Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology

Epidemiology and molecular characterization of *Mycobacterium bovis* in humans and cattle and assessment of its zoonotic importance in central Ethiopia

By

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A Thesis Submitted to Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Microbiology

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
aDNA	ancient DNA
BCG	bacille Calmette-Guérin
bTB	bovine tuberculosis
CDC	Centers for Disease Control and Prevention (United States)
DFWs	dairy farm workers
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FDA	United States Food and Drug Administration
FNA	fine needle aspirate
HIV	human immunodeficiency virus
IFN	interferon
IGRA	interferon-gamma release assay
L-J	Löwenstein-Jensen medium
MAC	*Mycobacterium avium* complex
MDR	multidrug-resistant
MDR/RR-TB	multidrug-resistant TB or rifampicin-resistant TB
DR-TB	multidrug-resistant TB
MIRU-VNTR	mycobacterial interspersed repetitive unit-variable number tandem repeats
MOTT	mycobacteria other than tuberculosis
MTBC	Mycobacterium tuberculosis complex
NAAT	nucleic-acid amplification tests
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
NGS	next-generation sequencing

NTM	nontuberculous Mycobacteria
OIE	Office International des Epizooties -In English: The World Organization for Animal Health
PCR	polymerase chain reaction
PPD	purified protein derivative
QC	quality control
RD	region of difference
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	real time PCR
SNPs	single nucleotide polymorphisms
SOP	standard operating procedures
TB	tuberculosis
TBLN	tuberculous lymphadenitis
TST	tuberculin skin test
UN	United Nations
WGS	whole genome sequencing
WHO	World Health Organization
ZN	Ziehl Neelsen
zTB	Zoonotic Tuberculosis

ABSTRACT

Mycobacterium bovis (M. bovis) is a member of the *Mycobacterium tuberculosis* complex (MTBC) and causes tuberculosis in humans (zoonotic tuberculosis-zTB) and animals, mainly in cattle (bovine tuberculosis-bTB). There were limited studies on zTB in Ethiopia but also a reliable estimate of bTB prevalence in cattle in central Ethiopia is missing. In addition no whole genome sequencing (WGS) based *M .bovis* studies had been performed in Ethiopia before this study. Also, there has been a limited effort in search for an alternative diagnostic method to culture to detect *M. bovis* in clinical specimens. Therefore, due to these research gaps, this study, which combined bTB and zTB, was conducted from 2018 to 2021 in central Ethiopia (Addis Ababa, Sebeta, Holeta, Sululta, Sendafa and Bishoftu) with the objective of generating epidemiological and molecular data to update our understanding on bTB/zTB. The bTB study in cattle involved a cross sectional one-stage cluster sampling survey of dairy farms in central Ethiopia using tuberculin skin testing and the collection of additional data by questionnaire to estimate the prevalence of bTB and identify potential risk factors contributing to bTB transmission. For the zTB part, surveillance of TB in humans was carried out among individuals working in bTB infected dairy farms, patients presented at selected health centers, and exposure to risk factors was assessed using questionnaire. From consenting TB suspected individuals, demographic and clinical information was collected by questionnaire. Sputum and Fine Needle Aspirates (FNA) samples were collected from suspected cases. In addition, isolation of *M. bovis* was done from raw milk collected from tuberculin skin test positive cows and from cattle tissue lesions. The genetic diversity of *M. bovis* isolates was examined using spoligotyping and whole genome sequencing (WGS) analysis. Furthermore, in this study the performance of a TaqMan real time PCR (RT-PCR) assay as an alternative diagnostic method to culture was evaluated. Two hundred ninety-nine (n=299) dairy herds in the six study areas were randomly selected, from which 5,675 cattle were tested. The overall prevalence of bTB after standardisation for herd-size in the population was 54.4% (95% CI 48.7-60%) at the herd level, and it was 24.5% (95% CI 23.3-25.8) at the individual animal level. A Generalized Linear Mixed Model (GLMM) was used to explore risk factors association with bTB status. We found that herd size, animal age, bTB history at farm, and breed were significant risk factors. With regard to zTB, among 110 DFWs in 73 bTB infected dairy farms, 41 had at least one of the symptoms that are typical for TB. Three DFWs had swollen nodes at their neck, a symptom typical for TB lymphadenitis. In

assessment of risk factors: raw milk consumption was practiced by more than two thirds of the DFWs with symptoms of TB (68.2%) and over half of DFWs with symptoms did not think TB could be transmitted via raw milk consumption. Overall in the surveillance of zTB (active and passive), a total of 167 specimens (sputum=131; FNA=36) were collected from 161 TB suspected individuals for the isolation of *M. bovis*. Of these processed specimens, three samples with *M. bovis* were detected in total (1.8%, n=167). And of these three, one *M. bovis* isolate was sequenced and the genotype was spoligotype SB1476 which was previously reported from cattle in Ethiopia suggesting possible zoonotic transmission. With regard to isolation and characterization of *M. bovis* from cattle, out of 827 cattle (abattoirs and dairy farms), 76 of them (9.2%) had tuberculous lesion. From these tuberculous lesions, 62 isolates (n=137 samples) from 42 animals were confirmed to be *M. bovis.* Similarly out of 975 milking cows which were tuberculin skin test positive (37.8%, n=2582), 490 composite raw milk samples were collected and of these 11 (2.2%) yield *M. bovis* isolates showing evidence that raw milk is not safe and can be a source of infection for human TB due to *M. bovis*. The genetic diversity of 74 *M. bovis* isolates (one being a human isolate) was assessed and ten different spoligotypes were recorded. In cattle spoligotype SB1176 was the most prevalent type (n=31, 44.3%) followed by SB0133 (n=11, 15.7%). Our WGS analysis with a total of 55 *M. bovis* isolates sequenced (one being a human isolate) showed three clonal complexes clearly segregating in the phylogeny: African 2 (Af2; n=47), European 3 (Eu3; n=7) and Unknown8 (n=1). In addition, the present study reported for the first time clonal complex European 3 (Eu3) from Ethiopia. With regard to TaqMan assay performance evaluation - the assay performance on 440 clinical samples was variable for different specimens and overall performed well for sputum samples for all targets.

In conclusion, this study recorded high prevalence of bTB in dairy cattle in central Ethiopia. *M. bovis* prevalence in humans in central Ethiopia was low; however, further investigation is needed in all regions at national level given bTB is endemic in cattle in Ethiopia. Knowledge gap on bTB and *M. bovis* isolation from milk, showed that there is a clear potential for zoonotic transmission and needs further investigation. The TaqMan RT-PCR assay is a promising methodology for the diagnosis of zTB and bTB, but with further validation works needed using different targets and specimens.

CHAPTER ONE

1.1. INTRODUCTION

Mycobacterium bovis (*M. bovis*) is acid fast, rod shaped zoonotic bacterium in the *M. tuberculosis* complex group (MTBC) causing tuberculosis (TB) in humans and animals. They include 12 closely related members of the *Mycobacterium* genus: *M. tuberculosis*, *M .bovis*, *M. africanum, M. microtii, M. canettii, M. caprae, M. pinnipedii, M. orygis*, *M. mungi*, *M. suricattae*, and the Dassie and Chimpanzee bacilli [1]. In cattle *M. bovis* causes chronic debilitating respiratory decease known as bovine tuberculosis abbreviated as bTB to differentiate it from human TB [2]. Although cattle are considered to be the true hosts of *M. bovis*, the pathogen infects a wide host range and has been isolated from domestic and non domestic animals including buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx [2]. This shows *M. bovis* has an exceptionally diverse host range.

Though global population growth is slowing, Africa and Asia will still see a large population expansion and urbanization and hence the demand for food [3]. According to FAO [4] livestock sustainability 2050 analysis for Africa, in the next 30-40 years the demand for livestock products such as milk and meat will increase. As a result, there will be significant investments in dairy farming in urban and peri-urban areas and this obviously will have animal, public and environment health impacts. In Ethiopia, particularly in Addis Ababa, the capital of Ethiopia, over the last decade an increase in the consumption of dairy products in general and of liquid milk in particular was seen. A recent study by Minten et al. [5] showed a 31% increase in milk consumption over the last decade in Addis Ababa. This high demand for milk parallel with a shift in dairy cattle population composition where urban farmers kept high milk yielding exotic or crossbred cows unlike rural farmers where 27 % of the rural dairy farmers had crossbred cows compared to 98 % in urban areas and 100 % for commercial farms [5]. This market opportunity for milk in urban and peri-urban areas though favored dairy farm expansion; it is challenged with land scarcity, which forced dairy farmers to keep large number of cattle in smallholdings and

lead to overcrowding [6]. Dairy intensification has the advantage of increasing milk production through larger herds, indoor housing/feeding, breeding technologies, and other inputs [7]. This mode of production is however, becoming environmentally unfriendly and is being associated with diseases of intensification such as bTB, human health, environmental health and animal welfare issues among other things [7, 8]. Globally the animal TB burden is highly variable across continents and countries due to a number of factors including the predominant host species, sample size, geographical location and diagnostic tests [9]. According to a recent review of animal TB by Ramos et al. [9] and specifically for cattle (other animals were also covered by the review) it was 10.3%, 13.8%, 17.8%, 33.6% and 20.5%, respectively for Africa, Asia, Europe, North America and South America. Based on this review, Africa is having the lower prevalence of bTB compared to other continents. This low report might be due to lack of resource in Africa to carry out a planned surveillance of bTB and this information may be obtained from cross-sectional studies only.

While there have been many small scale prevalence surveys in Central Ethiopia which recorded a high prevalence of bTB over the last two decades [10], the representativeness of these studies was limited. Generally the prevalence of bTB in Ethiopia ranges from 3.1% in smallholder production systems (rearing mainly zebu cattle) up to 48% in intensive dairy productions [11]. The burden in Central Ethiopia is far in excess of the national average recently estimated to be ~ 5.8% [11]. These studies contributed in estimating the burden of bTB in Central Ethiopia; however, had limitations in identifying the contributing risk factors for the spread of the disease to inform the development of potential control strategies. In addition to tuberculin skin test (TST) based surveys; there were studies on isolation and characterization of *M. bovis*-genotyping in central Ethiopia [12]. These previous studies employed conventional typing methods including spoligotyping and Mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) to report the true picture of the *M. bovis* prevalence [12]; this contributed to the definition of the African 2 clonal complex of *M. bovis* (Af2) confined to Ethiopia and East Africa [13] and to explore epidemiological links. These conventional typing methods, however, are limited in discriminatory power and identical spoligotype patterns can be found in phylogenetically unrelated strains [14]. Whole-genome sequencing (WGS) and SNP-based analyses can provide more detailed and discriminating phylogenetic analyses [15]. WGS based analysis of *M. bovis* were lacking in Central Ethiopia in particular and Ethiopia at large. To

address questions around transmission and control of bTB in the highly affected dairy sector in Central Ethiopia, WGS based phylogenetic analyses would be helpful.

WHO is publishing a global TB report every year since 1997. Globally in 2020, an estimated 9.9 million people fell ill due to TB. There were 1.3 million TB deaths among HIV-negative people and an additional 214, 000 deaths among HIV-positive people [16]. The End TB Strategy sets targets of a 90% reduction in TB deaths and an 80% reduction in the TB incidence rate between 2015 and 2030 [17]. In line with this global target, it is important to know the real burden of zoonotic tuberculosis (zTB) particularly in low- and middle-income countries as these countries may have minimal to no bTB-control program. Although the global distribution of zTB caused by *M. bovis* is believed to be generally low [18], there is a suggestion that the burden of *M. bovis* might be underestimated. zTB is indistinguishable clinically or pathologically from TB caused by *M. tuberculosis*. Differentiation between the causative organisms may only be achieved by sophisticated laboratory methods involving bacteriological culture of clinical specimens, followed by typing of isolates. All this makes it difficult to accurately estimate the proportion of human TB cases caused by *M. bovis* infection, particularly in low income countries [19]. According to WHO 2017 report there were an estimated 147000 new human cases of zTB globally, and 12,500 deaths due to zTB [20]. End TB strategy cannot be realized by only targeting TB which is mainly caused by *M. tuberculosis* and human TB caused by *M. bovis* needs to be also addressed. Although the attention given to zTB was inadequate, there were some studies in high income as well as middle and low income countries. Globally zTB contributed to 1.4% of new active cases of TB and the African region carries the heaviest burden of disease and death due to zTB, followed by the South-East Asian region [20, 21]. In Latin America such as Argentina 2% of pulmonary TB were due to *M. bovis* and human to human transmission was also reported [22]. In Africa zTB due to *M. bovis* was estimated to be 2.8 % [18] though there were reports as high as 5% in Nigeria [23]. *M. bovis* has also drug resistance concern for instance in Spain from 1994 to 1999, 13 pleuropulmonary infections caused by *M. bovis* were diagnosed and seven patients, including four HIV-positive patients who died, had multidrug-resistant *M. bovis* infection [24]. A resistance to Isoniazid was reported in immunocompetent patient in Ireland [25].

In Ethiopia intensive and extensive milk production is a major Emerging Livestock System around several urban centers and the prevalence of uncontrolled *M. bovis* infection in cattle in this system is high [26]. Furthermore, most of the milk in the Ethiopian market system is not sold through the large dairies where pasteurization is practiced, but direct from small farmers in an unpasteurized form. In Ethiopia and elsewhere in resource poor countries, removal of infected animals through test-and slaughter policies is economically unfeasible and, as the milk market grows, so will the risk of zoonotic transmission. In the last decade, numerous cross sectional surveys on bTB prevalence was undertaken by the investigators. These studies recorded consistent prevalence of bTB of >30% in exotic and cross bred cattle in peri-urban intensive dairy farms in central Ethiopia [27, 28]. However, only few studies were conducted on zTB to see whether this high bTB in Central Ethiopia has public health significance. Furthermore, studies that tried to assess the knowledge and practices of risk groups related to zTB and identify the main routes of transmission under Ethiopian setting are lacking. In literature, there are three potential routes of transmission of zTB: ingestion of contaminated dairy products (animal-to-human), inhalation of infectious droplet nuclei (animal-to-human or human-to-human), and direct inoculation of the skin (animal-to-human) [29]. Assessment of which one of these routes is/are the main driver/s of transmission would be helpful for implementing different public health interventions. One of the reasons mentioned for the under reporting of zTB is the diagnostic challenges. Specifically in paucibacillary specimens, the conventional microbiological TB diagnostic methods - acid-fast staining and mycobacterial culture might not be helpful for detecting such a rare pathogen [30]. In order to overcome this problem, evaluation of the performance of PCR based methods such as real time PCR can be considered as an alternative or complementary test to microbiological culture. The rapid detection and identification of mycobacterial species during early-stage of the disease is essential for patient management and infection control. To the best of our knowledge, there are no studies of such kind in Ethiopia.

1.2. LITERATURE REVIEW

1.2.1. Historical Background

Tuberculosis (TB) is an ancient disease of human andbovine types of the tubercle bacillus and has plagued humankind. Typical skeletal abnormalities of tuberculosis have been found in Egyptian mummies dating back to 2400 BC [31]. Tuberculosis was also known in the ancient Greece which Hippocrates referred to as Phtisis (phthisis is from the Greek word with the same meaning of decay or wasting). Hippocrates described Phthisis as a "weakness of the lung" with fever and cough which was commonest disease of the period and usually as being fatal. Over the years different names were given to TB. During the Middle Ages (5-15th century), TB was often referred to as Scrofula; infection of the lymph nodes in the neck. It was also known in England and France as "king's evil", and it was widely believed that persons affected could heal after a royal touch [32]. During this period Monarchs were believed to have magical or curative powers. It was also known by the name Consumption literally to mean the disease consumed the individual where weight drastically dropping as the disease progressed. These different names were finally unified to a single word 'tuberulosis' by Johann Lukas Schönlein in 1839 from the Latin “tuberculum,” meaning “small, swelling bump or pimple [33]. TB has claimed a lot of life's throughout human history. *Mycobacterium tuberculosis* may have killed more persons than any other microbial pathogen. During earlier 18^{th} century, TB has claimed many lives in Europe and America (800-1000 deaths per 100,000 inhabitants per year) before control interventions [33]. The major progress about the disease was made after Francisus Silvus defined the pathology of tuberculosis in 1671[34].

