelak, B., Marcus, S.A., Talaat, A.M., 2010. Transcriptional Profiling of *Mycobacterium tuberculosis* During Infection: Lessons Learned. Front. Microbio. 1. doi:10.3389/fmicb.2010.00121

Waters, W.R., Hope, J.C., Hamilton, C.A., Palmer, M.V., McNair, J., Skuce, R.A., Allen, A.R., Buddle, B.M., Villarreal-Ramos, B., Vordermeier, H.M., 2015. Immunopathogenesis of *Mycobacterium bovis* infection of cattle., in: Mukundan, H., Chambers, M.A., Waters, W.R., Larsen, M.H. (Eds.), Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria. CABI, UK, pp. 136–167. doi:10.1079/9781780643960.0136

Waters, W.R., Maggioli, M.F., Palmer, M.V., Thacker, T.C., McGill, J.L., Vordermeier, H.M., Berney-Meyer, L., Jacobs, W.R., Larsen, M.H., 2016. Interleukin-17A as a Biomarker for Bovine Tuberculosis. Clin Vaccine Immunol 23, 168–180. doi:10.1128/CVI.00637-15

Wei, M., Wu, Z.Y., Lin, J.H., Li, Y., Qian, Z.X., Xie, Y.Q., Su, H., Zhou, W., 2015. Regulation network of serum cytokines induced by tuberculosis-specific antigens reveals biomarkers for tuberculosis diagnosis. Genet. Mol. Res. 14, 17182–17192. doi:10.4238/2015.December.16.18

Whelan, A.O., Clifford, D., Upadhyay, B., Breadon, E.L., McNair, J., Hewinson, G.R., Vordermeier, M.H., 2010. Development of a Skin Test for Bovine Tuberculosis for Differentiating Infected from Vaccinated Animals. J. Clin. Microbiol. 48, 3176–3181. doi:10.1128/JCM.00420-10

WHO, 2019. Global tuberculosis report. Geneva: World Health organization.

WHO, FAO, OIE and The Union, 2017. ZOONOTIC TUBERCULOSIS.

Williams, G., Allen, D., Brewer, J., Salguero, F., Houghton, S., Vordermeier, M., 2024. The safety of overdose and repeat administrations of BCG Danish strain 1331 vaccine in calves and pregnant heifers. Heliyon 10, e34683. doi:10.1016/j.heliyon.2024.e34683

Williams, G.A., Scott-Baird, E., Núñez, A., Salguero, F.J., Wood, E., Houghton, S., Vordermeier, H.M., 2022. The safety of BCG vaccination in cattle: results from good laboratory practice safety studies in calves and lactating cows. Heliyon 8, e12356. doi:10.1016/j.heliyon.2022.e12356

WOAH, 2022. Mammalian tuberculosis (infection with *Mycobacterium tuberculosis* complex), In: Manual of diagnostic tests and vaccines for terrestrial animals World Organisation for Animal Health, Paris.

WOAH, (World Organisation for Animal Health), 2024. Guidelines for the control of *Mycobacterium tuberculosis* complex in livestock: Beyond test and slaughter [WWW Document]. URL https://www.woah.org/en/document/guidelines-for-the-control-of-mycobacterium-tuberculosis-complex-in-livestock/ (accessed 10.24.24).

Yahyaoui-Azami, H., Aboukhassib, H., Bouslikhane, M., Berrada, J., Rami, S., Reinhard, M., Gagneux, S., Feldmann, J., Borrell, S., Zinsstag, J., 2017. Molecular characterization of bovine tuberculosis strains in two slaughterhouses in Morocco. BMC Vet. Res. 13, 272. doi:10.1186/s12917-017-1165-6

Zinsstag, J., Schelling, E., Roth, F., Kazwala, R., 2006. Economics of Bovine Tuberculosis, in: *Mycobacterium bovis* Infection in Animals and Humans. Blackwell Publishing Ltd., Oxford, UK, pp. 68–83.

Willgert, K., Cliff, M., Meinke, S., Messina, D., Broom, D.M., Wood, J., Conlan, A.J.K., 2025. Burden of Bovine Tuberculosis on Animal Health, Welfare and Production: A Systematic Review. Transboundary and Emerging Diseases 2025, 6541298. doi:10.1155/tbed/6541298

APPENDIX

Appendix 2.1: Supplementary materials and methods

Experimental design and chain binomial model to estimate vaccine efficacy

Our experimental design allows for the estimation of vaccine efficacy in terms of both the impact on the risk of infection of vaccinates (direct protection or reduction in susceptibility) and infectiousness by measuring the relative rates of transmission between and to different combinations of vaccinated and unvaccinated animals. Phase I of the experiment allows the comparison of the rate of transmission of bTB to vaccinated and unvaccinated animals from a group of tuberculin (CCT) and IGRA test positive animals sourced from naturally infected herds (Figure 2.1). By separating the sentinels from Phase I by treatment type to use as seeders for a second challenge period we can then directly estimate the impact of vaccination on infectiousness as well as susceptibility.

Phase 1 includes two separate experimental replicates housed in distinct barns to control for any potential differences in exposure due to heterogeneity in the infectiousness of the seeder animals. Each experimental group was composed of an equal number *(~34)* of adult infected animals (seeders, S) and naïve susceptible calves (sentinels) to maximize the rate of transmission. Sentinels, differing by their vaccination status (Unvaccinated controls, U; Vaccinates, V), were introduced to seeders two months post-vaccination, maintaining contact for 12 months. During this period, blood was collected from every sentinel animal every two months for IGRA, and skin tests were conducted every four months. A peptide-based defined antigen skin test (DST) was used in both IGRA and skin test formats alongside standard tuberculins (PPDs). The surviving sentinel animals from Phase I were then revaccinated and crossed-over, as described above, to act as seeders for two new experimental groups (3,4) with a fresh set of vaccinated and unvaccinated sentinel calves which were then followed using the same schedule of tests for a second 12-month period of natural exposure.

For the purpose of analysis, the infectious status for each animal is imputed through the response to each of these candidate DIVA (Differentiates Infected from Vaccinated Animals) tests. For each

variant of the DIVA test (DST1, DST10) we construct a logical infection history where animals are considered to be infected - and potentially infectious - from the first positive test. The number of new infections per observation period is therefore calculated as the number of animals that switch diagnostic status within that interval. Tuberculin-based tests such as the single intradermal tuberculin (SIT) and comparative cervical tuberculin (CCT) tests, are not suitable for this purpose as the comprised specificity in vaccinated animals would lead to a different apparent rate of transmission for vaccinates and controls. Animals are assumed to remain positive for the duration of the experiment following their first positive test (and potentially contributing to transmission).

In Phase II, seeder animals were tested at a lower frequency than the sentinel animals. As such, we needed to impute number of infectious seeders animals in groups 3 & 4 based on their status at the end of Phase I and II. Given the small number of animals that changed status between these two points we do not expect this to affect our results, but to carry out a sensitivity analysis to our assumptions we considered three methods of imputation. The default method (main results) we change the status of seeder animals in Phase II only at the point that additional tests were carried out. To set absolute upper and lower bounds we also considered the cases where seeders were assumed to be fixed to their status at the end of Phase I ("minimum") or at the end of Phase II ("maximum"). As expected, these three methods of imputation all lead to statistically indistinguishable estimates of vaccine efficacy (Supplementary Table 2.2).

From the imputed time-series of infection states we can estimate the rate of transmission to control and vaccinate animals respectively using a chain binomial model (26,36) where we assume that the rate of susceptible animals acquiring infection between two tests is constant and given by the number of infected (unvaccinated) animals I and infected vaccinates I_V at the earlier test:

$$R(S \rightarrow I) = \beta \frac{(I + \varepsilon_I I_V)}{N}$$

Where N is the total number of animals within the experimental group, β can be interpreted as the per capita transmission rate per infectious animal and ε_I measures the reduction in infectiousness of vaccinated animals that become infected (relative to unvaccinated controls).

As per the experimental protocol several (a total of 16) seeder and sentinel animals were removed over the course of the experiment distributed between the four experimental groups (Figure 2.1). For analysis we assume that the number of infectious animals for each interval between successive tests consists of the total number of seeder animals and sentinel animals with a previous positive test result that were present within the barn for the whole period. Sentinel animals that were removed before testing positive will not contribute to estimates of transmission rates except with respect to the size of the group which is updated in the same wave for each observation interval. In Phase II, delays in the recruitment of calves meant that there also were two parallel entry points into Groups 3 and 4. As a consequence the numbers of infectious (and surviving) animals between successive tests are different for these two batches of animals (I and II) and for analysis are treated as two subgroups with the number of infectious animals present within each exposure period calculated again based on the entry and exit times of the animals to the experimental barn.

We can define the (constant) rate that vaccinates acquire infection between successive tests as:

$$R(V \rightarrow I_V) = \varepsilon_S \beta \frac{(I + \varepsilon_I I_V)}{N}$$

Where (ε_S) is the proportionate reduction in the risk of vaccinates becoming infected compared to unvaccinated animals.

Vaccine efficacy is typically reported as 1 - the relative risk of infection of vaccinates compared to controls (21), thus for reporting we define the direct (VE_D), indirect (VE_I) and total (VE_T) efficacy of vaccination in terms of our model parameters as:

$$VE_D = 1 - \varepsilon_S$$
$$VE_I = 1 - \varepsilon_I$$
$$VE_T = 1 - \varepsilon_S \varepsilon_I$$

Using these assumptions, the probabilities (P) of transmission within a discrete time interval Δt are:

$$P(S \to I) = 1 - e^{\Delta t \beta \frac{(I + \varepsilon_I I_V)}{N}}$$

$$P(V \to I_V) = 1 - e^{\Delta t \varepsilon_S \beta \frac{(I + \varepsilon_I I_V)}{N}}$$

The number of new cases (C) in the interval between t and t+Δt for susceptible will then be:

$$C(t, t + \Delta t) \sim \text{Binom}(S_t, P(S \to I))$$

and likewise, for vaccinates:

$$C_V(t, t + \Delta t) \sim \text{Binom}(V_t, P(V \to I_V))$$

As the probability of infection takes the same functional form as the complimentary log-log transformation, this chain binomial model can be estimated using a generalized linear model (GLM) with binomial response (and complementary log-log link function). If we specify an offset term of $\log(\Delta t\,(I + \varepsilon_I I_V)/N)$ then the baseline transmission rate (β) will be the estimated intercept. The direct vaccine effect (ε_S) can be estimated as the slope of an indicator variable that represents the treatment (T_t, with vaccinated = TRUE). The reduction in infectiousness ε_I can be estimated as the slope of a final explanatory variable equal to the ratio of the infected vaccinates to total vaccinates ($I_V/(I + I_V)$).

We use the rstanarm package (37) in R (38) to estimate the model within a Bayesian framework using Hamiltonian Markov Chain Monte Carlo (MCMC) and obtain posterior estimates for the direct vaccine efficacy to reduce susceptibility (1-ε_S), efficacy to reduce infectiousness (1-ε_I) and average base transmission rate (β) within the two experimental barns. Convergence was assessed using standard MCMC diagnostics (Rhat and effective sample size) and model fit was assessed using posterior predictive checks (specifically by forming Bayesian predictive p-values for the proportion of zeros, maximum and mean values).

An interim analysis was carried out at the end of Phase I to ensure that the one year contact time was sufficient to satisfy the stop/go conditions established by the simulation study carried out by Conlan *et al.* to inform the sample sizes and power calculations for the design (25). These were that:

1. The experimental R was > 1.5
2. At least half of the unvaccinated controls converted to be test-positive

The experimental R was estimated using a reduced version of the regression model described above with vaccine treatment as the only explanatory effect. Here, we estimate this reduced model separately for each experimental group to explore how the effective attack rates to vaccinated and unvaccinated animals differ between the experimental groups (Supplementary Figure 2.3).

Relationship between efficacy to reduce transmission and endpoint estimates

In the manuscript we discuss how estimates of vaccine efficacy based on fixed endpoints are not directly comparable to the estimates of efficacy based on transmission rates reported in this study. However, we can illustrate the systematic relationship between the mechanistic vaccine effects we measure ($\varepsilon_S, \varepsilon_I$) and endpoint estimates using a simplified deterministic version of the compartmental transmission model for bTB transmission we will use for scenario analyses in Section 3 below.

$$\frac{dS(t)}{dt} = -\lambda(t)S(t)$$

$$\frac{dI(t)}{dt} = \lambda(t)S(t)$$

$$\frac{dV(t)}{dt} = \varepsilon_s\lambda(t)V(t)$$

$$\frac{dI_V(t)}{dt} = \varepsilon_s\lambda(t)V(t)$$

Where S, I, V and I_V are the number of susceptible (unvaccinated), infectious (and unvaccinated), vaccinated and infected vaccinated animals respectively. The force of infection within the group

$$\lambda(t) = \frac{\beta(I(t) + \varepsilon_i I_V(t)}{N(t)}$$

depends on the mechanistic effects of BCG on transmission ε_s, ε_i as estimated in this study. Endpoint estimates define the vaccine efficacy in terms of the relative risk of infection (RR) of vaccinates compared to controls as $1 - RR$.

We consider a scenario with 30 initial vaccinates and controls held in contact for 12 months with 15 vaccinates and controls introduced to 30 (unvaccinated) seeder animals and three levels of transmission (low, mid and high force of infection (FOI) corresponding to β = 0.12, 0.24, 0.48) and with vaccine effects on transmission of $\varepsilon_s = 1 - 0.6$ and $\varepsilon_i = 1 - 0.75$ corresponding to a direct vaccine efficacy of 60% and reduction in infectiousness of 75%. At any point in the scenario, we can calculate the endpoint efficacy in terms of the relative risk of infection in the vaccinated and control groups:

$$\text{Endpoint Efficacy} = 1 - ((I_V/(I_V + V))/(I/(I + S)))$$

Supplementary Figure 2.1. illustrates how the endpoint vaccine efficacy for a chronic infectious disease such as bTB is biased downwards by the cumulative exposure of sentinel animals over the course of the experiment. The longer the exposure the lower the estimated vaccine efficacy will be, with this rate of decrease becoming faster the higher the rate of transmission within the population. Endpoint efficacy is a function and property of the population within which the vaccine is being used as well as the individual protective effects of the vaccination and thus should perhaps better be considered as a measure of vaccine *effectiveness* rather than efficacy. This limitation of relative risk measures is a specific issue for chronic infections – for acute infections the potential for bias is mitigated by the continual removal of infectives from the population. For challenge studies, corresponding to the vast majority of published estimates of BCG efficacy in cattle, this

issue is also less relevant due to their relatively short duration of exposure minimising the potential for transmission to bias estimates.

