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Bovine tuberculosis in Central Ethiopian slaughterhouses and the identification of causative mycobacteria by multiplex real-time PCR

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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 real-time PCR (mRT-PCR). 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 risk 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,

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24.2% (95% CI = 20, 29) were culture and mRT-PCR positive. All the culture-generated isolates were positive for *M. bovis* in mRT-PCR. 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 mRT-PCR. 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

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 RT–PCR 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 S1). 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 real-time PCR 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.

Materials and methods

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 Fig. 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 km 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.

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

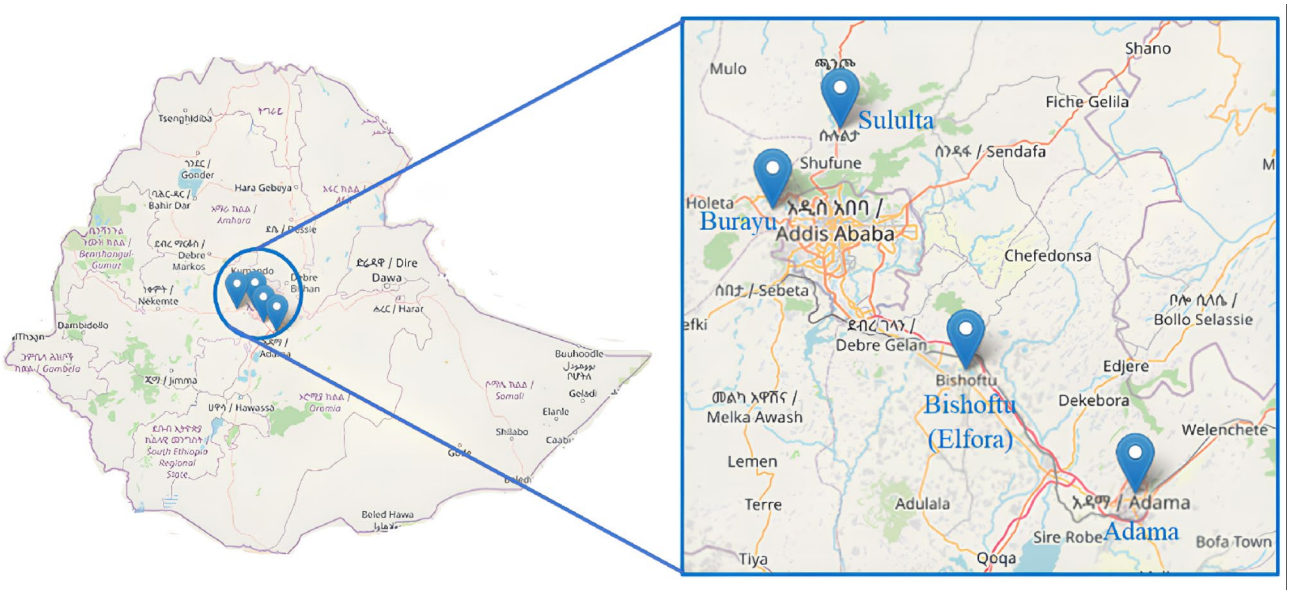

Fig. 1 Locations of the study slaughterhouses produced and marked using the leaflet package in R: Left) Study locations are indicated by blue ballons within the circle. Right) Study areas zoomed with expanded view

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.

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. The meat inspectors were educated to BVSc (Bachlor of Veterinary Science or DVM (Doctor of Veterinary Medicine) degree level. These inspectors were specifically trained by the Ethiopian Ministry of Agriculture to examine animal carcasses intended for the food supply, ensuring their safety before public consumption. 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 (Fig. 2). The