Mycobacterium tuberculosis was first discovered by Robert Koch, a German Doctor who communicated his discovery on 24 March 1882, to the Berlin Society of Physiology [35]. And to commemorate this, every year 24 March is declared by WHO as World TB Day to raise public awareness about the devastating health, social and economic consequences of TB and to step up efforts to end the global TB epidemic [36]. The view that tuberculosis might be a contagious disease was unbelievable until the nineteenth century as it was considered to be inherited or congenital or spontaneous. Jean Antoine Villemin, a French army doctor in Paris was the first to demonstrate tuberculosis was contagious disease in 1865 [35]. He showed that tuberculosis was transmitted from human to human, from human to animal or from animal to human. Preventive

measure using vaccine was started in 1921 after the discovery of tuberculosis vaccine called BCG (Bacille Calmette Guérin). Albert Calmette, a bacteriologist working together with Camille Guérin, veterinarian started research working at Pasteur Institute in Lille for developing vaccine for TB [37]. After over 13 years of research they were able to attenuate the virulence of *M. bovis* of bovine origin by sub-culturing more than 200 times and finally the BCG vaccine was introduced in 1921. Bacille Calmette-Guérin (BCG) is the only vaccine available today and has been used with good safety results for more than 99 years now. It is recommended primarily for preventing the most severe forms of tuberculosis, including disseminated tuberculosis and meningeal tuberculosis in children; however, its efficacy in preventing pulmonary tuberculosis and TB reactivation in adults has been questioned [37, 38].

In 1943–44 a soil microbiologist Selman A. Waksman who won Nobel Prize in 1952 in Physiology or Medicine and his PhD student Albert Schatz discovered the potent antimicrobial agent streptomycin in the growth medium of the soil microorganism *Streptomyces griseus*. Streptomycin demonstrated its specific effect in inhibiting tuberculosis in both animals and people [39].

The discovery of streptomycin in 1944 led to other important anti-tuberculous drug discoveries including isoniazid and pyrazinamide (1952), ethambutol (1961), rifampicin (1963) and after over 40 years gap bedaquiline got approved in 2012 [40, 41]. These chemotherapeutic interventions resulted in decline of tuberculosis cases. However, this decline of tuberculosis cases caused a decreased attention to tuberculosis control and poor public health infrastructure worldwide which led to a resurgence of tuberculosis in the late 1980s and early1990s and challenges of multidrug resistance (MDR; resistance to at least isoniazid and rifampicin); and occurrence of extensively-drug-resistant (XDR) strains, resistant to at least isoniazid and rifampicin, as well as to any fluoroquinolone and to any of the three second-line injectables (amikacin, capreomycin or kanamycin) [41]. Still TB remains the world's most deadly infectious disease that needs to be addressed.

History of bTB is ancient like human tuberculosis. *Mycobacterium tuberculosis* complex DNA was detected from an extinct Bison dated 17,000 years before the year 2001[42]. The earliest evidence concerning tuberculosis in animals is of Indian origin [43]. Cattle (*Bos brachyceros*) were first domesticated in Asia (India) and were later brought to Europe where they were

probably eventually crossed with the wild European cattle (*Bos primigenius*) [43]. According to Iyer [44] there is overwhelming evidence in the ancient Hindu literature (2,000 B.C. or earlier) that tuberculosis was common in elephants at that time. The advent of molecular techniques has helped to better estimate the time of origin of mycobacteria. The surviving ancient mycobacterial DNA (aDNA) can be amplified from bone by PCR. Taylor and colleagues [45] did a study on five Iron Age individuals with spinal lesions recovered from the cemetery of Aymyrlyg, South Siberia and genotyping indicated that cases were due to infection with *M. bovis.* The authors believe this to be the first report of *M. bovis* causing Pott's disease in archaeological human remains. Iron Age is believed to be the period that began between 1500 and 1000 B.C. [46]. From the early 1800s bTB has been described in cattle in slaughterhouses. bTB was known in Europe in the 1880s and widely spread in the 1930 and spread to other countries. Herd prevalence at that time exceeded 25% and about 10% of human cases of TB were caused by *M. bovis* [47]. In the middle of the twentieth century, effective government funded control programs were carried out in most industrialized countries. Today, bTB is re-emerging and threatens the livestock industry in industrialized countries with wildlife reservoirs like the white-tailed deer (*Odocoileus virginianus*) in the USA or the European badger (*Meles meles*) in the UK [48, 49]. However, in most developing countries, particularly in Africa, they lack the means and capacity for effective control. bTB in Africa is emphasized here because of the disproportionately high burden, the particular difficulties to control it and the distinctive importance of multiple transmission interfaces between wildlife, livestock and humans [50, 51].

Following the identification of the tubercle bacillus in 1882 by Koch and his believe that this same organism was causing tuberculosis in man and animals, there was a public fear and concern that it might be transmitted through milk and meat to the extent of calling milk the 'white poison'[52]. For instance in United Kingdom three Royal Commissions were established between 1890 and 1911 with the objective of ascertaining the connection between tuberculosis in animals and the disease in human [53]. And after extensive ten-year project work, the commission produced a series of reports, with the final one published in 1911 concluding that 'Man must therefore be added to the list of animals notably susceptible to bovine tubercle bacilli'. In 1898 *M. bovis* was identified 16 years after Kotch identified *M. tuberculosis*. Theobald Smith in 1898 isolated tubercle bacillus from tuberculous cattle and was the first to distinguish this organism from human tuberculosis [54]. He did this by studying its culture and pathogencity

properties where the bovine tubercle bacillus was slow growth (dysgonic) and with great pathogenicity for rabbits. Later on other cultural characteristics which helped identification were discovered such as production of niacin [54]. The Judicial Commission of the International Committee on Nomenclature of Bacteria has accepted the name of the species *M.bovis* in the seventh edition of Bergey's Manual of Determinative Bacteriology [54].

1.2.2. The Agent: *Mycobacterium bovis*

1.2.2.1. Morphology and general characteristics

The hypothesis that the same organism that causes TB in humans might not be responsible for animal tuberculosis was finally cleared when Theobald Smith in 1898 isolated tubercle bacillus from tuberculous cattle, *M. bovis* which showed distinct characteristics that is different from *M tuberculosis* [55]. *M. bovis* is a non motile, non spore forming, facultative intracellular, aerobic and zoonotic bacterium of the MTBC group with a wide host range causing tuberculosis in humans and animals [56]. Mycobacteria are within the order Actinomycetales, which shares with bacteria such as *Corynebacterium*, *Nocardia*, and *Rhodococcus*. These bacteria also express unique mycolic acids in the cell envelope that play a critical role in the structure and function of the cell wall [56]. The basic structure of the cell wall is typical of Gram-positive bacteria (cytochemically) but do not take up the dyes of Gram-stain because the cell walls are rich in lipids; mycolic acid making the bulk of these [56, 57]. This cell wall richness in lipids, make the surface hydrophobic and the mycobacteria resistant to many disinfectants and common laboratory stains. Once stained, the rods also cannot be decolorized with acid-alcohol solutions; hence the name acid-fast bacteria; a feature that can be exploited to identify mycobacteria via the Ziehl-Neelsen staining technique [58]. The lipids of the cell wall have a potent biologic activity and are thought to play a crucial role in pathogenesis [57]. Mycosides, phospholipids and sulpholipids are thought to protect the tubercle bacilli against phagocytosis. *M. bovis* is slow growing (generation time of roughly 20 hours) and require incubation for up to 8 weeks in egg based Lownstein-Jensen (LJ) media. Pyruvate (0.4%) supplement enhances its growth. *M.bovis* can be identified based on cultural and molecular tests [57]. Works on optimization of isolation of *M.bovis* especially from paucibacillary specimens such as milk and extrapulmonary specimens is inadequate and needs further investigation.

1.2.2.2. Virulence factors of *Mycobacterium bovis*

Mycobacteria lack classical virulence factors such as toxins, which are typical of other bacterial pathogens [59]. Most studies of mycobacterial virulence determinants have been performed with *M. tuberculosis* rather than with *M. bovis*. However, the presence of homologous genes in *M. bovis* and the close relationship between these microorganisms indicate the use of similar virulence determinants and mechanisms of pathogenicity [60]. *M. tuberculosis*, the human tubercle bacillus, produces progressive generalized disease in nonhuman primates, dogs, swine, and certain exotic animals; cats, rabbits, and cattle are quite resistant. *M. tuberculosis* may induce tuberculin skin sensitivity in cattle and other animals. Guinea pigs are susceptible to *M. bovis* and *M. tuberculosis* and develop progressive lesions following infection [61]. The structure and biologic functions of the glycolipid-containing cell wall of mycobacteria have been extensively investigated. In the discovery of anti-TB drugs, inhibition of cell wall assembly has proven useful; drugs such as ethambutol, isoniazid, ethionamide, and D-cycloserine successfully target the synthesis of its various components [62]. The cell wall core of mycobacteria is composed of three covalently attached molecules: peptidoglycan (PG) which is present in almost all bacteria, providing shape, rigidity, and osmotic stability to both gram-negative and gram-positive bacilli [63], arabinogalactan which is composed predominantly of galactose (Gal) and arabinose (Ara) sugar residues, and mycolic acid- long-chain α-alkyl-β-hydroxy fatty acids (C_{70-90}) form an integral component in the arabinogalactan - peptidoglycan complex (AGP) and contribute to the fluidity and permeability of the cell wall [62]. Phosphatidyl-*myo*-Inositol Mannosides (PIM), Lipomannan (LM), and Lipoarabinomannan (LAM)- these noncovalently linked are abundant in the inner and outer membranes of all *Mycobacterium* species [62].

Mycolic acids such as cord factor (trehalose dimycolate) are glycolipids that are extracted with petroleum from the cell wall of viable pathogenic mycobacteria in the 1950s; they are implicated in the granuloma formation of mycobacterial disease [64]. Glycolipids and sulfur-containing glycolipids (sulfatides) inhibit phagolysosome formation and avoiding virulent tubercle bacilli exposure to hydrolytic enzymes present in the lysosomes and promote survival within macrophages [65]. Sulfolipids induce changes in phagocytic cell function that may be important in decreasing the ability of phagocytes to respond efficiently to *M. tuberculosis*. Blanc and his colleagues [66] conducted an experiment and showed how *M. tuberculosis* circumvents innate immunity. They reveal sulfoglycolipids inhibit NF-κB activation and subsequent cytokine

production or costimulatory molecule expression by acting as competitive antagonists of Toll-like receptor 2, thereby inhibiting the recognition of *M. tuberculosis* by this receptor. However, sulfated lipids are absent from the envelope of *M. bovis* because the glycolipid sulfotransferase and arylsulphatase genes are disrupted or deleted. This difference may also contribute to determine the host range and tissue tropism of *M. bovis* [61].

LAM has been known to inhibit phagosome maturation by inhibiting the Ca^{2+}/calmodulin phosphatidyl inositol-3-kinase hvps34 pathways [67]. LAM inhibits T-cell proliferation and bactericidal activities of macrophages. LAM is scavenger of cytotoxic oxygen-free radicals produced by macrophages and nitrogen (reactive nitrogen intermediates, RNI) reactive intermediates [61]. Given that *M.bovis* has exceptionally wide host range and now with the availability of whole genome sequencing, it is important to explore the virulence factors and their pathogenecity in at least economically important hosts such cattle breeds and epidemiologically important hosts (reservoirs -wildlife).

1.2.2.3. Pathogenesis of *Mycobacterium bovis* infection

Study of the pathogenesis of tuberculosis in humans is restricted by the inability to conduct controlled experimental infection studies. Whelan and colleagues [68] used an experimental bovine infection model to test the virulence of well-characterized strains of *M. tuberculosis* and *M. bovis* in cattle, choosing the genome-sequenced strains *M. tuberculosis* H37Rv and *M. bovis* 2122/97. Cattle were infected with approximately 10^6 CFU of *M. tuberculosis* H37Rv or *M. bovis* 2122/97, and sacrificed 17 weeks post-infection. IFN-γ and tuberculin skin tests indicated that both *M.bovis* 2122/97 and *M.tuberculosis* H37Rv were equally infective and triggered strong cell-mediated immune responses, even though with some indication of differential antigen-specific responses. Postmortem examination revealed that while *M. bovis* 2122/97–infected animals all showed clear pathology indicative of bovine tuberculosis, the *M. tuberculosis*–infected animals showed no pathology. Culturing of infected tissues revealed that *M. tuberculosis* was able to persist in the majority of animals, even if at relatively low bacillary loads.

A comparative analysis studies in humans, cattle, zebrafish, and nonhuman primates has helped to better understand granuloma formation in TB pathogenesis [60]. *M.bovis* is an intracellular

pathogen that spreads in the body in two stages; the primary complex and post primary dissemination [69, 70]. Inhalation of the respiratory droplets that contain TB bacilli is the common route of entry (95%) for *M.bovis* and primary foci develops in lymph tissues associated with lung. The mucociliary clearance in the upper respiratory passages provides defense against infection by inhalation of mycobacteria. However, mycobacteria on small particles such as dust and water droplets that do not attach to the mucociliary layer can pass through terminal bronchioles, thus gaining access to alveolar spaces. The estimated size of terminal endings of bronchioles is about 20 μm as compared to 0.2 to 0.6 × 1 to 10 μm in size mycobacteria [71, 72].

1.2.2.4. Immunity of *Mycobacterium bovis* infection

1.2.2.4.1. Innate Immunity

Innate immunity is the first response to mycobacterial infections and is a preparatory step for adaptive immunity. By virtue of their position at the crossroads between the innate and adaptive immune response, macrophages play an essential role in the control of bacterial infections. The role of macrophages in mycobacterial infection include fusion between phagosomes and lysosomes, production of reactive oxygen and nitrogen species, antigen presentation and major histocompatibility complex class II expression and trafficking, as well as autophagy and apoptosis [73, 74]. Paradoxically, macrophages serve as the natural habitat to *Mycobacterium* where mycobacteria subvert the macrophage's mechanisms of intracellular killing and antigen presentation, leading ultimately to the development of tuberculosis disease. Mycobacteria survive in nonactivated macrophages by inhibition of fusion of phagosomes and lysosomes (Fig. 1). Internalized *M. tuberculosis* resides in a phagosome, where, in resting macrophages, the bacteria block maturation, lysosomal fusion, and acidification [71]. Entry of mycobacteria into alveolar macrophages can be mediated by either non-specific pinocytosis or through a well-defined variety of receptors [71]. *M.bovis* in the lung are phagocytosed by alveolar macrophages and interaction with the host begins though the outcome of the infection is dependent on different factors including the agent, host and environment [75]. Jensen and colleagues [75] compared the interaction between bovine monocyte derived macrophages (bMDM) and two *M. bovis* strains, AF2122/97 and G18, representing distinct genotypes circulating in the United Kingdom. The two investigated *M. bovis* strains interact in significantly different ways with the host macrophage. AF2122/97 strain demonstrated an increase in the

number of viable intracellular AF2122/97 organisms rapidly for the first 24 h, induced a greater transcriptional response by bMDM, also induced more bMDM cell death, with characteristics of necrosis and apoptosis, more inflammasome activation, and a greater type I interferon compared to G18. Aldwell and colleagues [76] observed inter-species variation (cattle, deer, possums, ferrets and mice) in controlling growth of *M. bovis* by alveolar macrophages.

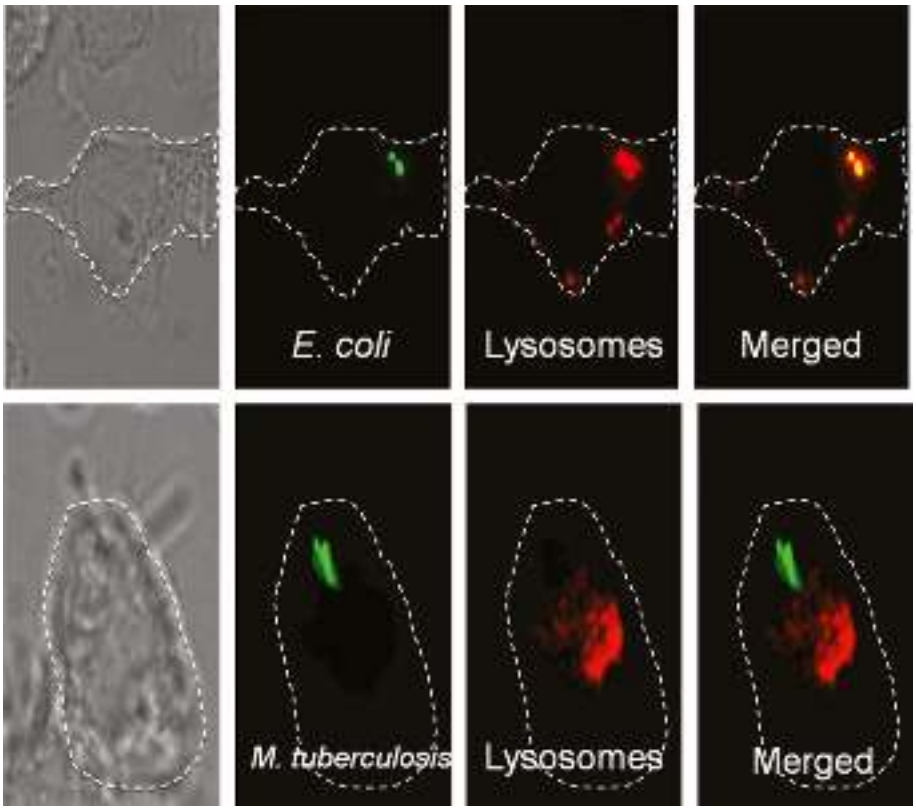


Figure 1. Confocal microscopy of THP-1 (designates a spontaneously immortalized monocyte-like cell line) macrophages infected with *E. coli* or Mtb.