Table 2.1 reports endpoint efficacies calculated based on the DST 1 (IGRA), DST 10 (Skin) and presence or absence of visible lesions, with 95% confidence intervals calculated by simulation. The lower estimated vaccine efficacy from visible lesions of 25%; 95% CI: 1-47% reported in this study is therefore entirely consistent with our higher estimates of vaccine efficacy and does not reflect any systematic issues with the ante-mortem DST test. Indeed, if we calculate the endpoint vaccine efficacy based on the DST 1 test results for groups 1-3 we get a consistent estimate of 39%; 95% CI 19-57% (within statistical uncertainty). The lower efficacy estimated by both of these methods therefore reflects the inherent bias in the use of endpoint estimates of vaccine efficacy from natural transmission (or field) studies. Estimates of the vaccine efficacy based on transmission can therefore also be considered to be statistically more robust as well as the more relevant measure to inform the likely effectiveness in the field.

Study Location and Subjects

The study took place at the Animal Health Institute, located in Sebeta, Ethiopia, about 20 km southwest of Addis Ababa. The premises had three separate barns, each capable of accommodating 80 adult cattle, with approximately 2,000 to 2,500 m^2 of fenced outdoor space. All relevant local biosecurity and safety procedures were adhered to.

The study incorporated 68 naturally infected adult cattle identified as seeders in the transmission experiment. Additionally, 68 Holstein-Friesian x Zebu crossbred male calves, age < three months and tested negative for bTB, were recruited as naïve sentinels from low-prevalence herds. The calves were randomly allocated to control and vaccinate groups using a double-blind lottery system.

Testing Procedures, Antigens and Vaccination

Eight weeks post-BCG vaccination, all sentinel animals were introduced to infected adult seeder herds. The seeders and sentinels were housed together in two separate barns. Every four months,

skin tests were conducted on sentinel animals using both Purified Protein Derivatives (PPDs) and the recently developed DST. Blood was drawn for IGRA every two months.

Bovine (PPD-B) and avian (PPD-A) tuberculins, were used as stimulating antigens in both skin and interferon-gamma (IFN-γ) release assays. A novel peptide cocktail representing the antigens ESAT-6, CFP-10 and Rv3615c was also utilized. We refer to the skin test format of the DST as DST10 and the IGRA as DST1 (corresponding to the units of antigen used respectively for each test). The calves of the vaccinate group were subcutaneously injected with 0.5 ml (1-4 x 106 CFU) of live BCG Danish Strain SSI 1331, while the controls received 0.5 ml of 0.9% normal saline (Supplementary Figure 2.4).

Interferon-gamma Release Assay (IGRA)

The IGRA was conducted using the commercially available BOVIGAM® kit. Around 5 ml blood samples were collected and stimulated with mycobacterial antigens (PPD and DST) within eight hours of collection. The levels of IFN-γ production by the lymphocytes were measured using sandwich enzyme-linked immunosorbent assay (ELISA) and expressed as the optical density at 450 nm (OD450) in the culture supernatant.

Skin Testing

For skin testing, both standard tuberculins and the DST were used. Skin thickness was measured at two sites on right and one site on the left using an Irish caliper before intradermal injection of 0.1 ml of antigen (Supplementary Figure 2.5). After 72 hours, the skin fold thickness was remeasured at the injection sites, and the increase in skin fold thickness was calculated. Diagnostic thresholds used to define positivity for this analysis are reported in Supplementary Table 2.1.

Ethical Approval

The study was approved and granted ethical clearance by the Institutional Review Board of Aklilu Lemma Institute of Pathobiology, reference number ALIPB IRB/44/2013/21.

Data Availability

Raw data, scripts and model code necessary to reproduce all results in the manuscript are available as supplementary information with this paper and publicly available at:

Supplementary Text

Mechanistic transmission model for the spread of bovine tuberculosis within the Ethiopian Dairy Sector

To assess the potential of vaccination as a control strategy for bTB in Ethiopia, we developed a mechanistic transmission model incorporating both within and between herd transmission calibrated using field estimates of the basic reproduction ratio R_0 and cattle movement data. In this supplementary text we define this model and describe the methods used to calibrate both the rates of within-herd transmission and frequency and pattern of cattle movements.

In **Section 1** we use a latent class model to estimate the basic reproduction ratio R_0 from a set of endemically infected herds in Ethiopia. We use these estimates to calculate within-herd transmission rates for our model which vary by herd, reflecting the considerable variation in herd-level prevalence seen in the Ethiopian dairy industry.

Cattle rarely show clinical signs of bTB in the early stages of disease, meaning cattle trade can facilitate transmission over long distances in the absence of systematic testing of herds. We therefore consider cattle movements to be the primary method of transmission between herds. Movements of cattle are not systematically recorded in Ethiopia. Limited samples of movement records available for a small number of herds in three regions with emerging dairy markets (Gondar, Hawassa and Mekelle) have previously been used to demonstrate that movements are locally clustered with measurable network characteristics (29). To simulate the potential rate of herd-to-herd transmission through movements of cattle between herds in Ethiopia, we require a probabilistic, generative model that can simulate synthetic networks with the same statistical properties as measured movement networks. To this end, in **Section 2** we use ERGMs to develop generative models of cattle movements in Ethiopia (44,45).

In **Section 3** we describe a parsimonious mechanistic model incorporating within-herd transmission rates estimated from Section 1 and synthetic cattle movement networks from Section 2 and define a set of transmission scenarios to evaluate the potential use of Bacillus Calmette–Guérin (BCG) vaccination as a control measure of bTB in Ethiopia.

1 Estimation of the within-herd basic reproduction ratio R_0 for bTB

1.1 Theoretical results for the relationship between R_0 and endemic prevalence within herds

For endemic infections, the basic reproduction ratio R_0, defined as the expected number of new infections when a single infectious individual is introduced into a fully susceptible population, can be related to the (true) prevalence of infection within a population. If we assume that the population is well mixed, then the effective reproduction ratio at equilibrium will be 1 and the prevalence P depends only on the value of R_0: P = 1-1/R_0. Thus, for a well-mixed population R_0 could be estimated crudely from the prevalence P as $R_0 \sim 1/(1-P)$.

However, for small populations (such as we have in our study) this estimator may be sensitive to stochastic effects. We can account for this uncertainty using an analytical result for the (quasi)-stationary probability distribution $P(I = i)$ of the susceptible-infected-susceptible (SIS) model (41):

$$P(I = i) = p(I = 1)\frac{(N-1)!}{i(N-i)!}\left(\frac{R_0}{N}\right)^{i-1}, i = 2, \dots, N$$

$$P(I = 1) = \left(\sum_{k=1}^{N}\frac{(N-1)!}{k(N-k)!}\left(\frac{R_0}{N}\right)^{k-1}\right)^{-1}$$

Where N is the size of the population, *I* the (true number) of infected individuals in the population. As the quasi-distribution only depends on a single parameter - R_0 – we can use the distribution as a likelihood to infer the value of R_0 given the observed number of infected individuals *I* within a herd. However, for bTB we lack a gold-standard definition of infection and must rely on estimates

of the apparent prevalence based on the tuberculin skin test, for which both the sensitivity and specificity is poorly characterised and has the potential to vary contextually due to levels of exposure to environmental mycobacteria, variations in the potency of tuberculin and the consistency of test operators. Thus, calculating the true prevalence of infection using estimates for the sensitivity and specificity of the SIT and CCT tests from the literature would not, we would argue, be appropriate and could underestimate the uncertainty in estimated transmission rates.

1.2 Walter-Hui latent class framework

The Walter-Hui latent class framework offers a potential solution to this problem (42,43). Providing at least two diagnostic tests are carried out on animals from at least two populations with different prevalence, the Walter-Hui latent class model provides a theoretical framework to infer the true prevalence of infection in the absence of a gold-standard test. Such latent class models are being increasingly used as a solution to evaluate the relative performance of bTB diagnostics (50). Here, however, we treat the unknown test-characteristics of the tuberculin tests as nuisance parameters and set out to use the Walter-Hui framework to integrate them out of our estimates – while still accounting for the uncertainty with respect to the true prevalence of infection.

The key assumption of the Walter-Hui model is that of conditional independence between tests, i.e., the probability of a test t being positive for individual (i), $P(T_{i,t} = 1)$ only depends on the latent (true) disease status of the individual ($D \in \{0,1\}$) and not the response of the other tests. For T tests there will be a set (E) of 2^T mutually exclusive combinations of positive and negative responses to each test, which we can use to define and simplify the notation for a Walter-Hui model with an arbitrary number of diagnostic tests (51). Let E_{et} indicate the response of an individual animal to test t (0 negative, 1 positive) then the probability of a particular observed pattern O in a population with true prevalence of disease θ will be:

$$P(O = E_e|s_t, p_t, \theta) = \prod_{t=1}^{T} \theta(E_{et}s_t + (1 - E_{et})s_t) + (1 - \theta)(E_{et}(1 - p_t) + (1 - E_{et})p_t))$$

Where s_t, p_t are the sensitivity and specificity of test *t* respectively. Each individual pattern of positive and negative results O_i from a set of A animals can then be considered as a sample from a Bernoulli distribution:

$$O_i \sim \text{bernoulli}(P(O_i|s_t, p_t, \phi))$$

Following (43,52) and to extend the model to account for the potential conditional dependence between the SIT and CCT tests, we reparametrize our model using a probit (Φ) link function for the sensitivity and specificity:

$$s_t = \Phi(a_{t,1})$$
$$p_t = \Phi(a_{t,0})$$

The shared dependence of the SIT and CCT test results on the bovine tuberculin reaction does not necessarily mean that the assumption of conditional dependence will be violated. However, as they could be considered as re-interpretations of the same test, we choose to model the trade-off in the interpretation of the two tests by parameterising the model using an explicit functional form for the receiver operating characteristics (ROC) curve that links the sensitivity and specificity of each test (53). Thus, we define:

$$s_t = \Phi(\Phi^{-1}(a_{t,0}) + \sigma)$$

With:

$$p_t = \Phi(a_{t,0})$$

This functional form arises from assuming that the values of $a_{t,0}, b_{t,0}$ for the two tests are normally distributed with equal variance leading to a symmetric ROC curve where the curvature (discrimination of the diagnostic test) is described by the parameter σ. This form allows us to explicitly model a ROC relationship between the SIT and CCT tests by estimating the specificity parameters for each test $a_{t,0}$, but calculating the sensitivities using the shared parameter σ.

To ensure numerical stability and to avoid the label switching problem common with this class of models, we restrict the value of σ to between [0,8] and the specificity parameters (on the probit scale) $a_{t,0}$ to the range $[-8,0]$, corresponding to the assumption that the specificity of both tests is > 50%.

1.3 Likelihood for R_0

If we recall from section 1.1 the likelihood for R_0 can be calculated from the true prevalence within the herd I. Given a set of test results O_i we can calculate the joint likelihood of the latent class and quasi-stationary model by marginalizing over the set of possible values of *I*:

$$L\left(R_0 \middle| O_i, a_{t,0}, \sigma\right) = \sum_{i=0}^{N} P(I = i) P(O_i | s_t, p_t, i)$$

We model the herd-to-herd variation in R_0 through a set of herd level uncentered random effects (η_g):

$$R_0 = \exp(R_m + \tau \eta_g)$$

Once again for numerical stability we restrict the range of η_g to [-8,8], $\tau > 0$ and R_m to between -8 and 3.5.

Normal (0,1) priors were used for all parameters except for the variance τ for which we used a (shrinkage) exponential (1) prior.

The model was implemented in Stan (15,16), estimated by Hamiltonian MCMC and analyzed in R using the RStan package (54). Full code and data are provided as a data supplement with this publication. Convergence was assessed through visual inspection of the chains and standard diagnostic statistics ($\hat{R} = 1$ for all parameters after $2{,}000$ iterations for 4 chains). Estimated parameters are presented as median posterior values with 95% Bayesian credible intervals (CrI).

1.4 Data and estimates of R_0

For this study we had access to tuberculin testing data collected under a consistent protocol from the Ethiopia Control of Bovine Tuberculosis Strategies (ETHICOBOTS) project (15,16), supplemented by a smaller data set of historical tuberculin testing data collected by the Animal Health Institute, Ethiopia (14). Test records were available for 9,464 animals from 415 unique herds – including many herds with no evidence of bTB infection. From this set of herds, we selected a study population of herds, with a herd size > 20 animals and apparent CCT prevalence of > 5%. This gives a study population of 3,319 animal tuberculin test records (bovine and avian) from 57 herds and for which we can define each animal's test status with respect to both the SIT and CCT tests. Herds came from a set of five study sites across Ethiopia including the dairy belt surrounding Addis Ababa and emerging markets in Gondar, Hawassa, Mekelle and Kombolcha (Figure 2.3).

Previous ETHICOBOTS publications (15,16) have discussed the high but incredibly variable level of prevalence seen in affected herds, demonstrated in Supplementary Figure 2.6 (left) which is also reflected in the variability in estimates of the within herd reproduction ratio Supplementary Figure 2.6 (right). In contrast to the assumption of density dependence often used for bTB transmission models, our estimates demonstrate no systematic relationship with herd size with a population average of $R_0 = 3.3;\ 95\%\ \text{CrI: } 2.7 - 4.0$. The level of variation in the apparent rates of transmission between herds has profound implications for the potential for any intervention, implying that for factors not currently ascertained (biological or management) control may be considerably more difficult (or easier) in some herds. To reflect this observed heterogeneity in our model simulations we assume that this represents a natural variation in the intrinsic herd-level reproduction ratio and sample values for our simulations from the full range of these posterior estimates of R_0.