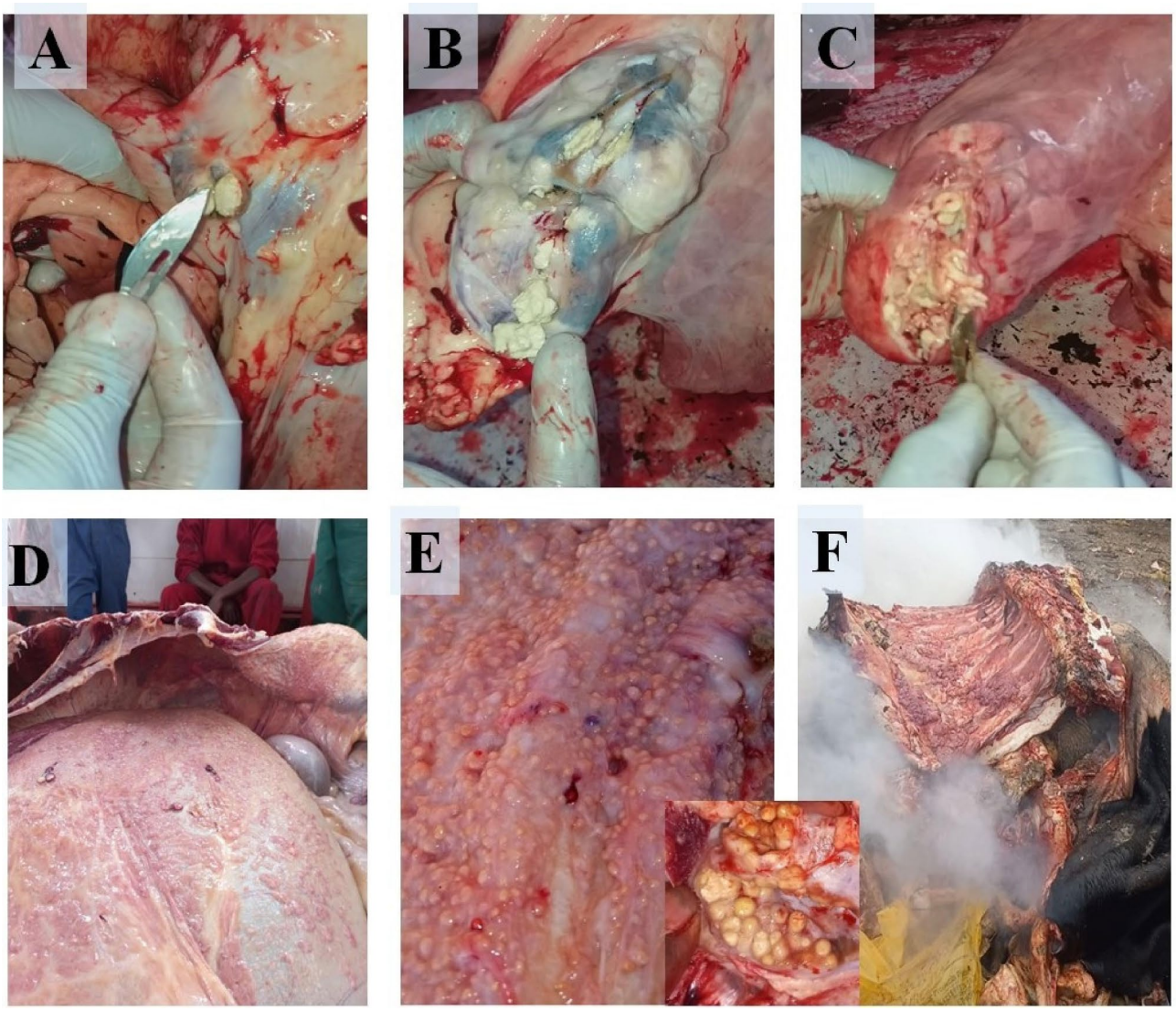

Fig. 2 Different types of bTB lesions observed. (**A**) A light yellow, dry caseous lesion in the left bronchial lymph node; (**B**) A white thick creamy caseous lesion within enlarged caudal mediastinal lymph node; (**C**) Cavitary tuberculous lesions with white creamy caseous abscess in the lung lobe (**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

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 4ºC and in the laboratory, stored at -20 °C until processing. Bacterial culture and isolation were performed at the same laboratory.

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 min 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, 2 N 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. 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.

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 min 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].

Detection and speciation of MTBC by mRT–PCR

The target genes used in the current multiplex PCR assay were tested and validated in previous studies [15, 16, 18, 21]. The DNA was amplified by two-step real-time multiplex PCR at AHI (Animal Health Institute), Sebeta, Ethiopia. In the first step of the multiplex PCR 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 PCR 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 S2.

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 µl (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 min at 95 °C for activation of the polymerase, 40 cycles of 15 s at 95 °C

for denaturation, and 40 cycles of 60 s 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.

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 mRT-PCR.

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 1) and the probability of *M. bovis* being cultured per animal (Table 2) and per tissue sample (Table 3). 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 (Tables 2 and 3) 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.

Ethical approval

Ethical approval was obtained from the Aklilu Lemma Institute of Pathobiology, Addis Ababa University,

Table 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% CI	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

Table 2 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 & mRT-PCR/ TBL positive animals	% (95% CI) culture & mRT-PCR 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 3 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 mRT-PCR /TBL positive tissues	% (95% CI) culture and mRT-PCR 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

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.

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 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 1).

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. 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.

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 mRT-PCR-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 mRT-PCR confirmation at both the animal (Table 2) and tissue (Table 3) levels. A multivariate logistic regression revealed significantly higher culture and mRT-PCR 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 2). Tuberculous-like lesions from male and crossbred cattle produced a significantly greater positive mycobacterial culture and mRT-PCR 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 mRT-PCR 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 3).

Table 4 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* and mRT-PCR 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

Multiplex RT–PCR

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) (Fig. 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 5; Fig. 3). However, none (0%) of the isolates tested positive for the *M. orygis*-specific PCR primer-probe sets.