Localization of *Eschercia coli* or *M. tuberculosis* with lysosomes is detected with immunofluorescence staining. Lysosomes are stained with Lysotracker Red dye (Red), and bacteria are labeled with fluorescein isothiocyanate (FITC) (green) before infection. While *M.tuberculosis* containing phagosome does not fuse with the lysosomes (Bottom Panel), the *Eschercia coli*-containing phagosome co-localize with the Lysotracker Red dye, indicating phagosome-lysosome fusion event (Top Panel).

Source: Hmama *et al*. [74]

Alveolar macrophages, through innate microbicidal mechanisms, attempt to control infection and limit bacterial replication through phagolysosome fusion, phagolysosome acidification, lysosomal proteolytic enzymes, and production of antimicrobial reactive oxygen and nitrogen

species [77]. In contrast with most phagocytized bacteria, mycobacterium prevents fusion of the phagosome with lysosomes. MTBC species have evolved mechanisms to escape this and at the same time, the phagosome is able to fuse with other intracellular vesicles, permitting access to nutrients and facilitating intravacuole replication [72]. If innate defenses fail, surviving bacilli establish intracellular residence within phagolysosomes. There, virulent mycobacteria use proteins such as early secreted antigenic target (ESAT)–6 to lyse phagolysosomal membranes and gain entry to the cytosol. In the cytosol, mycobacteria multiply, eventually causing macrophage cell death that induces the release of chemokines and cytokines, thus recruiting other cells to the site of infection.

1.2.2.4.2. Adaptive Immunity

The adaptive immunity has two components: humoral and cell mediated immunity (CMI). Of the two, it is the CMI which has great role in defending against *M. bovis* infection [78]. Dendritic cells containing bacilli exit the primary site of infection and migrate to local lymph nodes where, through antigen presentation and cytokine production, naive T cells recognize their cognate antigens, proliferate, and gain effector functions. The Th1 based CMI response lead to the production of cytokines and chemokines - tumor necrosis factor-α (TNF-α), IL-12, IL-6, and IFN-γ by APCs which are essential for the control of mycobacterial infection [79]. $CD8^+$ T cells role is in the lysis of infected cells. Primed, antigen-specific T cells migrate to the site of infection and there activate monocytes and macrophages; the recruitment of these cells contributes to the developing granuloma. The formation of granuloma is the hallmark lesion of tuberculosis in man and animals. Within the granuloma, the host interacts with the pathogen, and disease outcomes are determined. The granuloma is essential for control of mycobacterial pathogens, yet paradoxically, the granuloma also provides a venue for bacterial survival, multiplication, latency, and dissemination [60, 77].

1.2.2.5. Evolution/Genetic diversity and host range of *Mycobacterium bovis*

M. bovis is a member of the MBTC a term given to the group of Mycobacteria that cause tuberculosis (TB) in mammals [80]. The other groups of mycobacteria which do not cause tuberculosis or leprosy are known as Nontuberculous Mycobacteria (NTM), also called environmental mycobacteria, atypical mycobacteria and mycobacteria other than tuberculosis (MOTT). *Mycobacterium avium* complex (MAC) is a member of the NTM. NTM are opportunistic pathogens of animals and human placing some groups at increased risk, including those with underlying lung disease or depressed immune systems. These pathogens are typically not transmitted person-to-person.

Other members of MBTC include *M. tuberculosis*, *M. africanum, M. canettii*, *M. microti*, *M. caprae*, *M. pinnipedii, M. orygis*, *M. mungi*, *M. suricattae,* dassie bacillus and chimpanzee bacillus that are genetically very similar [1]. All members of the MBTC exhibit a 99.9% sequence similarity and their 16SrRNA are also conserved, with the exception of *M. canetti* and yet exhibit distinct host preference, indicating that this low level of genetic divergence holds major implications for host–pathogen interactions [81]. *M. bovis* displays the broadest spectrum of host infection, affecting humans, domestic or wild bovines and goats. *M. caprae* has been isolated only from goats. *M. microti* is a rodent pathogen, usually isolated from voles (rodents of the genus Microtus and related genera) that can also cause disease in immunocompromised human patients, *M. orygis* (antelopes), the “dassie bacillus” (rock hyrax), *M. mungi* (mongooses), *M. suricattae* (meerkats), and the “chimpanzee bacillus” (chimpanzees) [82, 83]. *M. pinnipedii* infects seals. MTBC also referred as human adapted and animal adapted groups depending on their host tropism (ecotypes) [83]. The lately included animal adapted MTBC include *M.mungi* (mongooses) and *M.orygis* (antelopes) [83]. Though *M.bovis* has a wide host range, their susceptibility varies (Table 1). Cattle are natural hosts for *M. bovis*.

Table 1. Susceptibility of animals to *M.bovis, M. tuberculosis, M. avium*

Host	*M.bovis*	*M. tuberculosis*	*M. avium*
Cattle	xxx	x	x
Goat	xxx	xx	xx
Pig	xxx	xx	xx
Cat	xxx		xx
Human	xxx	xxx	xx
Sheep	xx'	xx	xx
Horse	xx'	xx	xx
dog	xx'	xxx	xx
Fowl	-		xxx
Camels	xxx		

Source: DAFF [84]; Mamo et al. [85]

Meaning of symbols:

XXX: susceptible - visible lesions develop

XX ': susceptible - visible lesions sometimes develop

XX: susceptible - visible lesions seldom develop

X: visible lesions usually do not develop but animals react to the tuberculin test

There was hypothesis that *M tuberculosis* causes TB only in human evolved from *M. bovis* by host adaptation [86]. This hypothesis was based on the fact that *M. bovis* infects many animals including humans opposite to *M tuberculosis* which infects only humans. However, with the advent paleomicrobiology, it was found that *M tuberculosis* is ancient and is ancestor of mycobacterium group including *M. bovis;* new findings indicated that in fact tuberculosis first emerged in humans and was subsequently transmitted to animals (Fig. 2) [87]. Recent studies suggest that the common ancestor of the *M. tuberculosis* complex emerged from its progenitor perhaps 40,000 years ago in East Africa [88]. Some 10,000–20,000 years later, two independent clades evolved, one resulting in *M. tuberculosis* lineages in humans, while the other spread from

humans to animals, resulting in the diversification of its host spectrum and formation of other *M. tuberculosis* complex member species, including *M. bovis* (Fig. 2). A recent study by Loiseau and colleagues [89] suggested that *M. bovis* likely originated in East Africa. The proportion of *M.bovis* from this region in the global collection might be small (recent) for a larger analysis. However, in this region particularly in Ethiopia and East Africa as a whole, there has been collaborative research by Ethiopian and international scientists that led to the discovery of 'Lucy'(*Australopithecus afarensis*- ***Dinkinesh***, which means "you are marvelous" in the Amharic language) - the origin of human kind dated to about 3.2 million years ago [90]. It would be interesting to undertake collaborative paleomicrobiology studies of *M.tuberculosis* and *M.bovis* and possibly on other members of MTBC on fossils or ancient DNA (aDNA) in this region so that it will generate additional data to the previous evolutionary findings.

Regions of difference (RD), deleted loci absent from one MTBC member relative to another, serve as unique markers to differentiate species with some having had functional roles ascribed. Mostowy et al. [91] selected *M. bovis* and *M. bovis*-like organisms isolated from various mammalian host types of diverse geographic origins, which present an array of genetic profiles and laboratory properties. The DNA of these organisms was analyzed (by PCR and sequencing) and the result revealed the previously unknown evolution of the MTBC that MTBC members have evolved from a common ancestor, provide markers for diagnostic testing and molecular epidemiologic assessment, and point to both geographic and host-specific forms of genomic variability. Genomic analysis has helped to identify 14 regions (known as regions of difference or RD1–14). These regions, present in the reference laboratory strain *M. tuberculosis H37Rv*, are absent from the vaccine strain *M. bovis* var BCG; thus, helping to pinpoint chromosomal genes related to pathogenicity [92]. In parallel, six regions, known as H37Rv deletion 1 to 5 (RvD1–5) and *M. tuberculosis* specific deletion 1 (TbD1), are absent from the *M. tuberculosis H37Rv* genome relative to other members. By contrast, *M. canettii* contains all of the RD, RvD and TbD1 regions and it is believed that this is the most closely related genome to that of the bacilli's ancestor. *M. africanum* strains mainly isolated from West Africa and lack the RD9 region. *M. microti* lacks regions RD7, RD8, RD9 and RD10. Some strains that have been isolated from voles missed also part of the RD5 region. The most common *M. bovis* strains, "classical *M. bovis*" isolated from bovines in Argentina, the Netherlands, United Kingdom and Spain, as well mice as from humans, showed the greatest number of RD deletions, lacking regions RD4, RD5,

RD6, RD7, RD8, RD9, RD10, RD12 and RD13. *M. caprae* is closely related to *M. bovis* except that it contains several nucleotide substitutions in the gyrB gene that are not found in other members of the MTBC. In addition, the lack of the deletion of RD1, RD2 and RD14 in *M. bovis* var BCG apparently occurred during and after the attenuation process.

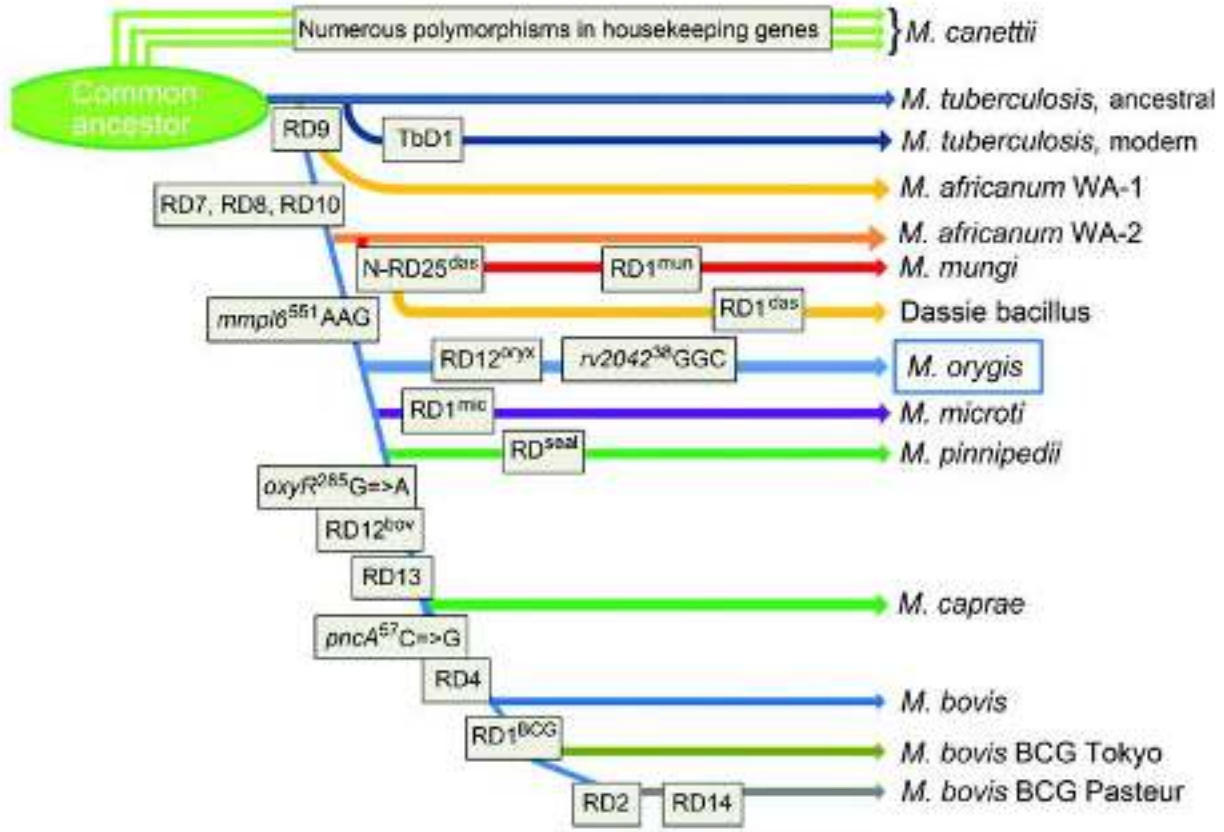


Figure 2. Updated deletion-based phylogeny of the MTBC

Source: Ingen et al. [93]

1.2.3. Bovine Tuberculosis (bTB)

Bovine tuberculosis is a chronic debilitating infectious disease caused by *M. bovis* that affects cattle, other domesticated animals and certain free or captive wildlife species [2]. It is usually characterized by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bTB can occasionally assume a more progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum.

1.2.3.1. The burden of bTB

1.2.3.2. Prevalence of bTB globally

Bovine tuberculosis is found throughout the world with variable burden, but many developed countries have reduced or eliminated bTB from their cattle population by implementing control strategies. However, significant pockets of infection remain in wildlife. The highest prevalence of bTB is in Africa and parts of Asia, but the disease is also found in Europe and the Americas [94]. But figures from a recent review differ to this statement. According to a recent review of animal TB prevalence by Ramos et al. [9] and specifically for cattle was 10.3%, 13.8%, 17.8%, 33.6% and 20.5%, respectively for Africa, Asia, Europe, North America and South America. bTB is an OIE-listed disease and must be reported to the World Organization for Animal Health (OIE) as indicated in its Terrestrial Animal Health Code chapter 8.11, Article 8.11.1 [95]. Nations currently classified as bTB-free include Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados and Israel. Eradication programs are in progress in other European countries, Japan, New Zealand, the United States, Mexico, and some countries of Central and South America (Fig.3)[96]. Although bTB has been eradicated from the majority of U.S. states, a few infected herds continue to be reported, and a few states may periodically lose their disease-free status. In particular, a focus of infection in wild white-tailed deer has complicated eradication efforts in Michigan. Similar problems exist with infected badgers in the UK and Ireland, and infected brush-tailed opossums in New Zealand. bTB is still widespread in Africa, parts of Asia and some Middle Eastern countries [96].