2 Generative model for cattle movements

2.1 Cattle movement data

Cattle movements between herds are not routinely recorded in Ethiopia. However, Mekonnen *et al.* (29), collected a cross-sectional sample of movement records in three study areas with emerging dairy markets (Hawassa, Gondor and Mekelle, Figure 2.3A). Collected between 2013 and 2018 social network analyses of these reported animal movements demonstrated local clustering with a small number of herds being highly connected, but with distinct regional differences in the network structure between the three study areas (29). Consequently, for this study we estimate separate generative models for the cattle movement data from each of these study sites and use the fitted models to define a set of plausible herd-to-herd transmission scenarios representative of the trading patterns within the three sample regions. After removing herds without an identification number, and only including movements with a known movement date, source and destination herd, movement records from 164 herds were included in the study, consisting of 63 herds in Gondar, 33 in Mekelle, and 68 in Hawassa.

2.2 Statistical analysis of cattle movement networks

ERGMs provide a framework for measuring structural network features, to infer the probability of connections between nodes of a network and simulate (static) networks with the same statistical properties (44,55). We fitted a directed ERGM of the observed cattle movement network for each of the three study areas using network characteristics with particular relevance for disease spread, namely: edges (movements) between herds as a measure of network density; geometrically weighted edgewise shared partnership (shared connections between two herds) as a representation of network clustering; geometrically weighted in-degree (the number of movements entering a herd) distribution, and geometrically weighted out-degree (the number of movements exiting a herd) distribution. In the geometrically weighted predictors, the added value of an additional shared connection, in- or out-degree decays at a geometric rate (44). The general form of ERGMs is:

$$P(Y = y) = \frac{\exp\{\theta^{\mathsf{T}} g(y)\}}{k(\theta)}$$

where Y is a set of potential networks and y is a particular version of Y, θ is the coefficient of the predictor statistics considered in the model (corresponding to edges, geometrically weighted

edgewise shared partnership, and geometrically weighted in-degree and out-degree distributions in our model), and the predictors are described in $g(y)$ as functions of network y. $k(\theta)$ is a summation of the numerator over all potential networks y (56). The preferred model and the value of the decay parameters for the geometrically weighted covariates were selected based on the Akaike's information criterion (AIC) and Bayesian Information Criterion (BIC) of the fitted networks for each region.

The MCMC diagnostics of the fitted ERGM were visually assessed for mixing and convergence. The goodness of fit was examined for the selected models by comparing the structural statistics of networks simulated from the fitted ERGM to the observed network (57). The ERGMs of the cattle movement networks were fitted using the ergm package (45,56,58) in R (38). The probability of movements between herds (edges) and the number of movements entering a herd (in-degree) were found to be significantly associated ($p<0.05$) with the empirical networks for all three study regions, while the number of movements exiting a herd (out-degree) was also significant only for Hawassa (Supplementary Table 2.3).

2.3 Simulated cattle movement networks

We used the estimated ERGMs to simulate synthetic cattle movement networks with similar features to the observed network data for each study region (Supplementary Table 2.3). For the purposes of validating the simulated networks, and for the scenario analysis defined below, we generated synthetic networks with the same number of herds as within the observed movement networks.

The ERGM models simulate the existence (and frequency) of edges (movements) between herds, but not the temporal dependence. In the absence of data on how rates of cattle movements change over time, we assumed that the network properties (estimated parameters of the ERGM) remain constant over time. We can use the fitted ERGM to simulate synthetic networks corresponding to a set of movements between herds (i.e. a set of n network Edges, $E = \{E_e\}$) within the same observation period from which the original network data was collected (t_{obs}). To explore transmission over longer periods for scenario analysis, we must simulate a table of animal

movements over the whole simulation time (T=50 years). While we can generate and concatenate repeated network fragments, the ERGM does not model the temporal order of movements which we must simulate separately. The empirical movement data has no clear temporal patterns with respect to the rates or ordering of movements, so we use a null model sampling the time ($t_{e,f}$ for each movement edge) uniformly within the range of the observation period. Finally, animal movements are often batched in groups of more than one animal. Thus, the number of animals ($B_{f,e}$) in a batch moving along each simulated edge e in fragment f is sampled from a negative binomial distribution of observed group sizes of movements for the given region. To avoid herds running out of animals, if 10 or less animals would remain in the source herd after removing the animals, the batch size is set to the next batch size where 10 animals remain in the herd after the scheduled movement. If there are less than 10 animals remaining in the herd, the batch size is set to 0.

So, in pseudocode, the set of animal movements E is the concatenation of a set of simulated network fragments $E_f = \{E_{ef}\}$, with e indexing the edges within fragment f and $H_{f,e}$is the herd size of the source herd associated with the edge.

for f in 1 to T/t_{obs}

$E_f \sim ERGM$

for e in $1{:}\,n$

$t_{e,f} \sim$ uniform$((365 * t_{obs} * f) - (365 * t_{obs} + 1), 365 * t_{obs} * f)$

$B_{f,e} \sim$ negbin$(O \ldots)$

End

End

Sequential network fragments corresponding to the same time horizon t_{obs} as the observed movement networks were simulated over 50 years using the ergm package. The steps taken to generate the synthetic movement networks are illustrated in Supplementary Figure 2.7. To compare the network structure of the generated networks to the empirical cattle movement networks and assure it remained stable over the simulated time period, networks were sampled from the 50-year simulation and assessed based on six network features: transitivity, edge density,

diameter, degree centrality, and betweenness centrality, and number of edges. Calculation and assessment of the network features was performed in R package igraph (59).

The network diameter (the length of the longest shortest path across the network (60) was consistently over-estimated in the simulated networks but the observed diameter remained within the simulated range for all three regions. In addition, the degree centrality (the number of edges connected to a node (60) was under-estimated in the simulated network for Gondar. For the other estimated network features, the observed network characteristics were within the range of the simulated movement networks (Supplementary Figure 2.8).

3 Simulation model for scenario analysis

3.1 Meta-population transmission model for bovine tuberculosis

A stochastic compartmental infectious disease model was developed with four possible epidemiological states (Supplementary Figure 2.9): susceptible (*S*), infected (*I*), vaccinated (*V*), and infected vaccinated (I_v) and eight events corresponding to birth, transmission and mortality (Supplementary Figure 2.9).

The deterministic skeleton of the model, which approximates the average rate of change in the number of animals in each compartment of a herd, is thus:

$$\frac{dS(t)}{dt} = (1-p)bN_0 - \lambda(t)S(t) - \mu S(t)$$

$$\frac{dI(t)}{dt} = \lambda(t)S(t) - \mu I(t)$$

$$\frac{dV(t)}{dt} = pbN_0 - \varepsilon_s\lambda(t)V(t) - \mu V(t)$$

$$\frac{dI_V(t)}{dt} = \varepsilon_s\lambda(t)V(t) - \mu I_V(t)$$

where p is the proportion of newborn calves that are vaccinated, b is the birth rate, N_0 is the population size at time $t = 0$ (corresponding to the present time), $\lambda(t)$ is the force of infection at time t, μ is the mortality rate, and ε_s is, as previously defined, the proportionate reduction in susceptibility for vaccinates.

The force of infection, $\lambda(t)$, is defined as

$$\lambda(t) = \frac{\beta(I(t) + \varepsilon_i I_V(t)}{N(t)}$$

where β denotes effective contacts, ε_i is the reduction in infectiousness for vaccinates and *N(t)* is the population size at time *t*.

Events and transitions between compartments (as described in Supplementary Table 2.4) and parameter descriptions and values are outlined in Supplementary Table 2.5. Each epidemiological compartment is further stratified by herd to form a meta-population model where transmission between infected and susceptible individuals only occurs within the same herd and herd-to-herd transmission only occurs through movements of animals between herds. Movements are pre-calculated using the method described in Section 2. During the simulation, the number of animals in a batch ($B_{f,e}$) to be moved between herds is modified to ensure the herd size ($H_{f,e}$) of the source herd does not fall below 10 animals:

if $H_{f,e} < 10$ then $B_{f,e} = 0$

if $H_{f,e} - B_{f,e} < 10$ then $B_{f,e} = H_{f,e} - 10$

The simulation model is implemented using the R package *SimInf* (39), which provides an epidemiological modelling framework for data-driven simulation of stochastic disease spread. Disease transmission within herds is modelled as a continuous-time Markov process and the simulated cattle movements and demographic processes, such as livestock births and deaths, are treated as scheduled (non-Markov) events (39).

The basic reproduction ratio (R_0) within each simulated herd is sampled from the empirical estimates obtained in Section 1. Thus, the value of R_0 is assumed to vary between herds, with the effective transmission rate β_i within each herd i in the meta-population calculated based on a sampled posterior estimate of R_0 as:

$$\beta_i = R_0(\mu + \rho)$$

where μ is the average mortality rate, and ρ the (average) movement rate of cattle for that herd.

3.2 Herd demography and initial infection status

The initial number of animals in a herd (N_0) was randomly sampled from recorded herd sizes in Ethiopia described in Section 1. The population size (N) of a herd was assumed to be constant and the birth rate (b) in a herd was balanced against the mortality rate (μ), and the average movement rate of cattle in the herd (ρ):

$$b = \mu + \rho$$

Herds were assumed to be either free from infection ($S_0 = N_0, I_0 = 0$) or endemically infected at the start of each simulation, with the frequency of affected herds specified by the observed herd prevalence of bTB in each study region. For bTB affected herds, the initial number of susceptible (S_0) and infected animals (I_0) in a herd was calculated from the sampled value of R_0 for that herd as:

$$S_0 = \frac{N_0}{R_0}$$
$$I_0 = N_0 - S_0$$

3.3 Control measures

The effectiveness of vaccination as a control measure for bTB was evaluated by comparing scenarios with no vaccination (proportion of newborn calves vaccinated $(p) = 0$) to those where

all newborn calves were vaccinated ($p = 1$)). Vaccinated animals have a reduced probability (ε_s) of acquiring infection and a reduced probability of transmission should they subsequently become infected (ε_i). For all scenarios, we assume that ε_s takes the value estimated from our natural transmission study. To account for the uncertainty in these estimates we sample the value of ε_s for each simulation from the posterior estimates obtained from the transmission trial (to give a direct vaccine efficacy VE_D with posterior range of 34-73%, median value of 58). Likewise, the reduction in infectiousness, ε_i is sampled from estimates to give an efficacy for an indirect vaccine efficacy of VE_I of 74%; 95% CrI: 46-89%.

To explore the sensitivity of our projections to this key variable we compare the estimated scenario to a range of indirect vaccine efficacies (reduction in infectiousness): 0%, 25% and 50%. The vaccination scenarios considered are summarized in Figure 2.3.

Any waning of vaccine protection over time was not considered and it was assumed that in any deployment animals would be annually revaccinated as required to maintain levels of protection.

3.3 Model simulation

For each scenario, outputs were simulated from 1,500 replicates.

Supplementary Table 2.1: Diagnostic thresholds. Diagnostic thresholds used to define positivity for skin (SIT, SCT, DST 10, in mm increase in thickness) and IGRA (DST 1, in optical density [OD] units) tests as used in this analysis.

Diagnostic Test	Format	Antigens	Measurement	Diagnostic Threshold
SIT	Skin	PPD-B	PPD-B	>= 4mm
CCT	Skin	PPD-B, PPD-A	PPD-B – PPD-A	> 4mm
DST 10	Skin	DIVA	DIVA	>= 2mm
DST 1	IGRA	DIVA	DIVA	>= 0.1 OD450 nm

Supplementary Table 2.2: Estimated vaccine efficacy for BCG in cattle by mode of action and method of imputation of infectious seeders in Phase II

Diagnostic Test	Imputation	Direct Efficacy (VE_D) (95% CrI)	Indirect Efficacy (VE_I) (95% CrI)	Total Efficacy (VE_T) (95% CrI)
DST 1 (IGRA)	**Default**	**58 (34-73)**	**74 (46-89)**	**89 (74-96)**
DST 1 (IGRA)	Maximum	57 (34-73)	77 (53-90)	90 (77-96)
DST 1 (IGRA)	Minimum	58 (34-73)	72 (42-88)	88 (71-96)
DST 10 (Skin)	**Default**	**46 (14-66)**	**67 (30-87)**	**82 (55-94)**
DST 10 (Skin)	Maximum	46 (14-66)	71 (39-88)	84 (61-94)
DST 10 (Skin)	Minimum	46 (14-66)	67 (30-87)	82 (55-94)

Supplementary Table 2.3: Variable estimates for the selected exponential random graph model of cattle movement networks in three study regions in Ethiopia.

Region	Variable	Estimate	SE	p-value
Gondar	Edges	-2.59	0.16	<0.001
	GW in-degree (fixed $\alpha\alpha$-decay = 0.75)	-2.82	0.36	0.001
Mekelle	Edges	-2.15	0.29	<0.001
	GW in-degree (fixed $\alpha\alpha$-decay = 0.75)	-2.30	0.56	<0.001
Hawassa	Edges	-2.68	0.27	<0.001
	GW in-degree (fixed $\alpha\alpha$-decay = 0.50)	-1.53	0.38	<0.001
	GW out-degree (fixed $\alpha\alpha$-decay = 0.75)	-1.20	0.42	0.004

GW = geometrically weighted; SE = standard error

Supplementary Table 2.4: Events and transitions between states of the stochastic transmission model.