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

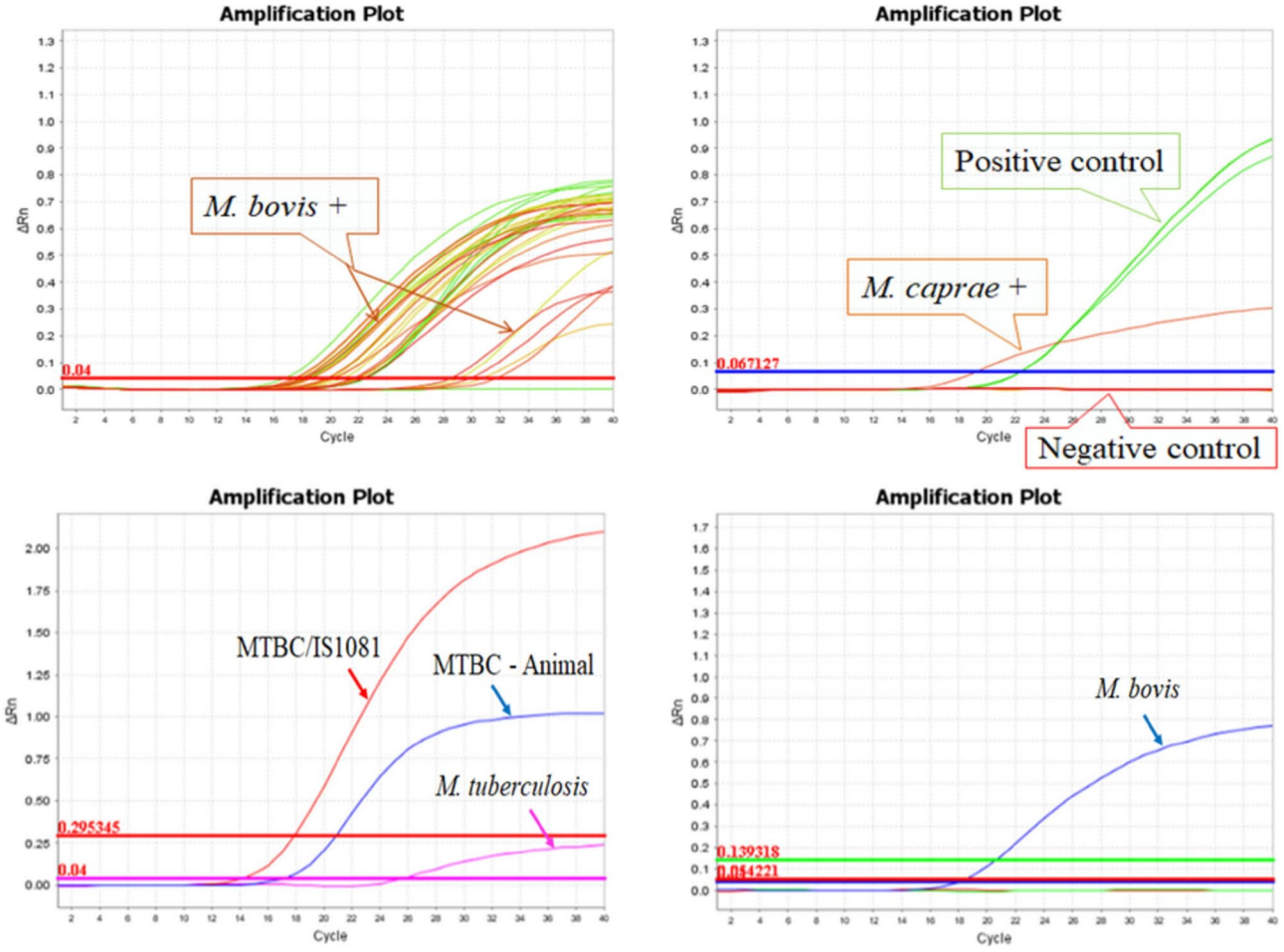

Fig. 3 Upper panel) Amplification plot showing partially selected PCR-positive *M. bovis* and single PCR-positive *M. caprae* samples. Lower panel) A sample with mixed *M. bovis* and *M. tuberculosis*, the plots of first step PCR are shown in the left and second step PCR in the right. The most abundant 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

Table 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

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 to 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 mRT-PCR 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 mRT-PCR positive. The higher culture and mRT-PCR 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 mRT-PCR positive, consistent with some previous studies [48, 56, 57]. All culture-positive isolates were identified as *M. bovis* by mRT-PCR, confirming its predominant role in bTB in this region. Based on culture and mRT-PCR 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 mRT-PCR 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, mRT-PCR 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 mRT-PCR 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 mRT-PCR 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 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 min), 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 mRT-PCR 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 bovine tuberculosis 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.

Supplementary Information

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Supplementary Material 1

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Author contributions
Conceptualization: VK, GA, and AJKC. Methodology: AF, AJKC, SS, VK, JLNW, DB, GA. Investigation: AF, SS, MZ, DW, ML, MGA, GB. Data analysis: AJKC, AF. Funding acquisition: AJKC, JLNW, VK, DB, GA. Supervision: VK, AJKC, BG, GA. Writing – original draft: AF, BG, GA. Writing – review & editing: All authors.

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Data availability
The data collected for the study and code required to carry replicate statistical analyses is available at: https://github.com/MonkeyMyshkin/fromsa_slaughterhouse.

Declarations

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

Consent for participation
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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