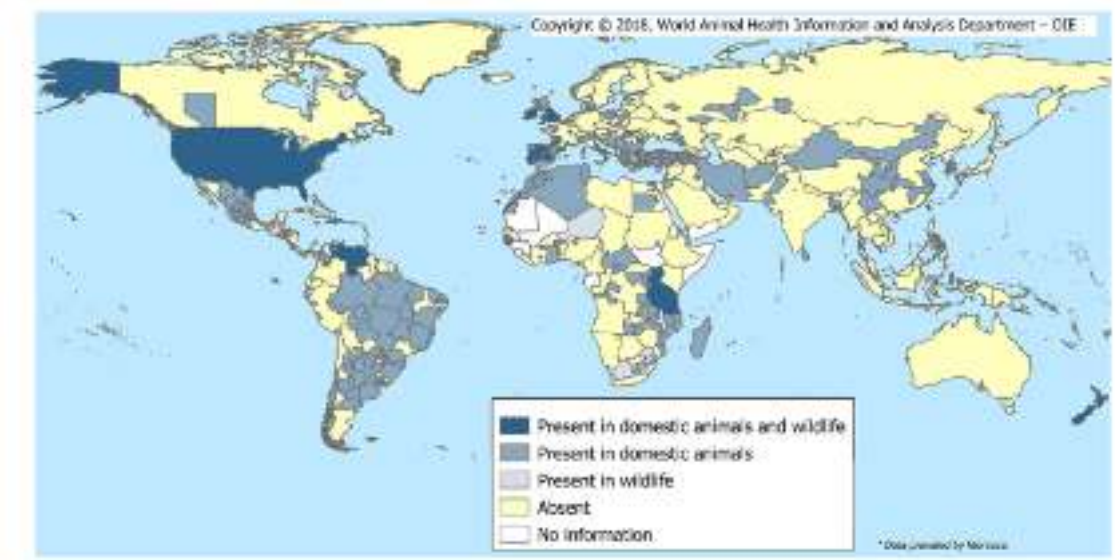


Figure 3. Global distribution of bTB in 2017 and first semester of 2018

Source: PANORAMA [96]

1.2.3.3. Prevalence of bTB in Africa

The presence of bTB in Africa was known in a period when the disease was known in Europe and Great Britain. In South Africa the disease was recorded for the first time in 1880 [97] and bTB was believed to be introduced into the local cattle population by the introduction of European breeds of cattle. However, molecular findings in the last decades and recent years challenged this hypothesis and proved bTB existed in Africa even before colonization. The detection of the presence of the indigenous *M.bovis* clonal complex African 1(Af1) and Af2 strains, respectively [13], which are restricted so far in Western and Eastern Africa. Also whole genome sequences showed that *M. bovis* likely originated in East Africa [89]. Prevalence of bTB in Africa used to be low due to the high proportion of local indigenous zebu cattle in the national herds which are less susceptible to bTB when compared to European breeds [98]. In addition difference in the farming contributed for low prevalence system where subsistence small scale and pastoral production systems were dominant which are free and less confined unlike the commercial farming [99]. However, over the last two-three decades in Africa there was increased urbanization and population growth and growing livestock farming around big cities [4]. Latino et al. [100] estimate in 2050 almost 70% of total meat and milk consumption will likely come from cities. The consequent changing livestock sector is characterized by among

other things change in the breed proportion of the national herds due to importation of European breeds for high milk production. This change in cattle breed proportion in Africa, lack of bTB control programs (except few countries like South Africa) and little attention given to bTB by authorities as veterinary authorities in Africa were concerned with the more economically important, rapidly fatal, and usually transboundary animal diseases such as contagious bovine pleuropneumonia (CBPP), East Coast fever, and anthrax lead to dissemination of bTB [11, 101].

bTB is now present in almost all African countries, affecting both domestic and wild animals. Daborn and Grange [102] reported that the disease was prevalent in 33 of 43 African member countries of the regional commission of the OIE, some member states fail to report the annual prevalence [103]. An example of the latest bTB prevalence on regional basis in Africa is provided below.

1.2.3.3.1. West and Central Africa

In West and Central Africa, bTB in cattle seems to be prevalent. In surveys that employ single intradermal comparative cervical test (SICCT), bTB in this region ranged between 3.6 % and 15.1% though higher prevalence as high as 50% were reported in few countries (Table 2). Similarly abattoir surveys using post mortem examination (pme) in countries in the region recorded a TB lesion prevalence ranging from 0.12 to 23.8% (Table 2).

Table 2. Prevalence of bTB in selected West and Central African countries

Country	Type of test[a]	Prev (%)	Reference
Nigeria	pme*,SICCT**, SICCT	9.1,15.08, 14.6	Okeke et al.[104]; Yohanna et al.[105]; Abubakar et al.[106]
Cameroon	SICCT, pme	3.59, 4-23.8	Awah-Ndukum et al.[107]; Egbe et al.[108]
Ghana	pme, SICCT	0.12, 13.8-50	Lopes et al.[109]; Bonsu et al. [110]
Chad	SICCT, pme	8, 11.3	Müller et al.[111]; Ngandolo et al.[112]
Burkina Faso	SICCT	6.05, 6.8	Boussini et al.[113]; Tarnagda et al.[114]
Niger	SICCT	3.6	Boukary et al. [115]

[a]Tests :*pme: postmortem examination; **SICCT; Single intradermal comparative cervical test

1.2.3.3.2. Eastern and Southern Africa

Bovine tuberculosis is also endemic in most of Eastern and Southern African countries (Table 3). Various researchers have reported prevalence of bTB from abattoirs and farms in Eastern and Southern region of Africa. Nalapa and colleagues [116] in Uganda conducted a cross sectional study and examined 1,576 slaughtered cattle in Mubende district municipal abattoir between August 2013 and January 2014 and reported 9.7% (153/1,576) TB-like lesions. Prevalence of bTB is dependent on the farming system (pastoral, mixed crop-livestock and urban and peri-urban systems) where bTB is low in pastoral area [117]. Mugambi and colleagues [118] conducted a cross-sectional bTB survey in three agro-ecological zones of Tanzania, namely the southern highlands zone (SHZ), eastern zone (EZ) and northern zone (NZ) and tested using SICCT a total of 391, 169 and 401 cattle in the SHZ, EZ and NZ, respectively. Results showed that a prevalence of 2.37% (n = 169) in EZ, SHZ 1.3% (n=391) and NZ, no positive result was recorded (n = 401) showing the farming system importance in bTB prevalence. In Kenya, 625 cattle from four sites within agro-pastoral and pastoral production systems were tested. In one area of Mwingi County, eastern Kenya, all the 161 cattle tested negative; while in the other three sites of Migori, in Nyanza, West Pokot and Laikipia in the Rift Valley, prevalence of 4-6% was obtained with SICTT. A study in Uganda by Inangolet et al. [119] conducted from July 2006 to January 2007 to determine the prevalence of bovine tuberculosis in the transhumant and agro-pastoral cattle herds in the border areas of Katakwi and Moroto districts using comparative intradermal tuberculin test revealed, 1.3% overall prevalence. In Eritrea in traditional livestock raising Ghebremariam and colleagues [120] screened 1077 cattle using SICCT and reported low prevalence of bTB (1.2%) comparable to Inangolet et al. [119]. These limited studies indicated estimation of the overall burden of bTB in Africa needs a comprehensive approach that includes the farming system, the type of diagnostic test, the breed etc. to come up with a representative figure otherwise will lead to under/over estimation as seen in the previous review paper discussed.

Table 3. Prevalence of bTB in selected Eastern and Southern Africa

Country	Husbandry type	Type of test[a]	Prev (%)	Reference
Tanzania		SICCT	2.4	Katale et al.[121]
Uganda		pme	9.7	Nalapa et al. [116]
Zambia		SICCT, SICCT	6.8, 4.8	Munyeme et al.[122]; Muma et al. [123]
Kenya		pme	18.95,	Gathogo et al. [124]
Sudan		pme	6	Asil et al.[125]

[a]Tests :*pme: postmortem examination; **SICCT; Single intradermal comparative cervical test

1.2.3.3.3. South Africa

Here South Africa's bTB outbreaks over 19 years (2000-2018, Table 4) is presented separate to show how bTB prevalence looks like in countries that have already implemented control program. South Africa is one of the biggest economies in Africa and South Africa started bTB scheme in 1969 probably the earliest bTB control programs in Africa [84]. Although measures to control bTB in domestic stock were in place, bTB is maintained in the wildlife, the buffalo (*Syncerus caffer*) and the Kafue lechwe [Marsh antelope] (*Kobus leche*) have been found to be maintenance hosts. The importance of other wildlife species is becoming apparent in South Africa [126]. The lesson from South Africa bTB control scheme for the rest of bTB endemic African countries and Ethiopia in particular, is that bTB control is not a quick fix and requires long term plan, resources and legal support.

Table 4. ***Mycobacterium bovis*** **cases reported in South Africa from 2000 to 2018**

Year	Outbreaks	Cases	Dead/culled
2000	10	174	181
2001	1	33	1
2002	4	123	32
2003	17	304	370
2004	11	1525	737
2005	14	747	856
2006	4	42	37
2007	6	102	50
2008	4	50	37
2009	18	36	1236
2010	8	18	7
2011	7	34	29
2012	3	90	0
2013	2	8	29
2014	8	102	66
2015	8	32	28
2016	3	247	0
2017	1	8	0
2018	3	4	3

Source: Meiring et al. [127]

1.2.3.4. Prevalence of bTB in Ethiopia

Understanding of the dairy farming systems is important as it likely affects the transmission of bTB and hence the prevalence in other farming systems. In Ethiopia, there are different dairy farming systems as described below.

1.2.3.4.1. Categorization of dairy farming systems

Ethiopia has the fifth largest national cattle herd in the world and the first in Africa with over 65.35 million heads, of which 97.76% are local zebu breeds [128]. The Ethiopian dairy systems can be categorized under three systems of operation [129, 130, 131, 132]: (a) *Mixed farming:* which is subsistence, mixed crop-livestock system, commonly practiced in the central highland regions. Cattle are the predominant species, with relatively large numbers of oxen used for traction on crop fields. (b) *Intensive farming:* such as in dairy (pure or cross-bred) farms in urban and peri-urban areas, or feedlots (fattening zebu cattle for local and export markets), and state-owned breeding centers where selected indigenous breeds are maintained for breed evaluation and for cross-breeding purposes. (c) *Pastoral/agropastoral farming:* is common in marginal, lowland areas such as in Borana, Somali, and Afar regions. Livestock kept in communally owned natural grazing ranges are at times driven far from settlements in search of good pastures and surface water. Cattle are the dominant species in this system in association with small ruminants and camels.

1.2.3.4. 2. Addis Ababa Milkshed

Brandsma and colleagues [132] conducted assessment of the major milksheds in Ethiopia and concludes that the eight milksheds rank as follows: 1. Addis Ababa 2. Adama-Asella-Ada/Debre Zeit 3. Hawassa-Dilla-Shashemene, 4. Bahir Dar-Gondar, 5. Ambo-Woliso 6. Mekele, 7. Dire Dawa and 8. Jimma. According to Brandsma and colleagues [132] Addis Ababa milkshed encompassed North Shoa Amhara, North Shoa Oromia, West Shoa and South West Shoa. This milkshed is considered the leading for dairy development in Ethiopia. Situated in an agro-ecological zone with high potential for roughage production and with plentiful by products, the area has received much public and international support. It has a relatively high number of crossbreds, and Artificial Insemination (AI) service is functioning relatively well, a large number of relatively strong cooperatives and unions for milk marketing, a growing number of milk

processors, and private sector interest to further invest in dairy processing and production (large scale producers, local and foreign investments for dairy plant establishment).

The Addis Ababa milkshed being highly intensified of all milksheds is challenged with diseases of intensification such as bTB. The first documented cases of bTB in Ethiopia were recorded in the 1960s in the annual abattoir meat inspection report of the Ministry of Agriculture [133]. Since then there were reports mainly from Universities and Research Institutions and international collaborative projects (summerized in *Ethiop.J.Health Dev*.Volume 22, Special Issue, 2008, 97-145) and these fragmented reports can hardly reflect the national burden of bTB at national level [11]. In Ethiopia there is no national bTB surveillance program like that of human TB national surveillance program which can determine an estimate of the overall prevalence of bTB in the country. Alehegne et al. [134] conducted a large scale cross sectional survey in major towns (excluding Addis Ababa and surroundings) and reported overall prevalence of 8.97% (0.82% - 24.05%) (Table 6). This bTB prevalence in major towns of Regional States was a bit higher compared to the rural and pastoral livestock farming system which ranges 0.8 to 2% [135, 117]. However, higher bTB prevalence was particularly true in the well established dairy belt/Addis Ababa milkshed in central Ethiopia where several studies have recorded over 25% bTB prevalence in animals (Table 5, Fig.4) and this could be a source of infection/threat to the emerging dairy industry in the regional towns via trade and other meanses of transmission. One of the major limitations of previous bTB prevalence surveys in central Ethiopia is the fact that they have recruited high proportion of larger herds and becuase of this recorded higher prevalences and can not be representative.

Table 5. Prevalence of bTB in Addis Ababa and surrounding towns

City/Town	Total examined	Test type[a]	Prev (%)	References
Addis Ababa	1241	SICCT	10.5	Asseged et al.[136]
	1350	pme	1.5	Asseged et al.[137]
	1869	SICCT	23.7	Elias et al.[28]
	1132	SICCT	34.1	Tsegaye et al.[27]
	2098	SICCT	18.7	Shitaye et al.[138]
	758	SICCT	25.5	Firdessa et al.[10]
	500	pme	5	Mekibeb et al.[139]
Sebeta	449	SICCT	54.6	Firdessa et al.[10]
Holeta	361	SICCT	10.2	Firdessa et al.[10]
	5424	SICCT	13.5	Ameni et al.[140]
Sululta	398	SICCT	47.5	Firdessa et al.[10]
Sendafa	341	SICCT	42.5	Firdessa et al. [10]
Bishoftu	649	SICCT	22.8	Firdessa et al. [10]
Sellalie	1041	SICCT	16.2	Ameni et al. [140]
Wuchale-Jida	763	SICCT	7.9	Ameni et al. [141]

[a]Tests :*pme: postmortem examination; **SICCT; Single intradermal comparative cervical test

In addition to the tuberculin surveys, abattoir surveys (postmortem examination) has been conducted at different abattoirs located in different towns of Ethiopia such as Addis Ababa, Adama, Melge Wondo, Awassa, Yabello, Gonder, Woldiya, Butajira, Jinka, Jimma and Hossana and bTB prevalence recorded in these abattoirs ranged from 3-10.2% [137, 142, 143]. At Addis Ababa abattoir Assseged et al. [137], Shitaye et al. [138] and Biffa et al. [142] examined 1,350, 984, and 600 cattle postmortem, respectively and tuberculous lesions detected in that order were 3%, 3.5%, and 15.2%. These studies do vary in their techniques they used for postmortem examination, where majority of them used the routine inspection technique, few studies like that

of Biffa et al.[142] also employed detailed postmortem examination which is a more sensitive method for the detection of tuberculous lesions.

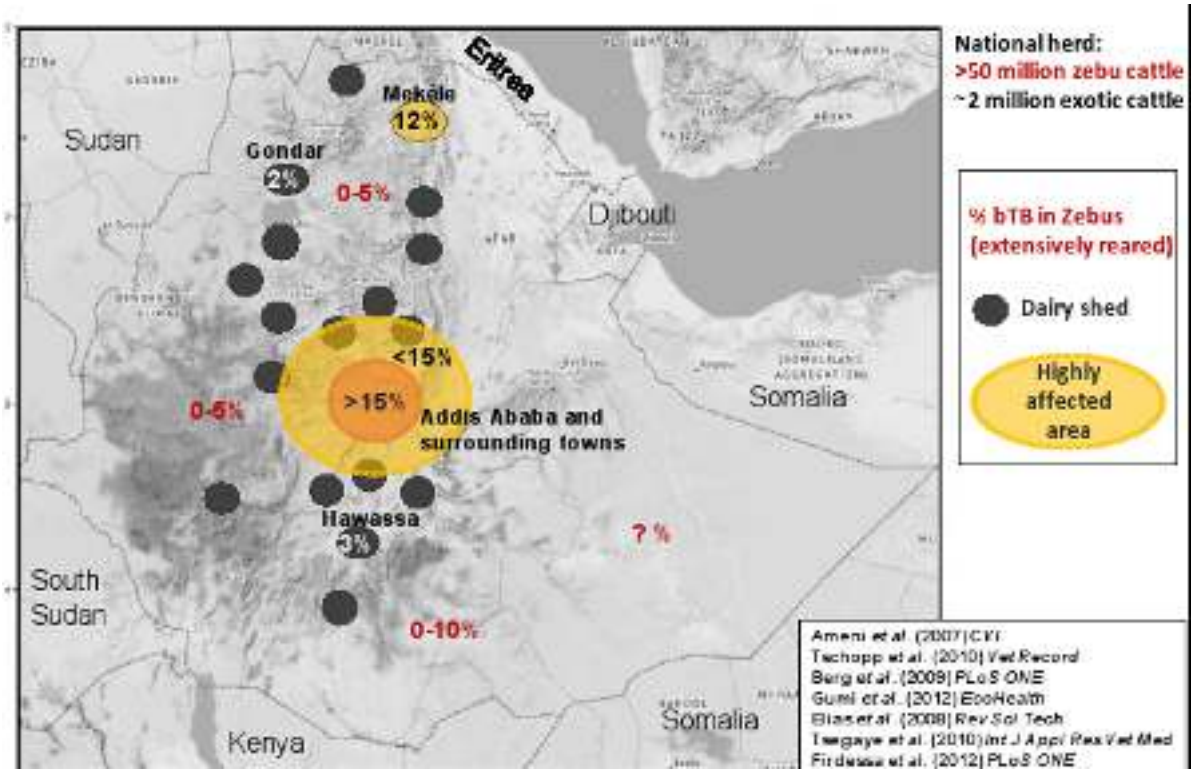


Figure 4. Distribution of bTB in Ethiopia (2007-2019)

Source: Berg [144]

Based on literature, there has been three sequencial review papers [11, 12, 138] specifically on prevalence of bTB in Ethiopia; the latest review paper by Romha et al. [12] included molecular epidemiology and zoonoses too. According to a comphrensive review by Sibhat et al. [11] a pooled prevalence estimate of bTB in Ethiopia was found to be 5.8% (95% CI: 4.5, 7.5). In both reviews the role of farming system/husbandry, breed and herd size were emphasized as factors that influence the prevalence of bTB. The prevalence of bTB in Holstein-Friesians, 21.6% (95% CI: 14.7–30.7), was higher than the prevalence in local zebus 4.1% (95% CI: 3.4–4.9). Cattle kept under intensive and semi-intensive production systems had higher prevalence, 16.6% (95% CI: 12.4–21.6), of bTB than those kept in extensive livestock production system, 4.6% (95% CI: 3.4–6.2) [11, 138].