Event	Effect	Probability per unit time
Birth (unvaccinated calf)	$S \rightarrow S+1$	(1-p)bN 0
Birth (vaccinated calf)	$V \rightarrow V+1$	pbN 0
Infection (unvaccinated animal)	$S \rightarrow I$	$\lambda(t)S(t)$
Infection (vaccinated animal)	$V \rightarrow I_V$	$\varepsilon s\ \lambda(t)V(t)$
Death	$S \rightarrow S-1$	$\mu S(t)$
	$V \rightarrow V-1$	$\mu V(t)$
	$I \rightarrow I-1$	$(\mu)I(t)$
	$I_V \rightarrow I_V-1$	$(\mu)I_V\ (t)$

Supplementary Table 2.5: Parameters and input values used in the transmission model. NA = not applicable

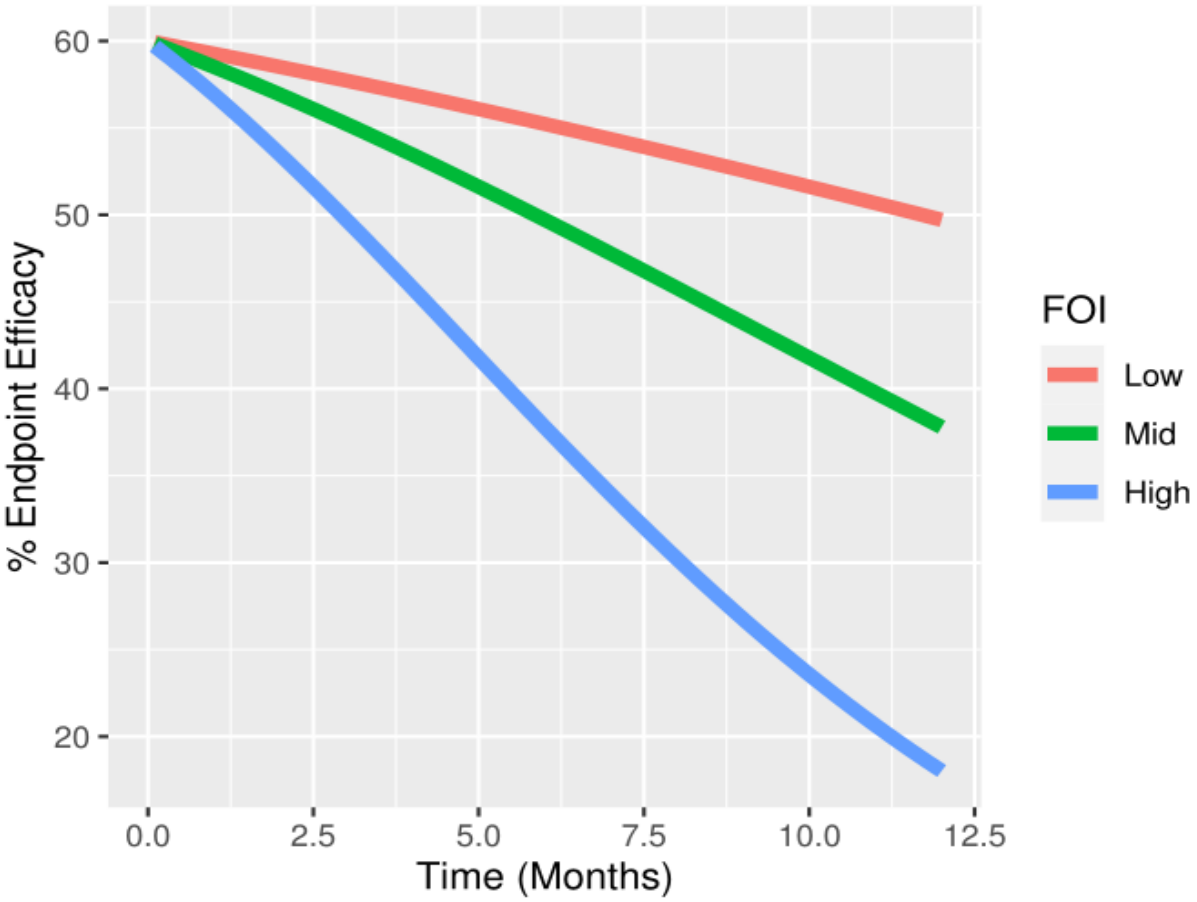
Description	Parameter	Value	Reference
Proportion newborn calves vaccinated	p	Two scenarios: 0, 1	NA
Susceptible animals in a herd at time t	S(t)		NA
Infected animals in a herd at time t	I(t)		NA
Vaccinated animals in a herd at time t	V(t)		NA
Infected vaccinated animals in a herd at time t	$I_V\ (t)$		NA
Herd size at time t = 0	N_0	Recorded herd sizes in Ethiopia	Described in Section 1
Herd size at time t	N(t)	S(t)+I(t)+V(t)+I V (t)	NA
Mortality rate	μ	Sampled from Normal distribution of estimated mortality rate per year in	Exponential rate estimated from age-structure of ETHICOBOTS herds

		Ethiopian dairy herds (mean = 0.27, standard deviation = 0.0049)	
Direct vaccine efficacy: reduction in infection rate of vaccinated individuals compared to unvaccinated individuals	εs	Sampled from posterior estimates of the direct vaccine efficacy (58%;95% CrI: 34-73%)	Natural transmission study
Indirect vaccine efficacy: reduction in infectiousness of vaccinated individuals who become infected compared to unvaccinated individuals	εi	Four scenarios: 0%, 25%, 50%, sampled from osterior estimates of the indirect vaccine efficacy (74%; 95% CrI: 46-89%)	Natural transmission study
Basic reproduction ratio	Ro	Sampled from posterior estimates of the basic reproduction ratio in infected dairy herds in Ethiopia and assigned to each herd	Described in Section 1
Movement rate	ρ	(animals moved out - animals moved in)/ N0	Simulated movements for each herd, herd size
Effective contacts	β	R0 *(μ+ρ)	R0 , mortality rate, movement rate for each herd
Birth rate	b	μ+ρ	Mortality rate and movement rate for each herd
Force of infection at time t	λ(t)	$\beta * (I(t)+(1-\varepsilon i) * Iv(t))/N(t)$	Effective contacts, infected animals, infected vaccinated animals, total number of animals in each herd, indirect vaccine efficacy

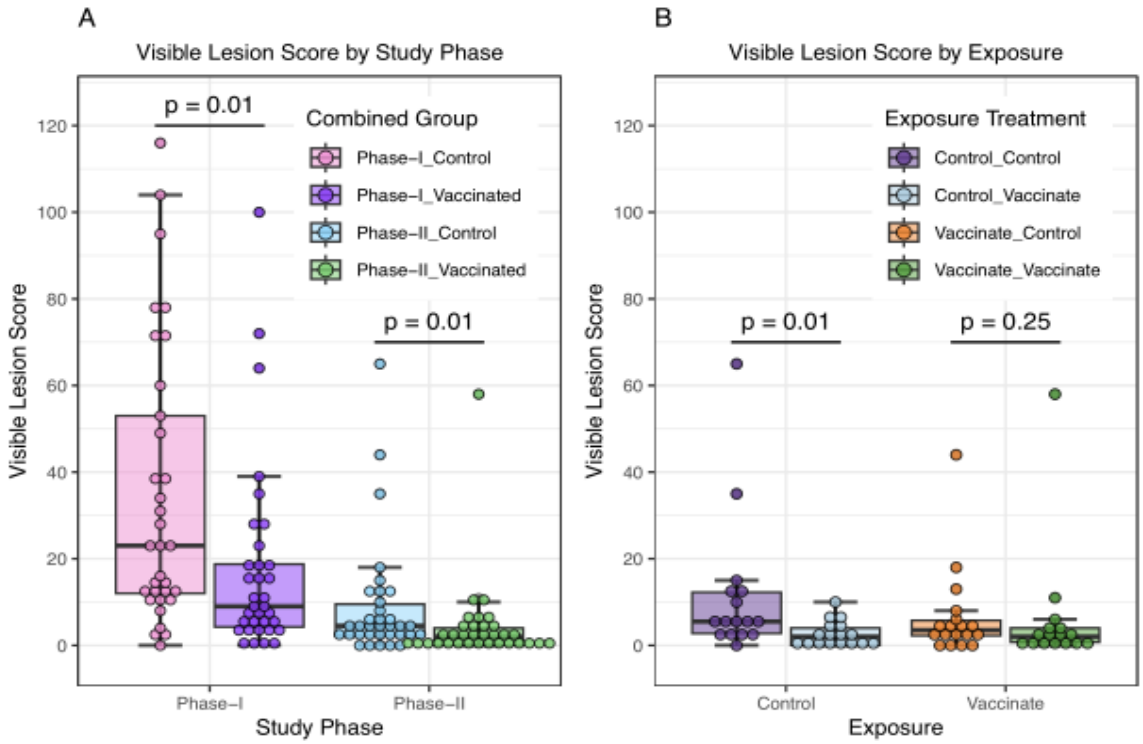
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Supplementary Table 2.6: Semi-quantitative systems of visible lesion scoring criteria

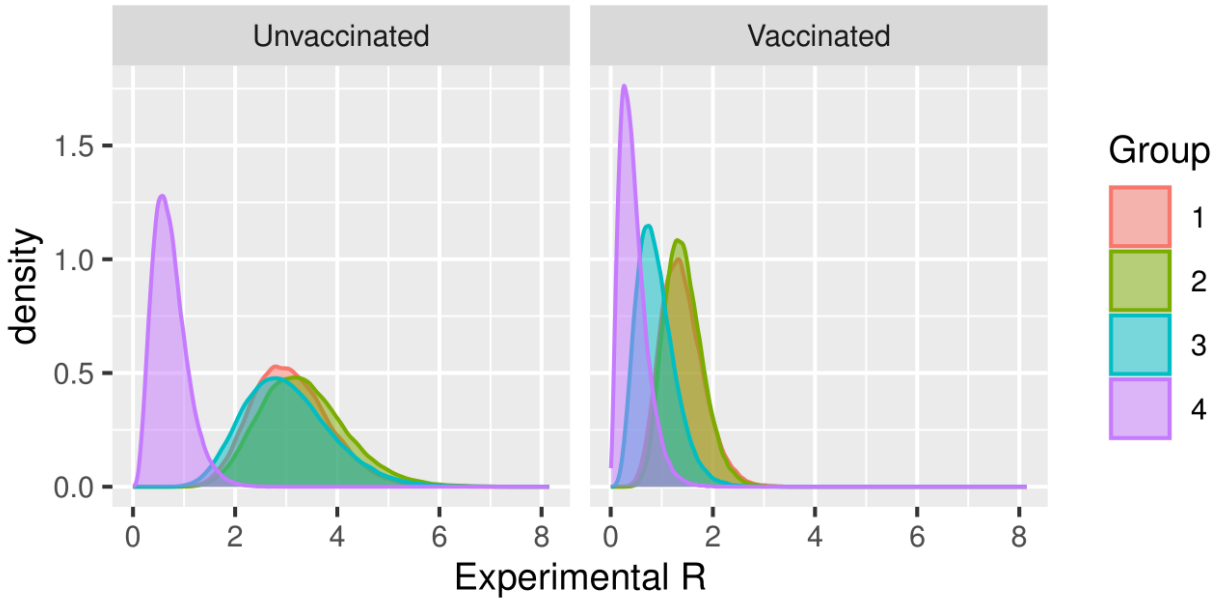
Organ	Scoring criteria	Score
Lung	No visible lesions	0
	A single small lesion, ≤10mm diameter	1
	2 to 5 small lesions, ≤10mm diameter	2
	Between 6 and 20 small lesions, ≤10mm diameter OR A single distinct lesion, >10mm diameter	3
	>20 small lesions, ≤10mm diameter OR >1 distinct lesion, >10mm diameter	4
	Large, affected area of lung containing lesions	5
Lymph nodes	No visible lesions	0
	A single small lesion, 1-2mm diameter	1
	2 to 5 small lesions, 1-2mm diameter	2
	Between 6 and 20 small lesions, 1-2mm diameter OR A single area at least 5mm by 5mm	3
	>20 small lesions, 1-2mm diameter OR >3 areas of at least 5mm by 5mm	4
	Consolidation of over 50% of lymph node	5
Other organs	No visible lesions	0
	A single small lesion, ≤10mm diameter	1
	2 to 5 small lesions, ≤10mm diameter	2
	Between 6 and 20 small lesions, ≤10mm diameter OR A single distinct lesion, >10mm diameter	3
	>20 small lesions, ≤10mm diameter OR >1 distinct lesion, >10mm diameter	4
	Large affected area of lung containing lesions	5



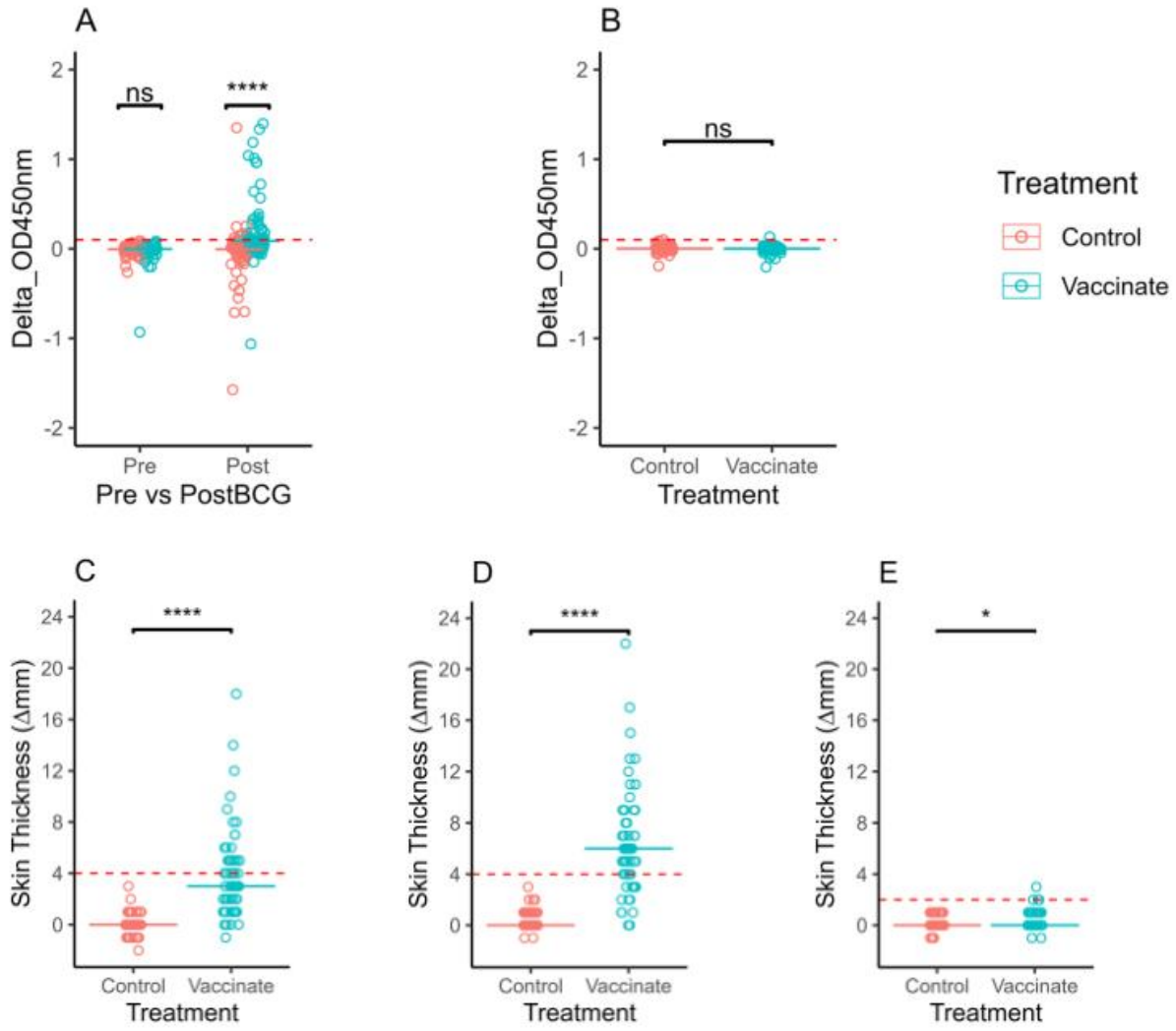
Supplementary Figure 2.1: The relationship between vaccine efficacy and endpoint estimates based on relative risk ratios within a modelled natural transmission study. The estimated endpoint vaccine efficacy is plotted as a function of time of exposure for three transmission levels corresponding to experiments with a low, mid or high force of infection (β = 0.12, 0.24, 0.48). The cumulative impact of exposure from the seeder animals leads to the endpoint estimate of efficacy of vaccination decreasing with the duration of the study, with this rate of decrease increasing with the rate of transmission within the population.



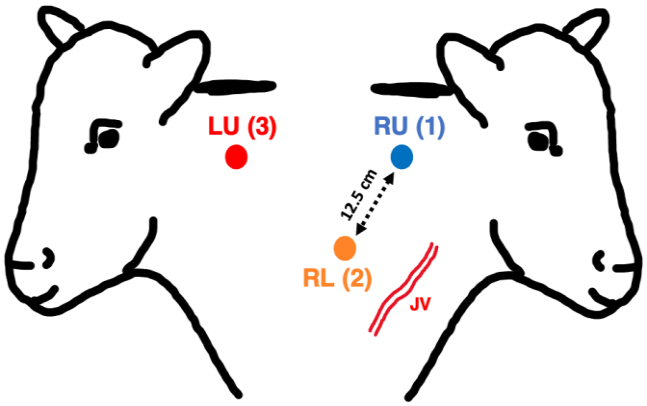
Supplementary Figure 2.2: Total Visible Lesion Scores as a Measure of Severity of bTB Pathology Across Two Study Phases and Treatment groups. In Phase I, sentinel calves were exposed to CCT and IGRA test positive cows and in Phase II, they were exposed to vaccinated (Vaccinate) or untreated (Control) sentinels from Phase I. Box plots illustrate the distribution of total visible lesion scores at the termination of Phase II. Boxes depict the interquartile range (IQR), with the median indicated by a horizontal line. Whiskers extend to the minimum and maximum scores within 1.5 times the IQR. Different colors distinguish between different treatment and exposure groups. The Mann-Whitney U test reveals statistically significant differences: between vaccinated and unvaccinated control sentinels exposed to CCT test-positive cows in Phase I (W = 780.00, p = 0.006; r= 0.39, 95% CI: 0.13-0.60), between pooled unvaccinated control and vaccinated sentinel groups in Phase II (W = 781.50, p = 0.005; r = 0.39, 95% CI: 0.14-0.60) and between treatment groups exposed to Control in Phase II (W = 212.50, p = 0.006; r = 0.56, 95% CI: 0.23-0.78). No significant difference was identified between treatment groups exposed to Vaccinates in Phase II (W = 177.50, p = 0.249; r = 0.23, 95% CI: -0.16, 0.56). 'r' denotes the rank biserial correlation, and CI represents 95% confidence intervals. This analysis suggests that BCG vaccination influences bTB pathology severity, as indicated by Total Visible Lesion Scores, in comparison to control groups across the two study phases.



Supplementary Figure 2.3: The estimated transmission rate between the different combinations of vaccinated and unvaccinated sentinel and seeder animals expressed as the experimental reproduction ratio R (the expected number of new infections in a fully susceptible population) of the given sentinel type (vaccinated or unvaccinated) when exposed for a duration of one year estimated independently from each experimental group (1-4, red, green, blue, purple respectively).

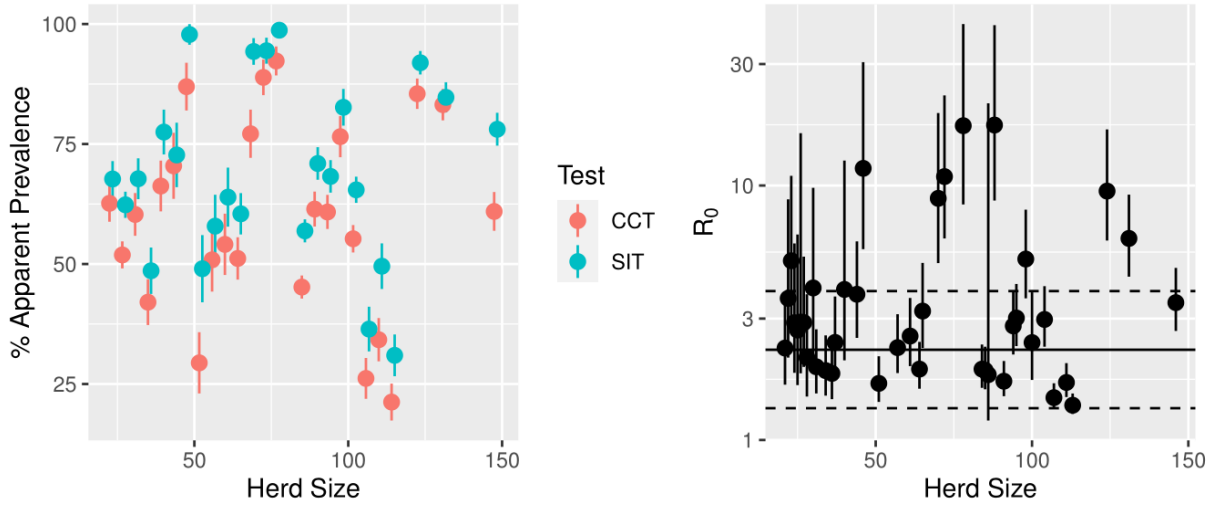


Supplementary Figure 2.4: Combined Phase-I and Phase-II. Test status of experimental sentinel animals, 67 controls and 67 BCG vaccinates, (A) prior to BCG vaccination (pre-BCG) and vaccine take confirmation at 8th week post-vaccination (post-BCG, just prior to seeder introduction) as indicated by standard IGRA PPD(B-A). At 8th week post-BCG, animals were also tested using (B) DST in IGRA (C) CCT (D) SIT and (E) DST skin test. Red dotted lines indicate respective cut-offs: 0.1 in A and B for standard BOVIGAM, 4 mm for CCT and SIT, and at 2 mm for DST skin test. The bars represent the median response to all respective antigens. Skin test data is reported as difference in skin thickness (Δ mm) between 72- and 0-hour readings. For IGRA, the background-corrected optical density (OD) values at 450 nm are shown. The statistical difference between the responses was determined using Mann-Whitney U test (****P < 0.0001).



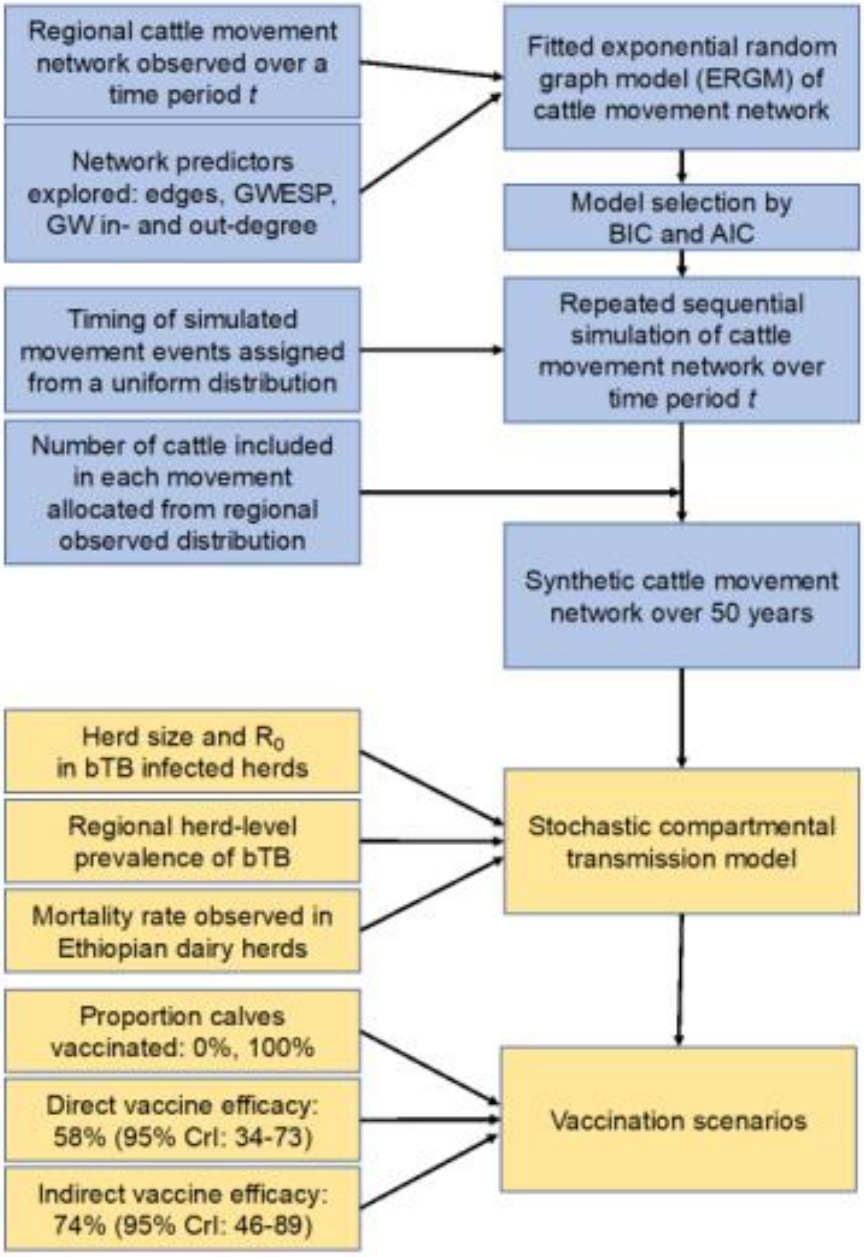
Randomization of injection sites in Latin Square formation			
Animal	Antigens		
	PPD-B	PPD-A	DST10
#1	RU (1)	RL (2)	LU (3)
#2	RL (2)	LU (3	RU (1)
#3	LU (3	RU (1)	RL (2)
#4	RU (1)	RL (2)	LU (3
.	.	.	.
.	.	.	.
#34	RU (1)	RL (2)	LU (3

Supplementary Figure 2.5: Intradermal injection sites for PPDB, PPDA and DST at right and left mid-cervical regions of the experimental calves. The site of injections on right side of the neck were marked 1, 2, and on the left side marked 3 (RU, right upper, RL, right lower, LU, Left upper mid cervical injection sites, JV, Jugular vein). Antigen injection sites on the right side were shaved at least 12.5 cm apart. The three antigens were injected in a Latin Square formation as shown in the table in the right side.

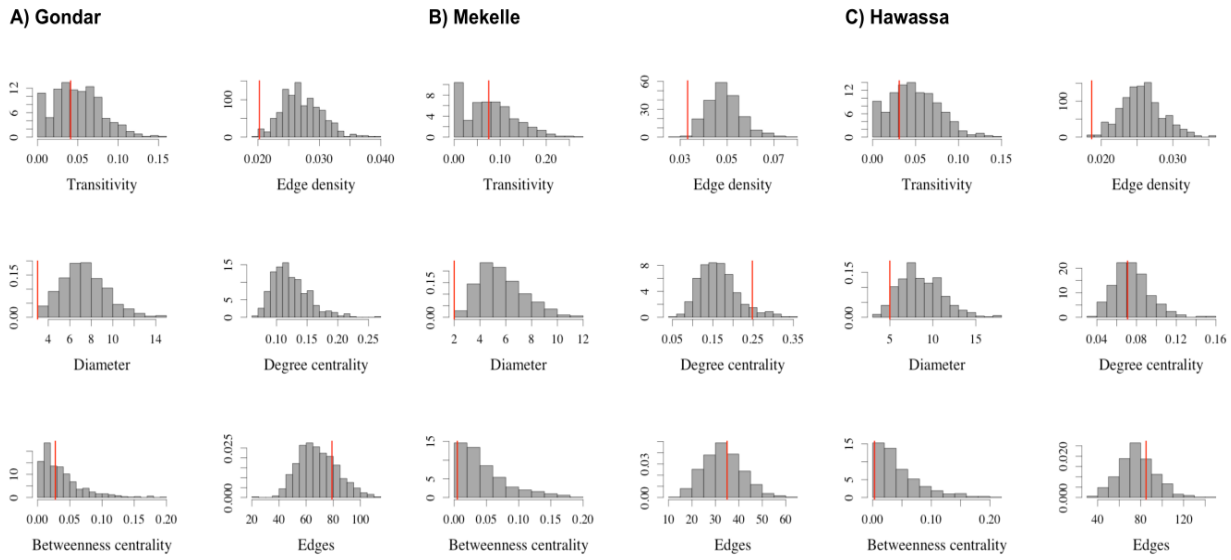


Supplementary Figure 2.6: (Left) Apparent prevalence plotted against herd size with respect to the SIT and CCT formats of the tuberculin skin test. Point values (mean) are presented along with naïve 95% (binomial) confidence intervals to reflect the uncertainty. (Right) Estimated values of the (within-herd) basic reproduction ratio R_0 for the same herds plotted against herd size, demonstrating no evidence of density (herd size) dependence and a high degree of heterogeneity in transmission between herds of all sizes. The critical value of R_0 (calculated as $1/(1-V_D)$, and

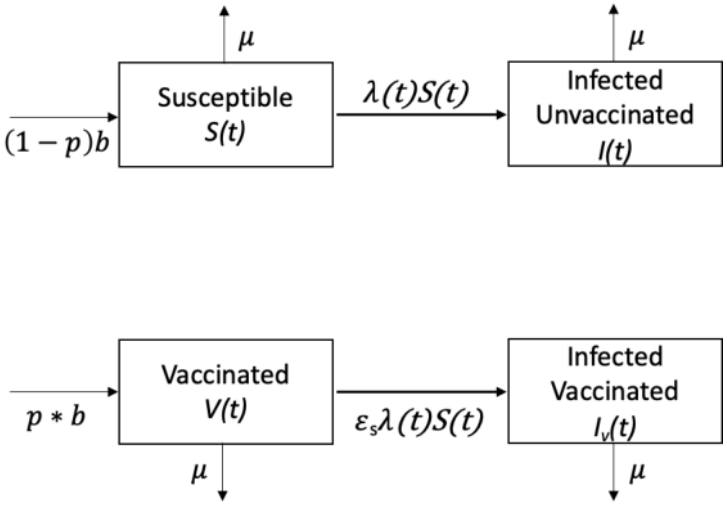
assuming 100% vaccine coverage) is plotted as a horizontal black line (dashed lines indicated 95% credible intervals) separating herds that would be below or above the elimination threshold for the estimated (direct) vaccine efficacy from our natural transmission study.