With regard to the scope of the studies in covering different domestic animals in screening for bTB in Ethiopia, it is almost entirely limited to cattle not surprising though as cattle are natural hosts for *M. bovis*. To the best of our knowledge no study so far in Ethiopia that attempted to explore whether there has been any spill over infection of wildlife with *M .bovis* from cattle as seen in other African countries. The domestic animals other than cattle where bTB was reported include camels, goats and pigs and sheep rarely [145, 146, 147, 148]. Mamo et al. [149] (Dire Dawa), Mamo et al. [150] (Akaki and Metehara) and Surafael et al.[151] (Akaki) examined 276, 906 and 420 dromedary camels postmortem, respectively and detected tuberculous lesions in that order in 5.1%, 10.04% and 4.52% of the examined animals. Similarly, Gumi et al. [117] examined 694 camels and 81 (11.7%) had tubercular lesions, a higher prevalence even compared to most cattle abattoir surveys an indication that camels are susceptible to *M. bovis*. This may need further studies that include both natural and experimental infection - using a similar protocol that was used for breed susceptibility study in cattle in Sellale [98]. Based on the tuberculin test (SICCT) Gumi et al. [117] tested 479 camels in the Somali region and reported a relatively low individual animal prevalence of 0.4% (95% CI, 0.1–3%) compared to Beyi et al. [152] who reported 6% (n=480) in a study that was carried out in Dire Dawa City Administrative Council (DDAC) and the Somali pastoral region from June 2007 to June 2008.

Table 6. bTB prevalence in dairy cattle in Regional States major towns during 2014

Study area	Regional State	Total no. of animals examined	Tuberculin +	Tuberculin doubtful	Tuberculin -	Tuberculin + (%)
Kombolcha	Amhara	424	102	48	274	24.5
Mekelle	Tigray	383	52	23	308	13.58
Butajira	SNNP	200	27	11	162	13.5
Nekemet	Oromia	218	24	17	127	11
Wukro	Tigray	103	7	12	84	6.8
Adigrat	Tigray	239	4	4	231	1.67
Assella	Oromia	240	13	50	177	5.42
Shashemene	Oromia	250	10	10	230	4
Yirgalem	SNNP	202	8	9	185	3.96
Ambo	Oromia	168	6	24	138	3.57
Dessie	Amhara	215	4	20	191	1.86
Debre Birhan	Amhara	245	2	9	234	0.82
Total		2888	259	237	2392	8.97

1.2.3.5. Risk factors of bTB

Humblet and colleagues [153] classified bTB risk factors in cattle as risk factors at the animal level (age, sex, breed, body condition, immune status, genetic resistance and susceptibility to bTB, vertical and pseudo-vertical transmissions, auto-contamination) and herd-level risk factors (history of bTB outbreak in the herd and human antecedent of tuberculosis in the household, herd size, type of cattle industry or enterprise, management, intensity of the farming system and housing of cattle, manure, feeding, supplementary feeding and feed storage, cattle-to-cattle transmission via the faeco-oral route). However, the contribution of each risk factor to bTB infection can differ from farm to farm, region to region or country to country due to difference in

management, type of the national herd etc... and hence their relevance varies [154]. Some are consistently identified across all settings such as age, herd size and bTB history.

1.2.3.5.1. Age

Several studies have identified age as an important risk factor for bTB here in Ethiopia or elsewhere where bTB is endemic [10, 155, 156]. McKinley et al.[155] analysed bTB detection in Great Britain from 1998-2013 in slaughter houses and found a strong effect of age with animals slaughtered at > 60 months of age having 5.3 times the odds of detection compared to animals slaughtered between 0–18 months of age. In Ethiopia Mamo et al. [157] screened 1087 cattle using SICCT in Afar and the age specific proportion of reactors was 4.9% for less than 2 years, 5.9% for 2 to 5 years, 13.1% for greater than 5 years to 9 years and 15.9% for greater than 9 years showing bTB infection increases with an increase in age. However, still there are controversies over whether the relationship of age and bTB infection is linear or like an epidemic curve shaped (meaning less in early age and in old ages i.e. anergy and high in the middle ages).

The relationship of age and bTB infection could be linear as reviewed by Broughan et al. [158] or like an epidemic curve shaped as seen in age specific reactor rates modeling [159]. It is complex issue and likely explanations were suggested for each scenario. Longevity and higher probability of contact or exposure overtime; more probability of responsive or unresponsive (anergy) for skin test at certain age level which again could be related to resistance with age are usually mentioned in explaining age and bTB relationship. One of the problems in study of age as a risk factor is how accurate and reliable the age estimation was. Here in Ethiopia there is no national cattle registry and tracing system - a system where every cattle will have ID and its birth date and other parameters get recorded and its where about can be traced and cattle movement also can be regulated. However, in reality age estimation at times depends on how good the owner can remember as few farms usually keep records. Therefore, it is important that data for risk factor analysis has to be obtained from commercial dairy farms where they usually keep records or at most effort has to be made to best estimate the age of each animal using a combination of parameters such as dentition.

1.2.3.5. 2. Breed

According to Central Statistical Agency of Ethiopia (CSA) [128], Ethiopia has 65.35 million heads of cattle which makes the country first in Africa and fifth in the world after Brazil, India, China and USA in that order [160]. By proportion the local zebu breeds make up the lion share of Ethiopian national cattle population (97.71%), followed by crossbreds (1.91%) and exotics (pure) makes only 0.32%.

Vordermeier and colleagues [98] conducted a well designed experimental study at field level where a total of 5,424 cattle of different breeds (925 Holsteins, 1921 cross-breeds and 2578 zebus) selected from the same husbandry system of Selalle (Northwestern Shoa) were compared for their susceptibility to *M.bovis* infection. These cattle were evaluated using tuberculin skin test (compares reaction sizes), post-mortem (severity, lesion distribution - assessed using a quantitative pathology scoring system) and in vitro immune responses from whole blood cultures measured using interferon-gamma (IFN-γ). In all of these parameters zebus were found to be less susceptible to *M.bovis* compared to Holsteins and their crosses. In observation study by Firdessa et al. [10] where 1837 crossbred cattle screened with the SICCT test and 62.8% were reactors where as local zebu thought small numbers were included in the study, was low prevalence (1.5%).

1.2.3.5.3. Herd size

Numerous studies in developed and under developed countries identified herd size as one of the major bTB herd-level risk factors [141, 161, 162, 163]. Whenever the number of animals in a farm increases, the probability of cattle-to-cattle contact increases and hence spread of bTB infection. According to a recent study in Bangladesh [163], the odds of bTB was 3.9 times higher in herds having more than four cows than those with 4 cows. Ameni et al. [141] in central Ethiopia and Kemal et al. [164] in Dire Dawa recorded significant association between herd size and bTB prevalence. Mamo et al. [157] and Dejene et al. [156] in Afar a region known for its pastoral production system identified herd size as a significant risk factor for bTB prevalence.

However, in a similar study by Gumi et al. [117] in Somali and Guji pastoralists of southeastern Ethiopia, herd size was not a significant risk factor. Gumi et al. [117] discussed their finding that in a pastoral system where animals constantly live in the open and under extensive conditions,

bTB, irrespective of the herd size, is rare. In agreement with Gumi et al. [117] but in the high land rural livestock production systems of Ethiopia, Tschopp et al. [135] found no significant association between herd size and bTB prevalence in their cross-sectional study that was conducted in three regional zones of three regional states (Oromia, Amhara, and Southern Nations, Nationalities and People Region (SNNPR)). It looks whether herd size is a confounding factor for type of dairy farming system or a risk variable on its own needs a properly designed study particularly in Africa where we see different types of livestock production systems.

1.2.3.5. 4. Dairy farming system

Bovine tuberculosis is highly associated with the types of farming system. bTB thrives in intensification. Brandsma and colleagues [132] has described the Ethiopian farming system. The farming system will determine contact between cattle -to- cattle, cattle -to-wildlife but also between cattle and contaminated environment. In the pastoral type of livestock production system, there is frequent movement of cattle and other animals covering vast areas in search of water and pasture where there will be only brief close contacts during congregation around water points. Ghebremariam et al. [120] in Eritrea assess the association of water points with bTB prevalence and found no significant association. Whereas the urban and peri-urban is characterized by keeping large number of improved breeds (commercial farming) leading to over-crowding particularly in Addis Ababa due to shortage of land and cows are kept indoors all the time. And due to the fact that bTB is a disease of intensification/high animal density, more surveys were conducted in the urban and peri-urban dairy production compared to mixed farming and pastoral production systems.

Ameni et al.[165] comparing the effects of zero grazing versus free grazing among 54 Holstein and 37 zebu cattle, it was reported that the severity of bTB was significantly higher (with significantly higher interferon-gamma levels and more severe lesions in cattle kept indoors at a higher population density than in cattle kept on pasture. In addition to close contact, stress caused by overcrowding or nutritional differences between housed and pastured animals was mentioned as contributing to the spread of the disease. Similar observation was made in Bangladesh, where the risk of bTB was 3.3 times higher in non-grazing cows than grazing cows [163].

1.2.3.5.5. bTB history

An area or herds with history of bTB have been consistently identified as being at significantly higher risk of future bTB outbreak than other areas or herds [166]. An extensive review of 4255 papers by Broughan et al. [158] concluded about these studies that: despite differences in study design and location, some risk factors are consistently identified, e.g. herd size, bTB history, presence of infected wildlife, whereas the evidence for others is less consistent and coherent e.g. nutrition, local cattle movements. Persistent infection could be due performance of the test (false negatives-lead to wrong decision by not in removing such animals), stage of the disease during the time of the test and anergy.

In summary previous risk factor analysis studies of bTB in Ethiopia has identified significant risk factors which has role in bTB transmission and are helpful for any future intervention strategy. However, most of these studies except few [135, 156] have problems in the study design and use of appropriate statistical test for the data analysis- these two are important in any epidemiological risk factor analysis study. How sample size was determined and how herds or individual animals were selected was not properly addressed. How cattle herds were selected proportional to the size of the cattle population is important for proper representation. Also how clustering effect (barn/herd/area etc...) was handled in data analysis is another area seen as a limitation. Therefore proper identification of potential risk factors requires proper study design and rigorous statistical analysis.

1.2.3.6. Transmission of bTB

The understanding of the routes of transmission is critical to effectively control bTB [101]. *M.bovis* transmission and establishment of infection is mainly by respiratory system. A single bacillus transported within a droplet nucleus is probably sufficient to establish infection within the bovine lung. Infected cattle should always be considered as potential sources of infection, since studies have demonstrated that a significant proportion of tuberculous cattle excrete *M. bovis* [167]. Postmortem examination of tuberculin test positive cattle in central Ethiopia showed that most had TB lesions in their lungs and/or lung associated lymph nodes [10]. Additionally calves are also exposed by ingestion of milk as adults which can be exposed via ingestion of *M. bovis* contaminated food and water and often develop primary foci in lymph tissues associated with the intestinal tract [78]. In both human and bovine tuberculosis, after entry through

inhalation or ingestion, bacilli localizes at the point of entry and produce typical tubercle in associated lymph nodes. Mostly pharyngeal, lung and mesenteric lymph nodes are affected [77, 79].

In addition to respiratory tract there are several other routes of transmission for *M. bovis* as reviewed and summarized by Drew et al. [168] (Table 7).

Table 7. Summary of the main routes of transmission of *Mycobacterium bovis*

Route	Description	Infective dose	Evidence
Inhalation (most common)	• Aerosols generated by coughing, sneezing • Direct inhalation of aerosols	Very low: single bacillus	• Experimentally demonstrated in a variety of species • Involvement of lymph nodes associated with the respiratory tract
Ingestion (common)	• Feed, water contaminated with mucous, nasal secretions, feces, urine • Milk from infected dam	High: several million bacilli	• Lesions in mesentric lymph nodes of lymph nodes of naturally infected animals • Experimentally demonstrated Epidemiological evidence
Transcutaneous (uncommon)	• Contamination of existing skin abrasions • Bite wounds	Unknown	• Humans handling infected carcasses, e.g., butcher's wart • Bite wounding in ferrets, badgers
Pseudovertical (rare)	• Consumption of milk from infected mothers • Close contact between mother and offspring	Unknown	• Epidemiological evidence in cattle, badgers, brushtail possums, and white-tailed deer
Vertical (very rare)	• Intrauterine from infected dam to offspring	Unknown	• Lesions in liver and portal system in calves from infected dams

Source: Drew et al. [168]

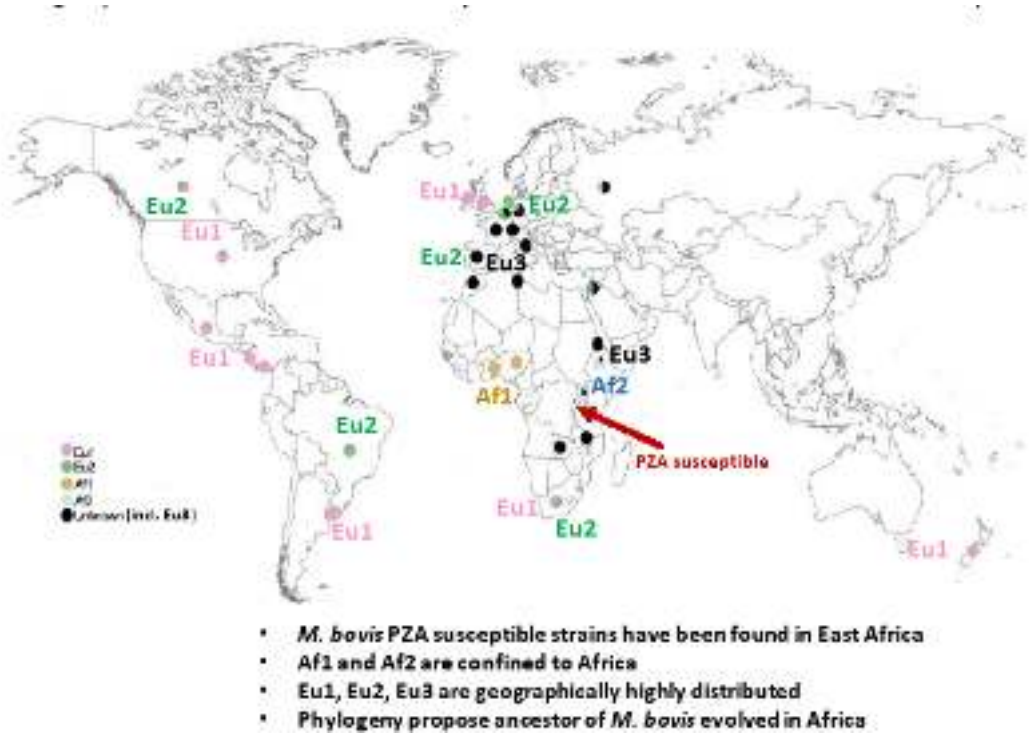
Within herd cattle-to-cattle, transmission may result due to aerosol transmission between animals in close contact. The locality of the primary lesions observed in postmortem examination provides the best indication of the route of infection. Tuberculous lesions are frequently found largely confined to the respiratory tract [10]. The source for this infection could be as a result of

introduction of purchased infected animals or contiguous spread [167]. bTB is also important in wildlife and serves as a reservoir of infection for cattle and it is a challenge for countries such as South Africa, UK and USA.

1.2.3.7. Molecular epidemiology of *Mycobacterium bovis*

Molecular epidemiology can be defined as the application of the techniques of molecular biology to the study of the epidemiology of disease in populations [169]. The term "molecular epidemiology" routinely appears in the titles of articles that use molecular strain-typing techniques—regardless of whether there is any epidemiologic application. What distinguishes molecular epidemiology is both the "molecular," the use of the techniques of molecular biology, and the "epidemiology," the study of the distribution and determinants of disease occurrence in populations [170]. Traditional typing systems based on phenotypes, such as serotype, biotype, phage-type, or antibiogram, have been used for many years. However, recent molecular methods that examine the relatedness of isolates at a molecular level have revolutionized our ability to differentiate among bacterial types and subtypes [171].