Supplementary Figure 2.7: Flow diagram of the components of the between-herd (in blue) and within-herd (in yellow) transmission model of bovine tuberculosis in Ethiopian dairy herds for each of the three study regions. GWESP = geometrically weighted edgewise shared partnership; GW = geometrically weighted.



Supplementary Figure 2.8: Comparison of the network characteristics of observed cattle movements (in red) and the distribution of network characteristics of simulated cattle movements (in grey) for three regions in Ethiopia with emerging dairy markets.



Supplementary Figure 2.9: Illustration of the compartmental transmission model consisting of four states: susceptible (S), infected (I), vaccinated (V) and infected vaccinated (I_V) cattle. Transition from S to I depends on the force of infection in the herd and the reduction in infectiousness in any vaccinated infected cattle (indirect vaccine efficacy). Transition from V to I_V depends on the force of infection, reduction in susceptibility to infection in vaccinated animals (direct vaccine efficacy), and the reduction of infectiousness in infected vaccinated animals (indirect vaccine efficacy).

Supplementary Table 3.1: 2 × 2 contingency table used to define measures of diagnostic test performance

Antemortem Test	Postmortem test		Row Totals
	Positive	Negative	
Positive	a (TP)	b (FP)	a + b
Negative	c (FN)	d (TN)	c + d
Column Totals	a + c	b + d	(a + b + c + d) = N

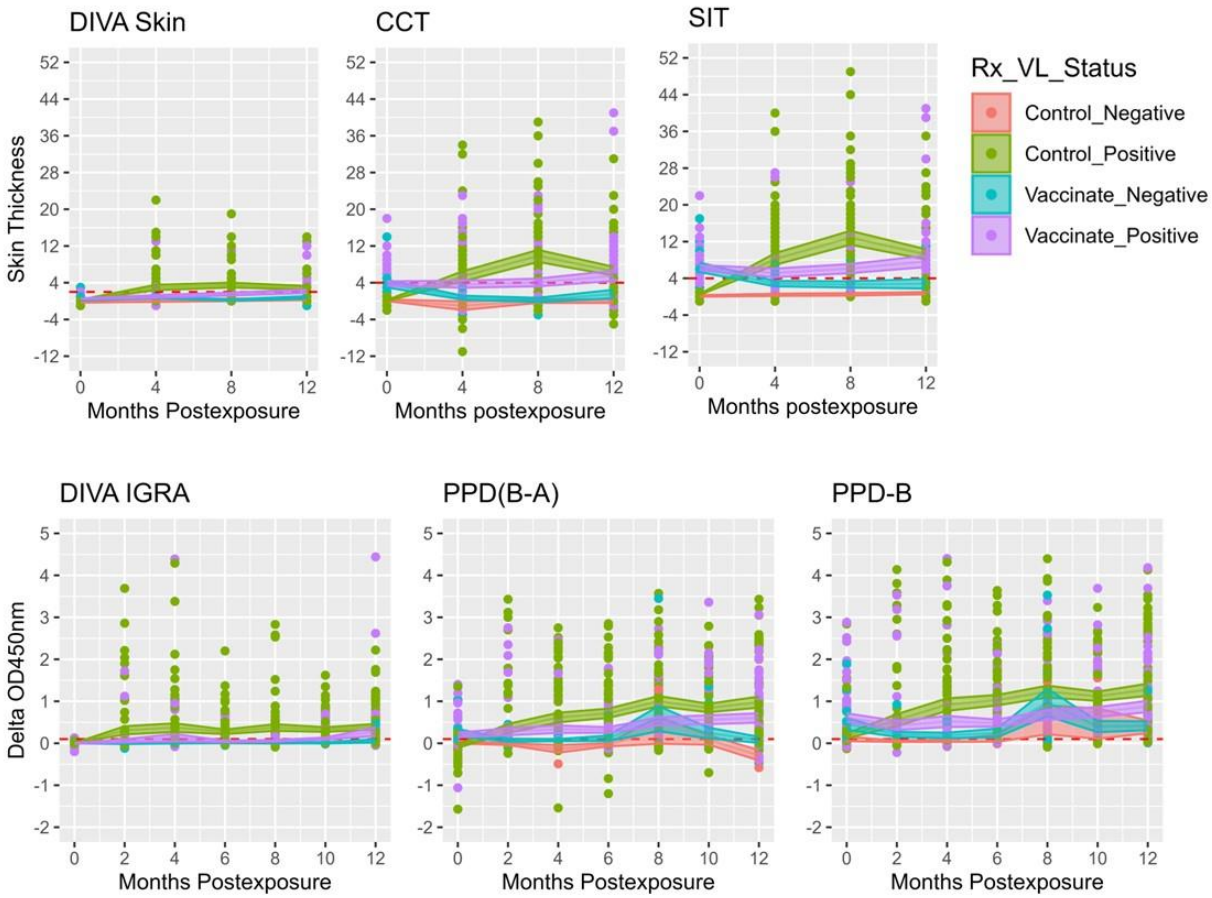
TP = True positive, FN = False negative, FP = False positive, TN = True negative

Supplementary Table 3.2: Relative sensitivity and specificity of IGRA and skin tests with respect to visible lesion (VL), culture or PCR positivity in BCG vaccinated and unvaccinated control animals at the 12th month postexposure

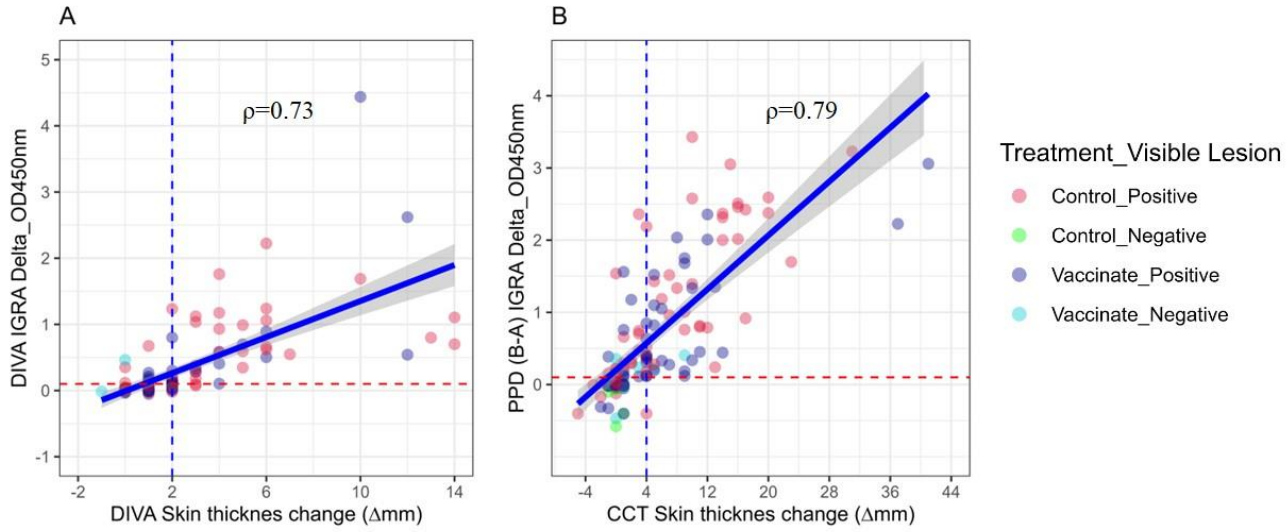
Treatment	Test	Antigen	Cutoff	Status	*M. bovis* Culture PCR or Visible Lesions		Sensitivity (95%CI)	LR+	LR-	Accuracy
					Positive	Negative				
BCG vaccinated	Skin	DIVA	≥ 2 mm	Positive	24	1	42 (30, 55)	2.11	0.72	0.45
				Negative	33	4				
		CCT	> 4 mm	Positive	23	0	40 (29, 53)	-	0.60	0.45
				Negative	34	5				
		SIT	≥ 4 mm	Positive	37	2	65 (52, 76)	0.4	0.59	0.65
				Negative	20	3				
	IGRA	DIVA	≥ 0.1	Positive	21	1	37 (26, 50)	1.84	0.79	0.40
				Negative	36	4				
		PPD (B-A)	≥ 0.1	Positive	39	1	68 (56, 79)	3.42	0.40	0.69
				Negative	18	4				
		PPD-B	≥ 0.1	Positive	50	5	88 (77, 94)	0.88	-	0.81
				Negative	7	0				
Unvaccinated controls	Skin	DIVA	≥ 2 mm	Positive	32	0	52 (49, 64)	-	0.48	0.54
				Negative	30	3				
		CCT	> 4 mm	Positive	28	0	45 (33, 57)	-	0.55	0.48
				Negative	34	3				
		SIT	≥ 4 mm	Positive	42	0	68 (55, 78)	-	0.32	0.69
				Negative	20	3				
	IGRA	DIVA	≥ 0.1	Positive	29	0	47 (35, 59)	-	0.53	0.49
				Negative	33	3				
		PPD (B-A)	≥ 0.1	Positive	48	0	77 (66, 86)	-	0.23	0.78
				Negative	14	3				
		PPD-B	≥ 0.1	Positive	56	3	90 (80, 95)	0.90	-	0.86
				Negative	6	0				

Supplementary Table 3.3: Summary of concordant test outcomes between antemortem bTB diagnostic tests to supplement the data presented in the Venn diagram in Figure 5

DST skin, DST IGRA and CCT Tests						**Concordance**				
	PM or PCR+		**PM or PCR -**		**Total**	**All three tests**		**Paired tests**		
Treatment	**Test+**	**Test-**	**Test+**	**Test-**	**Tested**	**Sum**	**Percent**	**DST IGRA & DST skin**	**DST IGRA & CCT skin**	**CCT & DST skin**
Controls	21	23	0	3	65	47	47/65 (72%)	56/65 (86%)	52/65 (80%)	56/65 (86%)
Vaccinates	12	26	1 +/- 1	3	62	41	41/62 (66%)	49/62(79%)	46/62 (74%)	51/62 (82%)
Total	33	49	1 +/- 1	6	127	88	88/127 (69%)	105/127 (83%)	98/127 (77%)	110/127 (87%)
SIT, IGRA PPD (B-A) or PPD-B Tests						All three tests		Paired tests		
Treatment	Test+	Test-	Test+	Test-	Tested	Sum	Percent	SIT & IGRA PPD (B-A)	SIT & IGRA PPD-B	PPD (B-A) & PPD-B IGRAs
Controls	41	6	0 or 3	0	65	47	47/65 (72%)	57/65 (88%)	48/65 (74%)	54/65 (83%)
Vaccinates	35	7	1, 2 or 5	0	62	42	42/62 (68%)	53/62 (85%)	46/62 (74%)	47/62 (76%)
Total	76	13	-	0	127	89	89/127 (70%)	110/127 (87%)	94/127 (74%)	101/127 (80%)

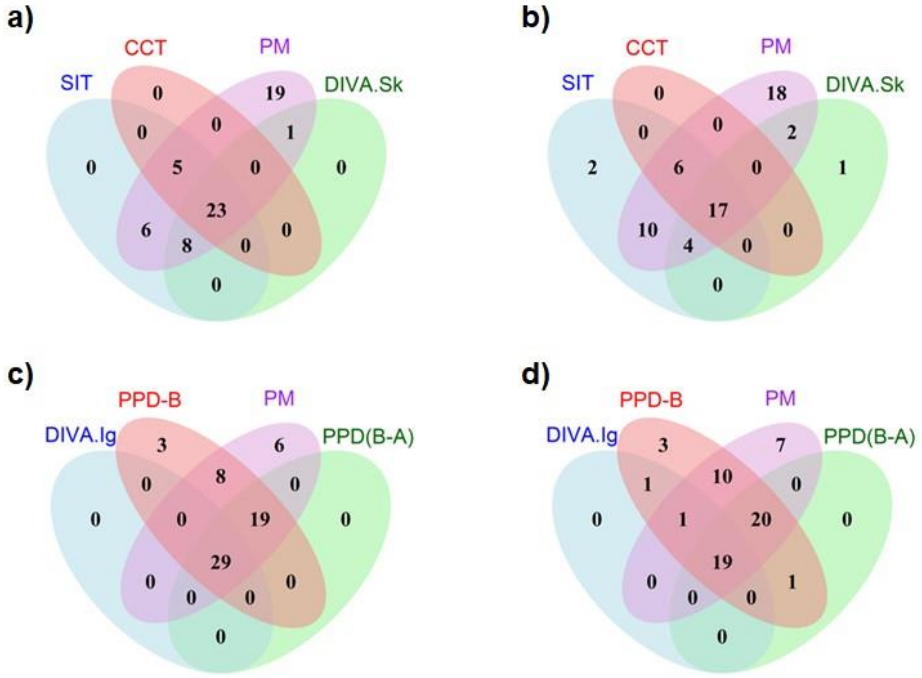


Supplementary Figure 3.1: Reduced skin test response at the fourth repeated test is suggestive of desensitization in the skin test (upper 3 panels) and not seen in the IGRA test results (lower 3 panels). The ribbon plots display the mean across all animals for each time point (line) and one standard error around the mean (shaded interval). Rx_VL_Status = Combination of treatment and visible lesion status of animals



Supplementary Figure 3.2: Correlation between OD450 nm levels of IFN-γ and skin test responses A) Between the DIVA skin and the DIVA IGRA tests. b) Between the CCT skin and bovine minus avian tuberculin IFN-γ tests. The solid-colored circles represent individual animals with treatment and visible lesions status combinations. The dashed horizontal red lines at 0.1 are the cut-offs used for tuberculin and DIVA IFN-γ responses. The dashed vertical blue lines at 2 and 4 are the cut-offs used for the DIVA and CCT skin thickness test responses, respectively.