The presence or absence of a Region of Difference (RD) has been used to infer the evolutionary process of members of the MTBC of which *M. bovis* is a member. These host-specific strains arose from a common ancestor by the successive loss of RDs of DNA [170]. *M. bovis* has further been classified into different clonal complexes based on deletion of specific, defined segments of its genome. Deletion of RDAf1, RDAf2, and RDEu1, respectively, characterizes the African 1 (Af1), African 2 (Af2), European 1 (Eur1) and European 2 (Eur2) clonal complexes, respectively, found in Western Africa, Eastern Africa, the British Isles and their former colonies and France and Italy [13, 111, 173, 174, Table 8](Fig.5)



- *M. bovis* PZA susceptible strains have been found in East Africa
- Af1 and Af2 are confined to Africa
- Eu1, Eu2, Eu3 are geographically highly distributed
- Phylogeny propose ancestor of *M. bovis* evolved in Africa

Figure 5. Geographic distribution of *Mycobacterium bovis* clonal complexes

Source: Loiseau et al. [89] (modified by Berg [144]

Table 8. Definition and description of the Af1, Af2, Eu1 and Eu2 clonal complexes of *Mycobacterium bovis.*

Clonal complex		Reference
African 1 (Af1)		Müller et al.[111]
Definition	Deletion of RDAf1 (5.3 kb, between Mb0586c and Mb0590c)	
Spoligotype marker	Absence of spacer 30	
Distribution	At high frequency in sub-Saharan West-Central Africa (Mali, Cameroon, Chad and Nigeria)	
African 2 (Af2)		Berg et al.[13]
Definition	Deletion of RDAf2 (14.1 kb, between Mb0599 and Mb0610)	
Spoligotype marker	Absence of spacers 3–7	
Distribution	At high frequency in East Africa (Uganda, Burundi, Tanzania and Ethiopia)	
European 1 (Eu1)		Smith et al.[173]
Definition	Deletion of RDEu1 (806 bp in TreY)	
Spoligotype marker	Absence of spacer 11	
Distribution	At high frequency in: the British isles, South Africa, Australia, New Zealand, The New World (except Brazil), Korea.	
European 2(Eu2)	Deletion of RDEu2	Rodriguez-Campos et al.[174]
Spoligotype marker	Absence of spacer 21	
Distribution	Present at low frequency in both France and Italy and absent from the British Isles	

Different molecular markers have been used for typing of *M. bovis* for tracing the source of outbreaks and assess the pattern of disease transmission [171]. Various studies have applied spoligotyping (most frequently used), mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) and whole genome sequencing (WGS) of *M. bovis* to identify geographical localization, routes of transmission and trace the origin of the outbreak [175, 176, 177, 178]. Taking the published data into consideration, SB0120 and SB0121 are predominant spoligotypes of *M. bovis* circulating among animals around the world [120]. According to various studies in Africa the prevalence and distribution of spoligotypes on regional bases looks like: SB0944 is localized in Western Africa [108, 111], SB0133 is prevalent in Eastern Africa [142, 179] and SB0120, SB0134, SB0140, SB0121, SB0130, and SB0268 are found in Southern and Northern Africa [180, 181, 182].

In Ethiopia according to a review by Romha and colleagues [12] of published scientific peer-reviewed articles from 1998 to 2017 exclusively conducted in Ethiopia, SB1176 spoligotype was found to be the predominant strain. This is unlike other east African countries where SB0133 is the dominant spoligotype. In Ethiopia more than 20 spoligotypes have been reported from different parts of Ethiopia and from different hosts but mainly cattle [183, Table 9].

Table 9 . Spoligotypes isolated from cattle and other animals in Ethiopia

Spoligotype	MTBC strain	Host species
SB0133, SB0134, SB0303, SB0912, SB0933, SB1176, SB1265, SB1468, SB1476, SB1477, SB1488, SB1489, SB1490, SB1491, SB1492, SB1517, SB1518, SB1519, SB1520, SB1521, SB1522, SB1941, SB1942, SB1983	*M. bovis*	Cattle
SB0133, SB1953	*M. bovis*	Camel
SB0933, SB1942, SB1983	*M. bovis*	Human
SIT1, SIT59, SIT149, SIT262, SIT1688, SIT1742	*M. tuberculosis*	Cattle
SIT149, SIT837	*M. tuberculosis*	Goat

Source: Bogale et al. [183]; Romha et al. [12]

Deletion typing studies conducted so far helped to know the predominant spoligotype/s and from the phylogenetic analysis, it was possible to examine the relatedness or clonality of the

isolates. However, there is no data in Ethiopia that tried to analyze the genetic diversity of *M.bovis* using WGS.

1.2.3.8. Diagnosis of *Mycobacterium bovis* infection

Diagnosis of bTB can be based on clinical diagnosis and laboratory examination.

1.2.3.8. 1. Clinical signs

Bovine tuberculosis may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs. The bacteria can also lie dormant in the host without causing disease for long periods. The usual clinical signs include: weakness, loss of appetite and weight, fluctuating fever, dyspnoea and intermittent hacking cough, signs of low-grade pneumonia, diarrhoea, enlarged and prominent lymph nodes [2].

1.2.3.8. 2. Diagnostic tests of bovine and zoonotic TB

Clinical signs of bTB are not only specific to bTB but shared by other diseases or syndromes and, therefore, do not enable to make a definitive diagnosis based on clinical signs alone. There are different laboratory based tests which are in use currently to confirm bTB though none of them can confirm for certainty (none with high sensitivity and specificity).

1.2.3.8.3. Tuberculin skin test (TST)-Field test

TST involves measuring skin thickness after injecting bovine tuberculin intradermal into the measured area and measuring any subsequent swelling at the site of injection 72 hours later [2]. TST can be conducted at different body sites but OIE recommends the caudal fold test (CFT), the (mid) cervical intradermal test (CIT), or the single intradermal comparative cervical test (SICCT) [2,184]. The skin of the neck is regarded to be more sensitive to a tuberculin related hypersensitivity reaction than the skin of the caudal fold. The single intradermal comparative cervical test (SICCT) is used to differentiate *M. bovis* infection in countries where non-tuberculous mycobacteria exposure (such as Para-tuberculosis or Johne's disease) is also possible [185]. TST is one of the standard diagnostic procedures for detecting *M. bovis* infections in cattle accepted by OIE for bTB international trade (export test), surveillance and eradication purposes;

the other being the IFN-γ assay [2]. TST though has limitation particularly in its sensitivity particularly in animals with the advanced stage of the disease, old age (anergy), stress and recently calved cows, hugely contributed for eradication of bTB. All countries which implemented test and slaughter policy for eradication of bTB used TST for screening purposes and those positive for TST were slaughtered.

Tuberculin (purified protein derivative-PPD) is used for bTB test currently. Like the tubercle bacilli it was discovered by Robert Koch in 1890. A glycerin extract of tubercle bacilli (initially known as Koch's old tuberculin (OT)), which Koch named tuberculin, when inoculated subcutaneously in guinea pigs that were infected with tuberculosis caused a reaction not seen in healthy animals. In humans, Koch noticed no reaction in healthy individuals, whereas in patients with active tuberculosis, a severe reaction occurred, characterized by fever, chills, and skin inflammation leading to necrosis. Koch believed that, in afflicted individuals, tuberculin produced a reaction that slowed or halted disease. He also believed that the reaction provided diagnostic evidence of acute tuberculosis [186]. Though it was later proved that tuberculin did not cure tuberculosis [187], its use for diagnosis was continued to be studied. Tuberculin preparations from the heat-treated products of growth (glycerol broth) and lysis of *M. tuberculosis or M. bovis* were known as human and bovine tuberculins, respectively. In the 1940s, the 'heat-concentrated synthetic medium tuberculins' or HCSM tuberculins, prepared from cultures in a synthetic liquid medium, replaced the 'old' tuberculins. The old and HSCM tuberculins have been replaced, almost world-wide, with the purified protein derivatives or PPDs. Seibert a biochemist purified the 'active principle' from Koch's Old Tuberculin, and she termed it purified protein derivative, which rapidly became known as PPD [188]. Currently Bovine PPDs is prepared from *M. bovis* production strain AN5 [2].

1.2.3.8. 4. Gamma-Interferon (IFN- γ) Assay

The IFN-γ assay is one of the tests used for diagnosis of bTB. It is developed in Australia in the late 1980s based on the same cell-mediated immune response as the TSTs and measures the production of IFN-γ by sensitized lymphocytes exposed in vitro to PPD or similar cocktails of antigens [189]. IFN-γ assay has been extensively trialed on more than 200 000 cattle since 1988 in Australia, Brazil, Ireland, Northern Ireland, Italy, New Zealand, Romania, Spain and the USA [190]. IFN-γ assay is approved by World Organization for Animal Health [2]. In this test system

whole blood is incubated with bovine PPD, avian PPD or negative control antigens, and IFN-γ released by sensitized lymphocytes in the supernatant plasma is then measured by enzyme linked immunosorbent assay (ELISA). Compared to TST, the IFN-γ assay has better sensitivity but lower specificity [191].

1.2.3.8. 5. Culture and molecular based tests

Despite our extensive knowledge on tuberculosis, disease diagnosis and the identification of the infecting mycobacterial species is not yet a simple matter. Methods used for the laboratory diagnosis of tuberculosis are continually evolving in order to achieve more rapid, less expensive, and accurate results. Acid-fast staining and culture for mycobacteria remain at the core of any diagnostic algorithm [192].

1.2.3.8. 5.1. Specimen collection

Specimen collection of tuberculosis disease in humans and animals will depend on the clinical manifestation of disease. The most common sources in humans are respiratory specimens including sputum, bronchial aspirates, and bronchoalveolar lavage fluid; however, other samples could be submitted for analysis [192]. Sputum is the most common specimen obtained for the diagnosis of pulmonary infection with MTBC or NTM. For *M. bovis* diagnosis the most commonly considered samples include sputum and FNA and tuberculos tissue lesions in animals. Milk, nasal swabs and faeces could be considered for molecular tests which are having high sensitivity [193,194]. Specimens should be collected in sterile, leak-proof containers and do not generally require transport media for preserving viability due to the hardy nature of mycobacterial organisms. Tissue may be placed in a small amount of sterile saline to avoid dehydration, while non-sterile water should be avoided due to the possibility of confounding contamination with environmental mycobacteria [195]. Most specimens should be re-frigerated during transport to the laboratory and up until the time of processing to maintain the viability of any mycobacteria present while preventing overgrowth of contaminating bacterial organisms. Mycobacteria become more concentrated in the sputum as patients sleep, so smear sensitivity increases with the use of early morning sputum [196].

1.2.3.8. 5. 2. Acid-fast stains for mycobacteria

Microscopic evaluation of stained smears is a rapid and in-expensive screening method for mycobacteria within clinical specimens. An acid-fast stain was developed in 1885 and still it is

an important diagnostic method in use [197]. When compared taking culture as gold standard, AFB stain has high specificity (98-99%); however, its sensitivity is low and it ranges from 22 to 80% depending on the burden of mycobacteria, the type of AFB stain used, and experience of the laboratory technician [195, 198,199].

1.2.3.8.5.3. Mycobacterial culture

Culture is the WHO-recommended gold standard for the diagnosis of TB disease. Organism isolation is not only important for definitive diagnosis but also for determining phenotypic drug susceptibility testing (DST) [200]. .Following decontamination, specimens may be cultured for the growth of MTBC and NTM. Culture is still considered as gold standard and performed on solid egg-based media, such as Lowenstein-Jensen (L-J) media. Despite the slow growth rate, culture for mycobacteria is approximately 100 fold more sensitive than AFB smear, requiring only 10–100 CFU/mL of specimen for reliable growth [196]. While some laboratories still use L-J media, many have transitioned to using more chemically defined agar-based media optimized for faster mycobacterial growth. The use of Middlebrook 7H10 or 7H11 agars, for example, allows for visible MTBC colony growth in 10–12 days as compared to 18–24 days with L-J media [195]. However, agar-based media is less stable and more prone to deterioration. For example, exposure to excessive heat or light may lead to the release of formaldehyde which is toxic to mycobacteria and may inhibit growth. Most *Mycobacterium* species, including MTBC, grow best at a temperature of 35–37 °C. Due to this slow growth rate, it may take several weeks for colonies to become visible on culture plates. Cultures are typically held for 6–8 weeks before being discarded and reported as negative [201].

1.2.3.8.5.4. Molecular diagnostics

Confirming the growth of MTBC in culture is important and unlike traditional biochemical tests, molecular methods allow for rapid species identification. There are several molecular technologies currently employed by clinical diagnostic laboratories to identify isolates from culture including nucleic acid hybridization probes, line probe hybridization assays, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), and DNA sequencing [195]. However, these tests require culture [202] and due to the slow growth rates of mycobacteria in culture, methods that rely on cultured organism lead to a delayed diagnosis despite their rapid result. And because of this there have been various efforts to

develop assays that can directly detect from the clinical specimen (Table 10). Khosravi et al. [203] assessed and recorded different results for the performance of five target genes (IS*1081*, IS*6110*, hsk65kd, mbp64 and mtp40) using nested PCR for detection of *M. tuberculosis* in different extrapulmonary specimens (lymph node, bone, wound, pleural effusion, urine, ascites fluid, CSF and others) but overall IS*1081* demonstrated high detection of *M. tuberculosis*..

Table 10. Sensitivity and specificity of real-time PCR assays for different targets and for different specimens

Target genes	Assay performance by Specimen type										Refs
	Overall		pulmonary		Extrapulmonary/FNA		AFB-negative		milk		
	Se(%)	sp (%)	Se (%)	sp (%)	Se (%)	sp (%)	Se (%)	sp (%)	Se (%)	sp (%)	
IS1081 and IS6110 (Xpert MTB/RIF Ultra assay)	87.5	98.7	87.5	98.7	ND	ND	78.9	98.7	ND	ND	Chakravorty et al [204]
whiB3 and *pstS1* (Multiplex real-time PCR-short$_{TUB}$ assay)	88	95	ND	ND	88	95	88	95	ND	ND	Alcaide et al.[205]
rpnB (Roche Cobas Amplicor MTB-PCR)	60	98	73	99	ND	ND	ND	ND	ND	ND	Abdeldaim et al.[206]
85B mRNA(RT-qPCR)	98.33	95.00	ND	ND	ND	ND	ND	ND	ND	ND	Demirci et al.[207]

Insertion sequence *6110* (IS*6110*) though widely used as a target for detection and typing of *M. tuberculosis,* it is with high polymorphism of this insertion sequence (0-20 copies) [175]. However, another multi-copy insertion sequence IS*1081* would perform better as it is specific and highly conserved and stable gene with four to six copies in the MTBC genome. Besides, the IS*1081* assay will detect rare *M. tuberculosis* strains that do not contain any IS*6110* genes- as shown in Chakravorty et al [204] study. Because of this merit IS*1081* was chosen as the target gene with the aim of improving the detection of all species/screening in the complex though it

will not specify exact species within the complex. For detection of *M. bovis* the TaqMan assay used the RD4 deletion as the RD4 deletion is unique for this species. No signal should be detected in the assay unless *M. bovis* genomes are present. And for the detection of *M. tuberculosis*, an assay that detects the presence of RD9 was used. The TaqMan assay for RD4 was based on the protocol described by King et al. [208] and the assay for IS*1081* was based on a protocol published by Taylor et al. [209].

For *M. tuberculosis*, the Xpert MTB/RIF test which is an automated, cartridge-based system that benefits from ease of use and a closed amplification system that reduces the potential for cross-contamination between specimens and is simple for laboratory technicians to perform and no advanced biosafety equipment is needed. And for these reasons, in 2010 WHO recommended use of the Xpert MTB/RIF for patients with suspected pulmonary tuberculosis in developing countries for detection of TB and rifampicin resistance [210]. For *M. bovis* there were various research outputs using amplification methods (RD-4 deletion typing by PCR) and Real Time PCR for direct detection of various specimens from humans and animals.