Concordance of the skin and IGRA tests on visible lesions, culture or PCR test status

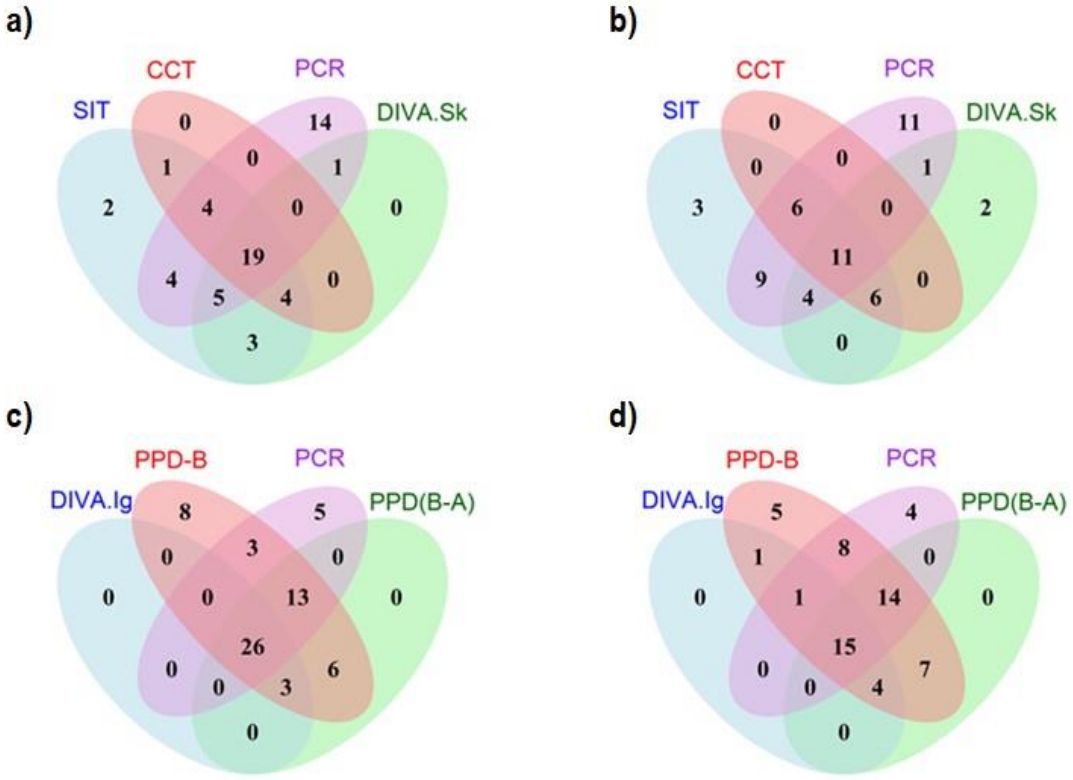


Supplementary Figure 3.3: Venn diagram comparing the number of cattle positives for visible lesions, culture or *M. bovis* PCR (PM) with the number of (**a**) unvaccinated control and (**b**) vaccinated cattle tested positive on the skin test formats: DIVA skin (DIVA.Sk), CCT or SIT (**c**) unvaccinated and (**d**) vaccinated cattle tested positive on the IGRA test format: DIVA IGRA (DIVA.Ig), PPD (B-A) or PPD-B. All numbers within the purple circle represent animals that tested positive in the postmortem test (62 unvaccinated and 57 BCG vaccinated). All numbers outside the purple circle represent animals that tested negative in the postmortem test (3 unvaccinated controls and 5 BCG vaccinated).

Supplementary Table 3.4: Supplementary to Figure 3.4

DIVA, CCT and SIT Skin Tests								**Concordance**		
	PM, culture or PCR+		**PM, culture or PCR-**		**Total**	**All three skin tests**		**Paired tests**		
Treatment	**Test+**	**Test-**	**Test+**	**Test-**	**Tested**	**Sum**	**Percent**	**DIVA & CCT**	**DIVA & SIT**	**CCT & SIT**
Controls	23	19	0	3	65	45	45/65 (69%)	56/65 (86%)	50/65 (77%)	48/65 (74%)
Vaccinates	17	18	3	2	62	37	37/62 (60%)	51/62 (82%)	39/62 (63%)	44/62 (71%)
Total	**40**	**37**	**3**	**5**	**127**	**82**	**82/127 (65%)**	**107/127 (84%)**	**89/127 (70%)**	**92/127 (72%)**
DIVA, PPD (B-A) & PPD-B IGRA Tests						**All three skin tests**		**Paired tests**		
Treatment	**Test+**	**Test-**	**Test+**	**Test-**	**Tested**	**Sum**	**Percent**	**DIVA & PPD (B-A)**	**DIVA & PPD-B**	**PPD (B-A) & PPD-B**
Controls	29	6	3	0	65	35	35/65 (54%)	46/65 (71%)	35/65 (54%)	54/65 (83%)
Vaccinates	19	7	4	1	62	27	27/62 (44%)	39/62 (63%)	28/62 (45%)	46/62 (74%)
Total	**48**	**13**	**7**	**1**	**127**	**62**	**62/127 (49%)**	**85/127 (67%)**	**63/127 (50%)**	**100/127 (79%)**

Concordance of the skin and IGRA tests on *M. bovis* PCR test status alone



Supplementary Figure 3.4: Venn diagram comparing the number of cattle positives for *M. bovis* culture PCR (PCR) with the number of (a) unvaccinated control and (b) vaccinated cattle tested positive on the skin test formats: DIVA skin (DIVA.Sk), CCT or SIT (c) unvaccinated and (d) vaccinated cattle tested positive on the IGRA test format: DIVA IGRA (DIVA.Ig), PPD (B-A) or PPD-B. All numbers within the purple circle represent animals that tested positive in *M. bovis* culture PCR (47 unvaccinated and 42 BCG vaccinated). All numbers outside the purple circle represent animals that were *M. bovis* culture PCR negative Note: The sum of *M. bovis* culture PCR negative animals outside the purple could be less than the maximum (18 unvaccinated controls and 20 BCG vaccinated) as they will be represented by 0 unless they are positive to the respective tests.

Supplementary Table 3.5: Supplementary to Supplementary Figure 3.4

DIVA, CCT and SIT Skin Tests						**Concordance**				
	PCR+		**PCR-**		**Total**	**All three skin tests**		**Paired tests**		
Treatment	**Test+**	**Test-**	**Test+**	**Test-**	**Tested**	**Sum**	**Percent**	**DIVA &CCT**	**DIVA & SIT**	**CCT & SIT**
Controls	19	14	10	8	65	41	41/65 (63%)	40/65 (62%)	37/65 (57%)	43/65 (66%)
Vaccinates	11	11	11	9	62	31	31/62 (50%)	34/62 (55%)	28/62 (45%)	37/62 (60%)
Total	**30**	**25**	**21**	**17**	**127**	**72**	**72/127 (57%)**	**74/127 (58%)**	**65/127(51%)**	**80/127(63%)**
DIVA, PPD (B-A) & PPD-B IGRA Tests						**All three tests**		**Paired tests**		
Treatment	**Test+**	**Test-**	**Test+**	**Test-**	**Tested**	**Sum**	**Percent**	**DIVA & PPD (B-A)**	**DIVA & PPD-B**	**PPD (B-A) & PPD-B**
Controls	26	5	17	1	65	32	32/65 (49%)	45/65 (69%)	34/65 (52%)	44/65 (68%)
Vaccinates	15	4	17	3	62	22	22/62 (35%)	36/62 (58%)	26/62 (42%)	33/62 (53%)
Total	**41**	**9**	**34**	**4**	**127**	**54**	**54/127 (43%)**	**81/127 (64%)**	**60/127 (47%)**	**77/127(61%)**

Appendix 3.1: Supplementary methods: Sample size determination

Our report represents a secondary analysis of data collected from a study where the primary objective was to estimate the efficacy of BCG vaccination to reduce transmission of bTB. For this primary objective the experimental reproduction ratio, defined as the expected number of new infections generated by a single infected animal over the course of the experiment, is the key design parameter. Given the chronic nature of bTB infection, R can be tuned by increasing (or decreasing) the duration of the experiment and thus the period of exposure between infectious and susceptible animals.

From previous studies we estimated that an in-contact period of 1 year would provide a long enough exposure to ensure an experimental R > 1 and produce sufficient infected seeder animals from Phase I to progress to Phase II (see https://doi.org/10.7554/eLife.27694 for full details). A conservative estimate of the sample size necessary to estimate a given total vaccine efficacy can be obtained using a final size method (https://doi.org/10.1017/S095026880600673X) for the total number of transmission events observed in the vaccinated and control groups. Considering each pair of susceptible and infected animals independently the probability of infection will approximately be:

$p_1 = \left(\frac{R_1}{R_1+2}\right)$ and $p_2 = \left(\frac{R_2}{R_2+2}\right)$

in the control and vaccinated groups respectively. R_1 and $R_2$are the experimental reproduction numbers in the control and vaccinated groups and $R_2 = \varepsilon_T R_1$ where the total vaccine efficacy to reduce transmission is $1 - \varepsilon_T$. The sample size can then be calculated using Fisher's exact test. When n is large (np > 0.5; n (1-p) > 5) the binomial distributions can be approximated by normal distributions leading to the following expression for n:

$$n = \big(p1(1-p) + p2\,(1-p2)\big)\left(\frac{Z_\propto + Z_\beta}{p_1 - p_2}\right)^2$$

Since we have two biologically replicated groups thus:

$$n = 2\big(p1(1-p) + p2\,(1-p2)\big)\left(\frac{Z_\propto + Z_\beta}{p_1 - p_2}\right)^2$$

Where:

- ✓ $Z_\propto \; and \; Z_\beta$ are the critical values of the standard Normal distribution for the two types of error, e.g. $Z_\propto$ = 1.96 for a 95% confidence level and Z_β = 0.842 (power 80%)

This method estimates that a group size of 52 animals (104 sentinel animals in total split between vaccinated and control groups) will provide 80% power to estimate an effect size (total vaccine efficacy) of 75% at the 95% significance level.

We aimed for a higher number (72 per group) than the calculated sample size required (72 per group) to account for potential losses and ensure robustness of the results. But when COVID-19 shutdown was declared, we were forced to start the experiment with the number of animals we already recruited that met the inclusion criteria, i.e. the 67 calves

Supplementary Table 4.1: Reported Prevalences of Tuberculous Like Lesions Based on Detailed Meat Inspections Conducted at Different Years and Slaughterhouses in Ethiopia.

Reference	Year	Study Method		Sample Size	TBL Prev	Abattoir's Location	Part of Ethiopia
(Ameni and Wudie, 2003)	2003	Abattoir	DMI	1125	5.2	Adama	Central Ethiopia
(Teklu *et al.*, 2004)	2004	Abattoir	DMI	751	4.5	Hossana	South Ethiopia
(Asseged *et al.*, 2004)	2004	Abattoir	DMI	1350	1.5	Addis Ababa	Central Ethiopia
(Yacob *et al.*, 2008)	2008	Abattoir	DMI	638	0.9	Bahirdar	N. West Ethiopia
(Biffa *et al.*, 2010)	2009	Abattoir	DMI	3322	10.1	Multiple	Many (Ethiopia)
(Berg *et al.*, 2009)	2009	Abattoir	DMI	32779	4.6	Multiple	Many (Ethiopia)
(Regassa *et al.*, 2010)	2010	Abattoir	DMI	1023	1.1	Hawassa	South Ethiopia
(Ameni *et al.*, 2010)	2010	Abattoir	DMI	1138	5.0	Kombolcha	N. Eastern Ethiopia
(Bekele and Belay, 2011)	2011	Abattoir	DMI	780	2.7	Jimma	S. West Ethiopia
(Tigre W., 2012)	2012	Abattoir	DMI	1102	5.4	Jimma	S. West Ethiopia
(Ewnetu *et al.*, 2012)	2012	Abattoir	DMI	720	5.8	Akaki	Central Ethiopia
(Mekibeb *et al.*, 2013)	2013	Abattoir	DMI	500	5.0	Addis Ababa	Central Ethiopia
(Zeru *et al.*, 2013)	2013	Abattoir	DMI	768	6.4	Mekelle	North Ethiopia
(Aylate *et al.*, 2012)	2013	Abattoir	DMI	1029	6.1	Woldiya	North Ethiopia
(Ayana *et al.*, 2013)	2013	Abattoir	DMI	487	9.3	Bako (TST+)	West Ethiopia
(Romha *et al.*, 2013)	2013	Abattoir	DMI	582	5.8	West Tigray	North Ethiopia
(Terefe, 2014)	2014	Abattoir	DMI	486	6.8	Adama	Central Ethiopia
(Woyessa *et al.*, 2014)	2014	Abattoir	DMI	1183	5.7	Nekemte	West Ethiopia
(Gebrezgabiher, 2014)	2014	Abattoir	DMI	768	2.6	Dilla	South Ethiopia
(J. Alemu *et al.*, 2016)	2016	Abattoir	DMI	500	13.2	Gambella	West Ethiopia
(B. Alemu *et al.*, 2016)	2016	Abattoir	DMI	500	10.6	Adama	Central Ethiopia
(Biru *et al.*, 2014)	2017	Abattoir	DMI	1107	3.5	Sululta	Central Ethiopia
(Tekle *et al.*, 2017)	2017	Abattoir	DMI	753	5.8	Hawassa	South Ethiopia
(Tintagu Gizaw, 2017)	2017	Abattoir	DMI	500	8.6	Gondar	N. West Ethiopia
(Woldemariyam *et al.*, 2021)	2021	Abattoir	DMI	384	4.7	Debre Birhan	Central Ethiopia
(Belete *et al.*, 2021)	2022	Abattoir	DMI	497	9.1	Gondar	N. West Ethiopia
(Ejo *et al.*, 2021)	2021	Abattoir	DMI	1458	4.3	Gondar	N. West Ethiopia
(Gemeda *et al.*, 2023)	2023	Abattoir	DMI	1020	7.8	Nekemte	West Ethiopia

Supplementary Table 4.2: The primer/probe sets used in this study for MTBC group and species-level identification with m-qPCR

Assay name	Target gene	Target organism	Primer/probe name	Sequence	Tm	Fragment length (bp)	Dye
IS1081	IS1081 (Rv1199c)	All MTBC	IS1081-F	GGCTGCTCTCGACGTTCATC	67.4	100	FAM
			IS1081-R	CGCTGATTGGACCGCTCAT	67.7		
			IS1081-P	CTGAAGCCGACGCCCTGTGC	71.7		
MTCAni	yrbE3A (Rv1964)	*M bovis, M orygis, M caprae, M tuberculosis* lineage 6	MTCAni-F	GGTTTCTCTTCAACGTCTTGCT	59.1	96	JOE
			MTCAni-R	CCGTCCCACGGCTTTGG	57.8		
			MTCAni-P	GGCTGTGCGATCTTCACCGTGAA	65.6		
MTCHum5433	mce3D (Rv1969)	*M tuberculosis*	MTCHum5433-F	CTTCGACAACAGCAACGGTG	60	77	TAMRA
			MTCHum5433-R	TCTTGACGATCTTGCCCACC	60		
			MTCHum5433-P	TGACGACGTGCTCATTCGG	60.4		
Morg	rskA (Rv0444c)	*M orygis*	Mory-F	ATTGTCGCGCCGAGACTG	60.5	88	FAM
			Mory-R	GTACCATCTTGGCCGAGCTG	60.8		
			Mory-P	CGTCCTCGGCTGACCC	59		
Mbov	RD4 (Rv1510)	*M bovis*	Mbov-F	AGCCGTAGTCGTGCAGAA	58.6	128	TAMRA
			Mbov-R	CCCGTAGCGTTACTGAGAAATTG	59.7		
			Mbov-P	CAACACTCTTGGAGTGGCCTACAACG	65.7		
McapRT	lepA	*M caprae*	McapRT-F	ACCGTGCGGATCTTG	52.9	153	JOE
			McapRT-R	CATGGAGATCACCCGT	52		
			McapRT-P	TATCGGGTACACAAAGACGA	56		

CURRICULUM VITAE

Biodata

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- PhD Candidate - Addis Ababa University (2018 – Present)
- MVSc in Veterinary Surgery and Radiology – Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India, 2006-2008 GC
- DVM – Addis Ababa University, Ethiopia, 1995-2001

Work Experience:

- **2014 - current:** Addis Ababa University (AAU), College of Veterinary Medicine and Agriculture (CVMA), Veterinary Surgeon and Associate Professor, teaching different veterinary surgery and medicine courses and doing research. Additionally, involved in administrative tasks and served as the Head of Veterinary Teaching Hospital (VTH) from 2014 to 2018 G.C.

- **2003 - 2014:** Jimma University, College of Agriculture and Veterinary Medicine (2001 to 2014 GC), Veterinary Surgeon and Associate Professor, teaching and research and worked as head of Continuous Education Department (CED) as well as Department of Biomedical Sciences at two different time points.
- **2001 – 2003:** Action Contre la Faim (Action against Hunger), Veterinarian and Assistant Animal Health Program Manager, Afar Region

Bovine tuberculosis related publications

1. **Fromsa**, A., Conlan, A.J.K., Srinivasan, S., Gumi, B., Bedada, W., Zeleke, M., Worku, D., Lakew, M., Tadesse, B., Bayissa, B., Sirak, A., Abdela, M.G., Mekonnen, G.A., Chibssa, T., Veerasami, M., Jones, G., Vordermeier, H.M., Juleff, N., Wood, J. L.N., Ameni, G. and Kapur, V. (2025). Comparative performance of tuberculin and defined-antigen cocktails for detecting bovine tuberculosis in BCG-vaccinated cattle in natural settings. Scientific Reports 15(1):4564. https://doi.org/10.1038/s41598-025-85389-1
2. **Fromsa, A**., Conlan, A.J.K., Srinivasan, S., Zeleke, M., Worku, D., Lakew, M., Abdela, M.G., Bahiru, G., Wood, J.L.N., Bakker, D., Gumi, B., Ameni, G., and Kapur, V., (2024). Bovine tuberculosis in Central Ethiopian slaughterhouses and the identification of causative mycobacteria by multiplex real-time PCR. BMC Microbiology, 24:394. https://doi.org/10.1186/s12866-024-03543-7
3. **Fromsa, A.,** Willgert, K., Srinivasan, S., Mekonnen, G., Bedada, W., Gumi, B., Lakew, M., Tadesse, B., Bayissa, B., Sirak, A., Girma Abdela, M., Gebre, S., Chibssa, T., Veerasami, M., Vordermeier, H.M., Bakker, D., Berg, S., Ameni, G., Juleff, N., de Jong, M.C.M., Wood, J., Conlan, A., Kapur, V., (2024). BCG vaccination of cattle reduces transmission of bovine tuberculosis, improving the prospects for elimination. Science **383** eadl3962. https://doi.org/10.1126/science.adl3962
4. Lakew, M., Conlan, A.J.K., Tadesse, B., Srinivasan, S., Yalew, B., Benti, T., Olani, A., Kinfe, G., Ashagrie, T., Abebe, A., **Fromsa, A**., Abdela, M.G., Bayissa, B., Gebre, S., Mihret, A., Mekonnen, G.A., Ameni, G., Ashenafi, H., Wood, J.L.N., Gumi, B., Kapur, V., 2025. Comparative performance and age dependence of tuberculin and defined antigen

bovine tuberculosis skin tests assessed with Bayesian latent class analysis. Sci Rep 15, 19728. https://doi.org/10.1038/s41598-025-05223-6

5. Lakew, M., Tadesse, B., Srinivasan, S., Aschalew, M., Andarge, B., Kebede, D., Etifu, A., Alemu, T., Yalew, B., Benti, T., Olani, A., Abera, S., Bedada, W., **Fromsa, A.**, Mekonnen, G.A., Almaw, G., Ameni, G., Ashenafi, H., Gumi, B., Bakker, D., Kapur, V., 2024. Assessing the feasibility of test-and-cull and test-and-segregation approaches for the control of high-prevalence bovine tuberculosis in Ethiopian intensive dairy farms. Scientific Reports 14, 14298. https://doi.org/10.1038/s41598-024-64884-x
6. Bahiru, G., Jena, P.K., Addissie, A., Behera, M.R., **Fromsa, A**., and Gumi, B. (2022). Zoonotic Tuberculosis in Occupationally Exposed Groups in the Adama Municipal Abattoir, Central Ethiopia. Ethiop. J. Health Dev. 36(1):5001. doi:10.20372/ejhd.v36i1.5001 https://doi.org/10.20372/ejhd.v36i1.5001
7. Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., **Fromsa, A.**, Ameni, G. Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., and Kapur, V. (2019): A defined antigen skin test forthe diagnosis of bovine tuberculosis. Science Advances; 5: eaax4899. https://www.science.org/doi/10.1126/sciadv.aax4899

Other publications

1. Dugassa, J., **Fromsa, A.**, Wirtu, A., and Telila, C. (2022). Inguinal herniorrhaphy in goat: The novel way of using vest-over-pants closure technique. Vet Rec Case Rep. 2022;10: e321. https://doi.org/10.1002/vrc2.321
2. Kitessa, J.D., **Fromsa, A.,** and Wirtu, A. (2021). A case report on ventrolateral herniorrhaphy in sheep: The novel way of using vest-over-pants closure technique. Vet Med Sci, 1–5. https://doi.org/10.1002/vms3.606
3. Fromsa, A., and Saini, N. S. (2019): Canine urolithiasis and concurrent urinary bladder abnormalities: Symptoms, haematology, urinalysis and comparative radiographic and ultrasonographic diagnosis. Vet Med Open J.; 4(1): 18-26. doi: 10.17140/VMOJ-4-132.
4. Dugassa, J., and **Fromsa, A.** (2019): Unilateral surgical amputation of horn due to suppurative sinusitis in cow. Vet Med Open J.; 4(1): 45-48. doi: 10.17140/VMOJ-4-135.

5. Jiregna Dugassa and **Abebe Fromsa** (2018): Review on dissociative anaesthetics and compatible drug combinations in veterinary clinical practice. Vet Med Open J.; 3(1): 21-30. doi: 10.17140/VMOJ-3-129.
6. Hassan, A., and **Fromsa, A.** (2017). Review on Chemical Sterilization of Male Dogs. International Journal of Advanced Research, 5(11): 758-770. doi: 10.21474/IJAR01/5828
7. Tadesse, M., Ayana, D., Kumsa, B., and **Fromsa, A.** (2020): Zoonotic helminth parasites of dog in Bishoftu Town, central Ethiopia: prevalence, dog owners' knowledge and control practice. Ethiop. Vet. J., 24 (1), 93-115.
8. **Fromsa**, **A.,** Saini, N.S., and Sood, N.K. (2016): Histopathological Studies on Renal, Prostate and Urinary Bladder Diseases in Dogs, Advances in Biological Research, 10 (3): 175-182
9. **Fromsa, A.,** and Jobre, Y. (2012): Estimated annual economic loss from organ condemnation, decreased carcass weight and milk yield due to bovine hydatidosis (Echinococcus granulosus, Batsch, 1786) in Ethiopia, Ethiopian Veterinary Journal, 16 (2): 1-14
10. Paulos, D., Addis, M., **Fromsa, A,** and Mekibib, B. (2012): Prevalence of gastrointestinal helminths among dogs and owners' perception about zoonotic dog parasites in Hawassa Town, Ethiopia, Journal of Public Health and Epidemiology, 4: 205-209, 2012
11. Gidey, A., Mulugeta, S and **Fromsa, A.** (2012): Prevalence of Bee lice Braula coeca (Diptera: Braulidae) and other Perceived Constraints to honey bee Production in Wukro Woreda, Tigray Region, Ethiopia, Global Veterinaria, 8(6): (631-635).
12. Sulayeman, M., and **Fromsa, A.** (2012): Lameness in Dairy Cattle: Prevalence, Risk Factors and Impact on Milk Production, Global Veterinaria, 8 (1): 01-07, 2012
13. **Fromsa**, **A.,** Meharenet, B. and Mekibib, B. (2011): Major Trematode infections of Cattle Slaughtered at Jimma Municipality Abattoir and the occurrence of the Intermediate Hosts in Selected Water Bodies of the Zone, Journal of Animal and Veterinary Advance, 10 (12):1592-1597.
14. **Fromsa, A.** and Mohammed, N. (2011): Prevalence of indigestible foreign body ingestion in small ruminants slaughtered at Luna Export Abattoir, East Shoa, Ethiopia, Journal of Animal and Veterinary Advance, 10 (12):1598-1602.

15. **Fromsa, A.** and Jobre, Y. (2011): Infection prevalence of hydatidosis (Echinococcus granulosus, Batsch, 1786) in domestic animals in Ethiopia: A synthesis report of previous surveys, Ethiopian Veterinary Journal, 15 (2), 2011
16. Addis, M., **Fromsa, A.,** and Ebuy, Y. (2011): Study on the prevalence of Lungworm Infection in Small Ruminants in Gondar Town, Ethiopia, Journal of Animal and Veterinary Advance, 10 (13):1683-1687.
17. Mulaw, S., Addis, M., and **Fromsa, A.** (2011): Study on the Prevalence of Major Trypanosomes Affecting Bovine in Tsetse Infested Asosa District of Benishangul Gumuz Regional State, Western Ethiopia, Global Veterinaria, 7 (4): 330-336.
18. **Fromsa**, **A.** Saini, N.S and Rai, T.S. (2011): Diagnosis, prediction and mineral analysis of uroliths in canine patients, Global Veterinaria, 7 (6): 610-617.

Conferences

Fromsa, A., Willgert, K., Srinivasan, S., Mekonnen, G., Bedada, W., Gumi, B., Lakew, M., Tadesse, B., Bayissa, B., Sirak, A., Girma Abdela, M., Gebre, S., Chibssa, T., Veerasami, M., Vordermeier, H.M., Bakker, D., Berg, S., Ameni, G., Juleff, N., de Jong, M.C.M., Wood, J., Conlan, A., Kapur, V., (2024). Oral presentation on Impact of BCG Vaccination on TB Burden in Livestock at Meet the Expert session and Effect of BCG Vaccination in Cattle at TBScience Session, The Union World Conference on Lung Health 2024 (WCLH 2024) held in Bali, Indonesia, from November 22-26, 2024.

Fromsa, A., Conlan, A.J.K., Srinivasan, S., Zeleke, M., Worku, D., Lakew, M., Abdela, M.G., Bahiru, G., Wood, J.L.N., Bakker, D., Gumi, B., Ameni, G., and Kapur, V., (2024). Bovine tuberculosis in Central Ethiopian slaughterhouses and the identification of causative mycobacteria by multiplex real-time PCR. Conference, Research Week at Aklilu Lemma Institute of Pathobiology, Addis Ababa University, May 2021. Poster presentation.