Seagar and his colleagues [211] in Scotland tried to evaluate two in-house *IS6110* real-time PCR assays for the rapid detection of *Mycobacteria* in clinical specimens. They processed totally 87 specimens (62 sputa, 15 bronchoalveolar lavages and one pleural fluid) and nine non-respiratory samples (four pus, two aspirates, one abscess fluid, one cerebrospinal fluid (CSF) and a tissue specimen). The sensitivities of the two assays range from 87 and 100% when compared with culture but both had similar specificity of 91.2%. In India Negi and his colleagues [198] processed 156 clinical samples for detection of *M. tuberculosis* by Ziehl Neelsen (ZN) smear examination, LJ medium culture, BACTEC radiometric culture and PCR tests. And the sensitivities were 74.4% for PCR test, 33.79% for ZN smear examination, 48.9% for LJ culture and 55.8% for BACTEC culture. However, there was no significant difference as far as specificity of different tests was concerned. Also Kim and his colleagues [212] in South Korea evaluated multiplex real-time PCR for direct identification of MTC and NTM on sizable number of reference isolates and clinical specimen. And a total of 1075 of 1142 (94.1%) MTBC and 141 of 143 (98.6%) NTM were isolated from respiratory specimens, and the remaining from nonpulmonary specimens. The target gene for MTBC was the insertion sequence *IS6110*. Primers and probes were designed manually or using the Primer3 program and five primer–probe

mixes were created. Mix I had primer–probe sets specific for MTBC, NTM and internal control. Mix II had primer–probe sets specific for *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. abscessus* and an internal control. Mix III had primer–probe sets specific for *M. chelonae*, *M. peregrinum*, *M. gastri*, *M. gordonae* and *M. terrae*. Mix IV had primer–probe sets specific for *M szulgai*, *M. kansasii*, *M. ulcerans*, *M. septicum* and *M. mucogenicum*. Mix V had primer–probe sets specific for *M. celatum*, *M. smegmatis*, *M xenopi*, *M. marinum/M. ulcerans* and *M. scrofulaceum*.

From bulk tank raw milk Zuma´rraga et al. [213] tried to detect *M. bovis* using IS6110 polymerase chain reaction (PCR) and evaluated 177 samples. They got no growth on culture but positive PCR results were obtained in 102 (40%) showing the importance of molecular tests in paucibacilliary specimens. And this if supported by spiked experiment could have ruled out the possibility of false positives. There were similar comparison tests for detection of *M. bovis* in milk in Argentina and Brazil which are currently working to eradicate the disease and conduct surveillance programs [192, 214]. From FNA samples (n=132) collected in Addis Abeba, Ethiopia, Zewdie et al. [215] reported 43.2% sensitivity and 87.9% specificity for ZN staining and 93.2% and 62.1% for direct PCR (RD9 and RD4 deletion typing based PCR) in that order. All the isolates were *M. tuberculosis* and no *M. bovis* was detected. In another study in south Ethiopia, FNA specimens from 40 patients presenting at a rural health and diagnosed as positive for TBLN on the basis of clinical and cytological criteria were analyzed for mycobacterial DNA by PCR (*pncA* gene) and among 35 PCR-positive cases of TBLN, 29 (82.9%) were caused by *M. tuberculosis* and six (17.1%) were caused by *M. bovis* [216]. These varying results clearly showed the importance of complementing clinical detection with experimental methods (spiked specimens).

1.2.3.8.5.5. Genotyping methods

Several systems have been proposed to classify MTBC strains into distinct lineages and families [217]. The first attempts to differentiate strains were based on phage typing and on the basis of lytic results with 11 mycobacteriophages, it was proposed that the species *M. tuberculosis* may be subdivided into at least 3 major phage types, A, B, and C, and into 2 subjects, Ax and A2 [218]. Nowadays different molecular typing methods are available and the most frequently used genotyping methods include, Spoligotyping and VNTR typing and some *IS6110* RFLP typing

[175, 178, 217, 219]. Whole genome sequencing and identification based on SNPs is now considered for higher resolution and this methodology is now common in most countries.

1.2.3.8.5.5.1. Restriction fragment length polymorphism (RFLP) typing

RFLP analysis - compares the subfragments produced when these molecules are cleaved by the same Restriction Endonuclease. ; Different numbers and/or sizes of fragments are produced from a given molecule if that molecule has lost or gained a restriction site or if it has undergone any insertion or deletion of nucleotides. The sets of subfragments from each sample are compared by gel electrophoresis and are manifested by the development of bands at different positions in the gel. Various types of samples can be examined by this method [175, 220, 221]. IS6110 is an insertion sequence belonging to the enterobacterial IS3 family and is a 1361 bp long sequence and considered as specific for *the MTBC*; different strains contain different numbers of copies of *IS6110* – e.g. the genome of the reference strain of *M. tuberculosis (H37Rv)* contains 16 copies, while some strains apparently lack this sequence. Strains of *M. bovis* generally contain a single copy but some may have up 20 copies [221], Table 11).

Table 11. Repetitive DNA sequences in *Mycobacterium tuberculosis* complex

Repeated sequence	Host range	Copy number	Polymorphism
IS6110	*M. tuberculosis*	0-20	High
	M. africanum	0-20	High
	M. bovis	1-20	High
	M. bovis-BCG	1-2	None
IS1081	*M. tuberculosis*	5-6	Low
	M. africanum	5-6	Low
	M. bovis	5-6	Low
	M. bovis-BCG	5-6	Low
DR cluster	*M. tuberculosis*	1	High
	M. africanum	1	High
	M. bovis	1	High
	M. bovis-BCG	1	High

Source: Kanduma et al. [175]

RFLP typing used to be considered as 'gold standard' particularly for *M tuberculosis* typing, however, it has its own limitation and is today replaced with other typing methods in most TB laboratories.. Interpretation of the results is difficult because the large numbers of fragments generated produce a complex pattern and only a small number of different RFLP types are observed. It has limited discriminatory power in isolates with five or less IS*6110* bands (most *M. bovis*). It is a lengthy process (require culture) and it is difficult to compare results between laboratories (reproducibility) [220, 222].

1.2.3.8. 5.5.2. Spoligotyping

The most common epidemiological molecular-typing method applied to *M. bovis* is spoligotyping. The name ‘spoligotyping’ derives from a contraction of the phrase ‘spacer oligotyping’ [221]. This method identifies polymorphisms among the spacer units in the direct repeat (DR) region of the chromosome. The DR region comprises multiple, virtually identical, 36-bp regions interspersed with DNA spacer sequences of a similar size (direct variant repeat

(DVR) units). The DR region can contain >60 DVR units. However, 43 of the spacer units were selected from the spacer sequences of *M. tuberculosis* reference strain H37RV and *M. bovis* BCG strain P3 and are used in the standard application of spoligotyping to strains of the *M. tuberculosis* complex. Polymorphisms in spoligotype patterns are visualized by the loss of hybridization signal from spacers**.** Each spoligotype pattern is given assigned an international name (for example, SB0140) by Mbovis.org data base [https://www.mbovis.org/ accessed Nov 20, 2020]. The advantages of 43 spacer-spoligotyping are that it is highly reproducible and, since it is based on PCR amplification, very little DNA (20–50 ng) is needed. It has even been used with DNA extracted directly from sputum from smear-positive patients, however, with mixed results. The main limitation is the inferior discriminatory power when compared with IS*6110*-RFLP and MIRU-VNTR typing [222]. Based on this spoligotyping method there were lots of various *M. bovis* spoligotypes reported. For instance to mention few predominant spoligotypes in some countries: In French out of 4,654 *M. bovis* isolates from 1978 to 2013 that were typed by spoligotyping, only 3 spoligotypes were predominant and accounted for more than half of the total strain population: SB0120 (26%), SB0134 (11%) and SB0121 (6%) [223]. In Mexico SB0121is dominant [224], in China SB0121 [225], in Great Britain SB0140 [219], while in the USA eighty-five percent (90/106) of the human *M. bovis* cases were clustered in two spoligotypes, SB0145 and SB1040 [178, 226]. In Ethiopia to the best of the author search, spoligotypes of *M. bovis* isolated from human were not reported except for Firdessa and his colleagues [194] who reported four human *M. bovis* isolates that showed typical bovine spoligotype profiles lacking spacers 3, 9, 16, and 39–43. In addition, they lacked spacers 4–7 and were deletioned for RDAf2 a deletion that defines the Af2 clonal complex. However, from animals more than 20 spoilgotypes of *M. bovis* were reported and SB1176 (41.2%) was found to be the most prevalent spoligotype in Ethiopia [194].

1.2.3.8.5.5.3. Variable Nucleotide Tandem Repeat (VNTR)

VNTR typing is the equivalent of mini-satellite typing and measures variation in the number of repeats at a series of loci dispersed throughout the genome [219]. The loci used in VNTR typing of mycobacterial isolates have been named as exact tandem repeat (ETR) loci and mycobacterial interspersed repetitive units (MIRU) loci. It has good discriminatory power compared to spoligotyping. Skuce and his colleagues [154] did VNTR and spoligotyping to a panel of 100

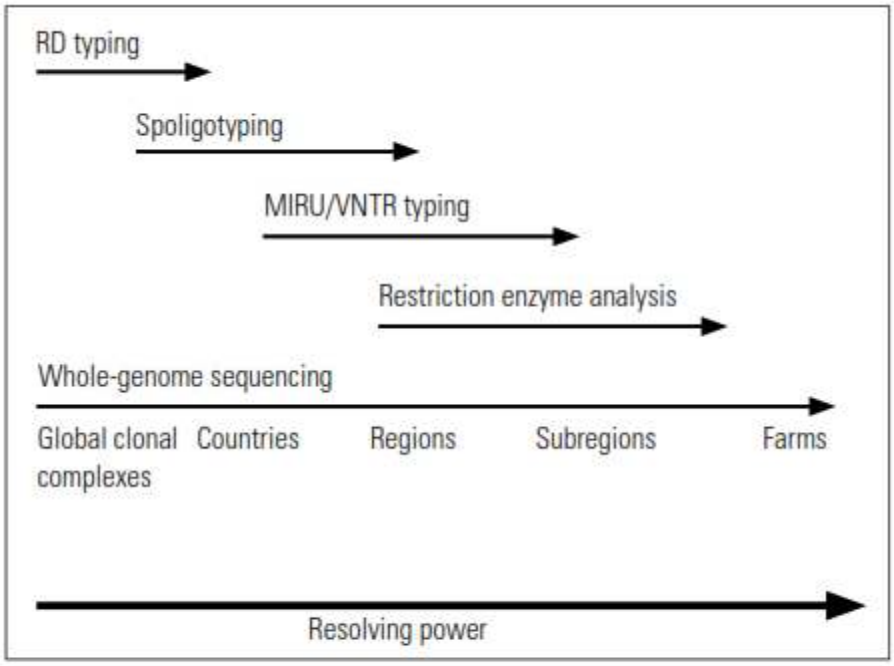
different *M. bovis* isolates and reported thirty-three allele profiles by the novel VNTRs and 29 for spoligotyping and other to other types.

1.2.3.8.5.5.4. Single-Nucleotide Polymorphism (SNPs) Genotyping/Whole genome sequencing

According to Singleton [221] SNP description- SNP is believed to have occured in the genome of organism, when a variant nucleotide which differs from that at the corresponding site in most other members of the population –but which occurs in at least 1% of other individuals within that population; for example, if 1% of the population has the sequence . . .TTTCAT. . . but the majority have . . .TTCCAT. . . then the minority group have a SNP at the third nucleotide in this sequence. The complete genome sequence of *M. bovis* was published [227] 5 years after the publication of the genome sequence of *M. tuberculosis* [228]. The genome of *M. bovis* is >99.95% identical to that of *M. tuberculosis* but has seven deletions. The region containing the deletions is called the region of difference (RD) and ranges from 1 to 12.7 kb. This finding, therefore, could suggest that, overall; the main evolutionary force shaping the genome of the *M. bovis* gene has been the deletion. Though major advances have been achieved, it is still difficult to explain how *M. bovis*, a microorganism whose genome has largely lost genomic regions and with no unique genes, inhabits a broader biological niche than its ancestor, *M. tuberculosis*. Probably, the presence of more than 1200 SNP in coding regions or genes that distinguish *M. bovis* from *M. tuberculosis* may, in part, explain this characteristic [229].

Coll and his colleagues [228] conducted a large scale study to investigate SNPs as robust (stable) markers of genetic variation for phylogenetic analysis and they examined 1601 strain from a diverse geographic spread and they have identified B92k SNP and they concluded the SNP-based phylogeny is consistent with the gold-standard regions of difference (RD) classification system. They identify a single panel of 62 SNPs that may be used to resolve all seven lineages and a total of 55 sublineages.

In summary Kao et al. [230] illustrated schematically the relative resolving power of different genotyping methods in Fig. 6 below.



RD: regions of difference
VNTR: mycobacterial interspersed repetitive-unit variable-number tandem repeat

Figure 6. Schematic Illustrations of the relative resolving power of different typing schemes

Source: Kao et al. [230]

1.2.4. Human tuberculosis due to *Mycobacterium bovis* infection (Zoonotic TB)

According to WHO, the World Organisation for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) zoonotic tuberculosis can be described as human infection with *M. bovis* of animal origin [231]. As described earlier *M.bovis* is a member of MTBC [219]. *M. bovis* identity i.e. its different from *M tuberculosis* was established shortly after the discovery of *M. tuberculosis* in 1898 [54]. However, its recognition as a zoonotic pathogen was debatable. The reason for this was that Robert Koch and others expressed doubt that humans could not contract TB from consumption of contaminated products of cattle and saw no need for controlling bTB in cattle [232]. This controversy triggered further investigation by British Royal Commission on Tuberculosis and others for over ten years and they proved that *M.bovis* can be transmitted via consumption of contaminated dairy products and therefore, pasteurisation of milk was introduced to break the route of transmission [45].

1.2.4.1. Prevalence of zoonotic tuberculosis

zTB continues to be reported globally which accounts for 1.4% of the global TB burden, with the largest disease burden in low- and middle-income countries [233]. There were reports from high-income countries too which has already applied bTB control program and pasteurize milk despite variation in burden. For example, in the USA, during 2006 - 2013 *M. bovis* accounts for 1.3% - 1.6% of human tuberculosis cases annually; however, in areas bordering Mexico the prevalence of *M. bovis* was even higher (2%) than the national estimate [234]. A retrospective analysis of 533 culture-positive cases from 2000 to 2015, in a Mexican tertiary-care centre, 372 (69.7 %) were caused by *M. tuberculosis* and 161 (30.2 %) by *M. bovis* [235].

Generally, it is believed that the world wide burden of zTB is underestimated due to lack of properly designed surveillance system in countries where bTB is endemic and absence of laboratory facility and expertise to diagnose and/or to differentiate *M. bovis* from *M. tuberculosis* in these countries [236]. Despite these gaps, according to WHO 2019 annual tuberculosis report, there were 143 000 new cases of zTB and 12 300 deaths due to *M. bovis* infection [237]. In the Western countries TB cases caused by *M. bovis* ranged from 1.4% to 3%. For instance in New Zealand combined epidemiological and laboratory investigation of human tuberculosis cases over the period 1995–2002 showed that *M. bovis* accounted for 2.7% (54/1997) of laboratory-confirmed human tuberculosis cases, a rate of 0.2/100 000 population [238]. In The Netherlands, 1.4% of tuberculosis (TB) cases were caused by *M. bovis* and was mainly extrapulmonary (n = 136; 58.9%). In Ireland *M. bovis* caused 3% of human tuberculosis cases in southwest during 1998–2006 and of 11 *M. bovis* strains genotyped, 9 belonged to common animal spoligotypes. Seven strains were from sputum and potential sources of human-centered disease transmission.

In developing countries bTB in cattle is largely uncontrolled. According to a review by Srinivasan et al. [239] India one of the 30 high TB burden countries has a higher pooled bTB prevalence estimate of 7.3% (95% CI: 5.6, 9.5) compared with Ethiopia of 5.8% [11]. In India out of 105 samples (collected from farmers, dairy workers, and livestock keepers) and screened by duplex PCR assay, 25 samples were positive for MTBC where *M. bovis* was detected in 12 (11.4%) and *M. tuberculosis* was detected in 13 (12.4%) cases [240]. In Latin America such as Argentina 2% of pulmonary TB were due to *M.bovis* and human to human transmission was also reported [241]. In Africa zTB due to *M. bovis* was estimated to be 2.8 % [18] though there were

reports of 5% (3/60 isolates) in Nigeria [25]. zTB has been reported from other African countries too despite lack of laboratory facility and expertise to differentiate *M. bovis* from the other members of MTBC (Table 12). In Ethiopia in the last decade, cross sectional surveys on bTB prevalence recorded consistent prevalence of bTB of >30% in exotic and cross bred cattle in peri-urban intensive dairy farms in central Ethiopia [10]. However, only few studies were conducted on zTB to see whether this emerging bTB in central Ethiopia has public health significance. Firdessa and his colleagues [194] attempted to explore the public health risk for bTB in different parts of Ethiopia and reported a lower contribution of *M. bovis* where only 4 (0.4%) of 964 isolates had undergone RD9 and RD4 deletions characteristic of *M. bovis*. However, in another study which had used direct molecular detection method reported a higher contribution. Out of FNA specimens from 40 patients presenting at a rural health center in South Ethiopia, 35 were PCR-positive of which 29 (82.9%) were caused by *M. tuberculosis* and six (17.1%) were caused by *M. bovis* [216]. This highlights the role of type of diagnostic method used to determine the prevalence of zTB in Ethiopia.

Table 12. A selection of reports of zTB in Africa

Country	Year	Study design/setting	Sample size	Sample type	Total MBTC	*M.bovis*	% *M.bovis*	Reference
Ethiopia	2004/2005	CS*/ Dera Woreda, North Showa	145	FNA	108	1	0.9	Seyoum et al. [242]
Ethiopia	2013/2015	CS/Jimma University Specialized Hospital	436	FNA	304	2	0.7	Tadesse et al.[243]
Nigeria		CS/Ibadan	70	sputum	7	2	2.8	Adesokan et al.[244]
Algeria	2017/2019	CS/Northern Algeria		Pulmonary & Exp.** samples	115	7	6.1	Damene et al.[245]
Tanzania		CS/four districts of Manyara region	457	FNA	65	7	10.8	Cleaveland et al.[246]
Uganda		Kampala	344		343	1	0.3	Asiimwe et al.[247]

CS*: Cross sectional study; Exp. **: Extrapulmonary

1.2.4.2. Transmission of zoonotic tuberculosis

Unlike *M. tuberculosis, M. bovis* is capable of infecting multiple hosts including domestic animals, cattle being main host, a wide of range of wildlife reservoirs and humans [2]. It is important to understand how zTB is transmitted from animals to human and even between human if any for designing better prevention and control program of End TB. Humans are most commonly infected through consumption of contaminated raw milk or unpasteurized dairy products [19]. Also ingestion of raw or undercooked meat sourced from generalized bTB can transfer *M.bovis* to humans [248]. Transmission can also occur via inhalation of aerosol generated during handling of carcass or air exhaled by animals infected with *M. bovis* [248, 249]. Direct contact to wound related to occupational exposure such as abattoir work during handling of infected meat can lead to infection with *M.bovis* [250, 251]. The potential for human to human transmission of *M.bovis* was rare, however, there were sporadic reports in Argentina [22] and a possibility in Spain was reported in HIV infected individuals [23].

1.2.4.2. 1. Cattle to human transmission

As cattle are the primary host of *M. bovis* and hence they serve as the main source of infection for zTB. Evidences of *M.bovis* transmission from cattle to human are obtained from risk factors analysis studies and recently from molecular epidemiology findings [252]. In a retrospective cohort analysis of human *M. bovis* cases in England, Wales, and Northern Ireland during 2002–2014, 357 cases were identified and for 74% of patients, exposure to risk factors accounting for *M. bovis* acquisition, most frequently consumption of unpasteurized milk, was known [253].

A whole genome sequence analysis of 172 isolates of *M. bovis* (155 from cattle and 17 from humans) in Mexico identified two predominant spoligotypes patterns seen in both cattle and humans: SB0145 and SB1040. The SB0145 spoligotype represented 59% of cattle isolates (n = 91) and 65% of human isolates (n = 11), while the SB1040 spoligotype represented 30% of cattle isolates (n = 47) and 30% of human isolates (n = 5) [178]. In this report none of the human patients had a history of contact with cattle, which is suggestive of dairy products as a possible source of their infections. In another study in Africa, eight of *M. bovis* isolates originated from sputum (n=1017) collected in two big national surveys in Zambia; one in urban-drug resistance survey and the second survey carried out in the pastoral area in Southern part of Zambia were found to be spoligotype SB 0120 showing bovine origin [254].

Theoretically, eating undercooked or raw meat from tuberculous animals could be a mechanism for human infection with *M. bovis*. However, according to a review by Rua-Domenech [19], the evidence for human *M. bovis* infection due to ingestion of meat from tuberculous animals is very weak or non-existent. But in countries such as Ethiopia where culture of eating raw meat is high and is prepared in different national dishes as *Kurt*, *Kitfo*, *Dulet* and *Milas Senber* and undercooked meat such as *Leb Leb*; the possibility of transmission of *M.bovis* particularly from disseminated form of tuberculosis cannot be ruled out. For example in a study in India that aimed to check the viability of mycobacteria in frozen buffalo meat destined for export, out of 45 frozen buffalo meat cultured on LJ media; 27 samples were positive of *mycobacteria*. Among them *M. bovis* positive in 24 samples, *M. tuberculosis* positive in one sample and *M. cosmeticum* positive in 5 samples [255].

Though transmission of *M. bovis* to humans is attributed mainly to consumption of contaminated unpasteurized dairy products, it can also occur via inhalation of air exhaled by infected animals.

Depending on the nature of their job/occupation, people can have different levels contact/exposure with cattle. A study in Mexico by Torres-Gonzalez et al. [256] tried to assess the association of different levels exposure (high, medium and low) and *M.bovis* infection in 311 dairy farm and abattoir workers using IGRA and spoligotyping of both human and cattle isolates. And IGRA positive test (latent TB) was found in 70.4%, 48.9% and 1.2% of individuals assigned to high, medium and low exposure groups, respectively. Categories of exposure were High risk for: direct contact with livestock in closed spaces (e.g. abattoir workers, vet professional performing cattle necropsies, milkers); Medium risk: direct contact with livestock in open spaces (e.g. breeders, feeders etc); Low risk: no direct contact with livestock (e.g. owners of the cow shade, admin workers, people involved in commercial). Therefore, this study proved an increased risk among those occupationally exposed in non-ventilated spaces.

Infection with *M. bovis* can occur via direct inoculation in rare cases. A slaughterhouse worker who developed a cutaneous granulomatous inflammatory reaction visited a hospital and it was found to be due to *M. bovis* infection [250].

1.2.4.3. Risk factors of zoonotic tuberculosis

The drivers in the transmission of *M. tuberculosis* are well studied. However, similar studies for *M. bovis* are limited. Davidson and colleagues [253] explored the epidemiology of *M. bovis* disease in England, Wales, and Northern Ireland, 2002–2014 in a retrospective cohort study. They considered demographic factors (age, sex, address (rural vs urban); clinical factors (site of the disease, previous diagnosis); social risk factors (current or past imprisonment, homelessness, drug and alcohol misuse); exposure (contact with TB patient, travel to a country with high TB incidence, consumption of unpasteurized dairy products, occupational contact with animals, physical contact with wild animals, physical contact with any animal having TB). The main finding in this study was most patients were >65 years of age and for 74% of patients, exposure to risk factors accounting for *M. bovis* acquisition, most frequently consumption of unpasteurized milk, was known. Unlike high income countries such as UK where bTB is low (though recently saw an increase due to wildlife reservoir), in low and middle income countries bTB is high and likely exposure of risk groups to *M. bovis* would also increase [248]. These occupational risk groups include livestock farmers, abattoir workers, veterinarians and their assistants, hunters, wildlife workers as well as other animal handlers are at different risk of contracting *M. bovis*

infection, depending on the nature of their jobs and how close is their interaction with infected animals [248]. In a cross-sectional study in Uganda that involved 43 livestock farmers with cervical lymphadenitis, three were positive for *M. bovis* and the spoligotpe patterns for these *M. bovis* isolates were identical with the isolates from the cattle [179]. In another study in south eastern Ethiopia by Gumi et al.[117] in pastoral setting where human (sputum=260; FNA=32) and animal (cattle, camels, goats) (tuberculous lesion=207) samples were investigated and three from sputum and 24 from cattle *M. bovis* isolates identified and in this study as well identical spoligotypes of human and cattle *M .bovis* were observed. With regard to abattoir workers, Khattak and colleagues [257] in Pakistan screened 141 abattoir workers of which 16 had chronic cough and sputum samples from these suspected pulmonary TB tested with PCR and four of them were positive for *M. bovis.* Occupational zTB studies involving veterinarian are generally few probably due to their overall small number. One large study in USA which involved 259 veterinarians that participated in at least five official bTB herd tests was unfortunately only aimed at looking veterinarian injuries associated with bTB testing not possibility of zTB acquisition [258]. Ullah et al. [259] in Pakistan processed 32 sputum samples obtained from 10 veterinarians and 22 veterinary assistants and all samples were negative for AFB, culture and PCR.

1.2.4.4. Symptoms, diagnosis and treatment of zoonotic tuberculosis

Clinically zTB cannot be distinguished from *M. tuberculosis*, although it may be more likely to cause extra-pulmonary disease [20]. When symptoms of TB disease due to *M. bovis* occur they are similar to the symptoms of TB caused by *M. tuberculosis*; this can include commonest symptoms such as fever, night sweats, and weight loss [260].

There could be other symptoms depending on the site of infection. For example cervical lymphadenopathy historically known as *scrofula* a term used to designate a chronic swelling of cervical lymph nodes; was once a very common form of tuberculosis in children who drank infected milk [261]. Butchers and slaughter workers when handle tuberculous meat can be infected through the skin and develop localized skin disease (“butcher’s wart”) [262]. Pulmonary TB due to *M. bovis* is commonly associated with reactivated infections [263] and the symptoms may include fever, cough, chest pain, cavitation and hemoptysis [260]. Diagnosis and treatment of TB due to *M. bovis* is the same as *M. tuberculosis.* CDC USA recommended treatment of TB

due to *M. bovis* consists of rifampicin, isoniazid and ethambutol and the duration is extended to 9 months due to the exclusion of pyrazinamide, since all strains of *M.bovis* are resistant to it [264].

1.2.5. One Health Concept

The term One Health is now used to describe the unified human and veterinary approach to zoonoses [265]. There are many definitions of it as reviewed by Häsler et al. [266]. A comprehensive description of One Health is provided by Destoumieux-Garzón et al. [267] as "The One Health concept: a holistic, transdisciplinary, and multisectoral approach of Health"(Fig. 7). Over the last decades zoonotic infectious agents such as SARS-CoV-2 (COVID-19) are becoming a health, social and economic challenge at national and global scale, which calls for closer cooperation between animal and public health experts/institutions [268]. In Africa the interaction of animals and humans is common and hence needs a collaborative approach in early detection, diagnosis and data sharing to tackle zoonotic diseases. As new findings emerge a new definition for zoonotic tuberculosis (human infection with *M .bovis* of animal origin.) has beensuggested as other member of the MTBC such as *Mycobacterium orygis* which causes tuberculosis in Antelopes and possibly in other animals, have been reported as the cause of tuberculosis in humans, especially in patients from India [269]. In this study out of 940 (548 pulmonary and 392 extrapulmonary samples) samples collected between Oct 1, 2018, and March 31, 2019, from tuberculosis suspected patients visiting the outpatient department at Christian Medical College (Vellore, India), seven *Mycobacterium orygis* and no *M.bovis* (wild type) had been isolated. This study further emphasized that the burden of zoonotic tuberculosis might be underestimated by surveillance studies restricted to *M. bovis*.

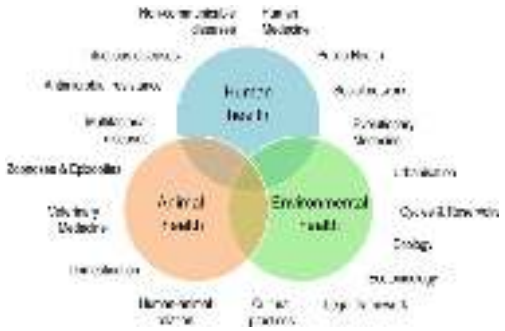


Figure 7. The One Health concept: a holistic, transdisciplinary, and multisectoral approach of health

Source: Destoumieux-Garzón et al. [267]

1.3. Statement of the problem

Previous studies of bTB in Ethiopia particularly in regional states were smaller scale - limited to few farms/study sites which led to over/under representation of farms and failed to show the best estimate of bTB prevalence and the associated risk factors for initiating control intervention [11]. In addition, latest burden of bTB was not known-the latest study in central Ethiopia, which was comparable to the present study, was conducted over 10 years to this study [10]. If this problem remains uncovered, there would be a risk of wider transmission of bTB and the huge cattle population of Ethiopia (over 65.35 million) would be at risk and affect the economy [128]. This transmission can also have a public health problem. Human TB due to *M. bovis* is largely neglected and because of this few studies were conducted so far in Ethiopia [194, 270]. The government and other stakeholders such as WHO focus are on TB due to *M. tuberculosis*. Almost all studies other than Ameni et al. [271] study were hospital based and no risk group based studies were conducted. bTB is high in central Ethiopia and the parallel public health impact was not known [11]. Understanding the genomic diversity is important for investigating routes of transmission and trace the origin of the outbreak. Previous genomic diversity studies in Ethiopia used spoligotyping and MIRU-VNTR [10, 272] and this has helped to identify prevalent genotypes but these techniques have limitation i.e. less discriminatory power (spoligotype) and reproducibility (MIRU-VNTR) [174]. WGS and SNP-based analyses can provide more detailed and discriminating phylogenetic analyses [273]. However, in Ethiopia there were no WGS based studies before this study.

In low and middle income countries that includes Ethiopia, the true incidence zTB is likely underestimated due to lack of laboratory and surveillance capacity and hence difficulty to differentiate *M. tuberculosis* and *M. bovis* as direct smear microscopy is the most common diagnostic method used [274]. Acid-fast staining is a simple and inexpensive method; however, it is less sensitive [30]. Culture is sensitive and still the 'gold standard' for *Mycobacterium* identification; however, it is time consuming and harsh decontamination process may render mycobacteria non-viable if exposed too long [275]. On the other hand, real-time PCR (RT-PCR) has eliminated the necessity of performing such analyses and increased sensitivity and specificity [276]. Evaluation of RT-PCR as an alternative method for direct detection of mycobacteria from

clinical (human and animal) specimens would be helpful in the early diagnosis, treatment and control of tuberculosis as well as zoonotic tuberculosis.

Due to these research gaps this study was initiated and conducted for generating data on these stated problems.

1.4. Hypothesis

- Prevalence of bTB in central Ethiopia might be generally high but likely to vary among dairy farms
- The burden of zoonotic TB in Ethiopia is underestimated and might be higher than previous reports
- There might be high genetic diversity amongst *M. bovis* in central Ethiopia due to ongoing transmissions over the years.

1.5. Objectives

1.5.1. General objective

- To assess *M. bovis* infection in humans and cattle in central Ethiopia

1.5.2. Specific objectives

- To estimate the prevalence and associated risk factors of bTB in central Ethiopia dairy cattle
- To determine the occurrence of zTB and assess potential risk factors in central Ethiopia
- To determine the genetic diversity of *M. bovis* isolates obtained from human and cattle specimens
- To evaluate the performance of RT-PCR as an alternative diagnostic method for direct detection of *M. bovis* in human and animal specimens.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1. General Outline of the Study

This study has two parts though conducted in the same study setting, the first being the bTB study and the second one was the human component termed zTB study and was conducted from 2018 to 2021 in central Ethiopia (Fig. 8). The bTB study had tuberculin skin testing of dairy cattle and the collection of additional herd and animal level data by questionnaire to identify potential risk factors contributing to bTB transmission. And the zTB study, had active surveillance of TB which was carried out among individuals working in bTB infected dairy farms referred in this study as "dairy farm workers**"(**DFWs**)**. Also passive surveillance of TB was conducted at selected health centers located in the study area. From consenting TB suspected individuals, demographic and clinical information was collected through an interviewed-administered questionnaire with expert in the field. Sputum and Fine Needle Aspirates (FNA) samples were collected from TB suspected cases. In addition, to assess the sources of infection for human TB due to *M. bovis*, pathogen of interest - *M. bovis* was isolated from cattle tissue lesions collected from slaughterhouses and also from culled bTB positive cattle from dairy farms. Also *M. bovis* was isolated from raw milk of tuberculin skin test positive cows. And finally all human and cattle isolates were subjected to whole genome sequencing for phylogenetic analysis and genetic diversity assessment in the two hosts. A diagnostic method evaluation study was conducted using a pooled specimen of cattle and humans to evaluate the performance of TaqMan real time PCR assay for direct detection of *M. bovis* in sputum, FNA and milk.

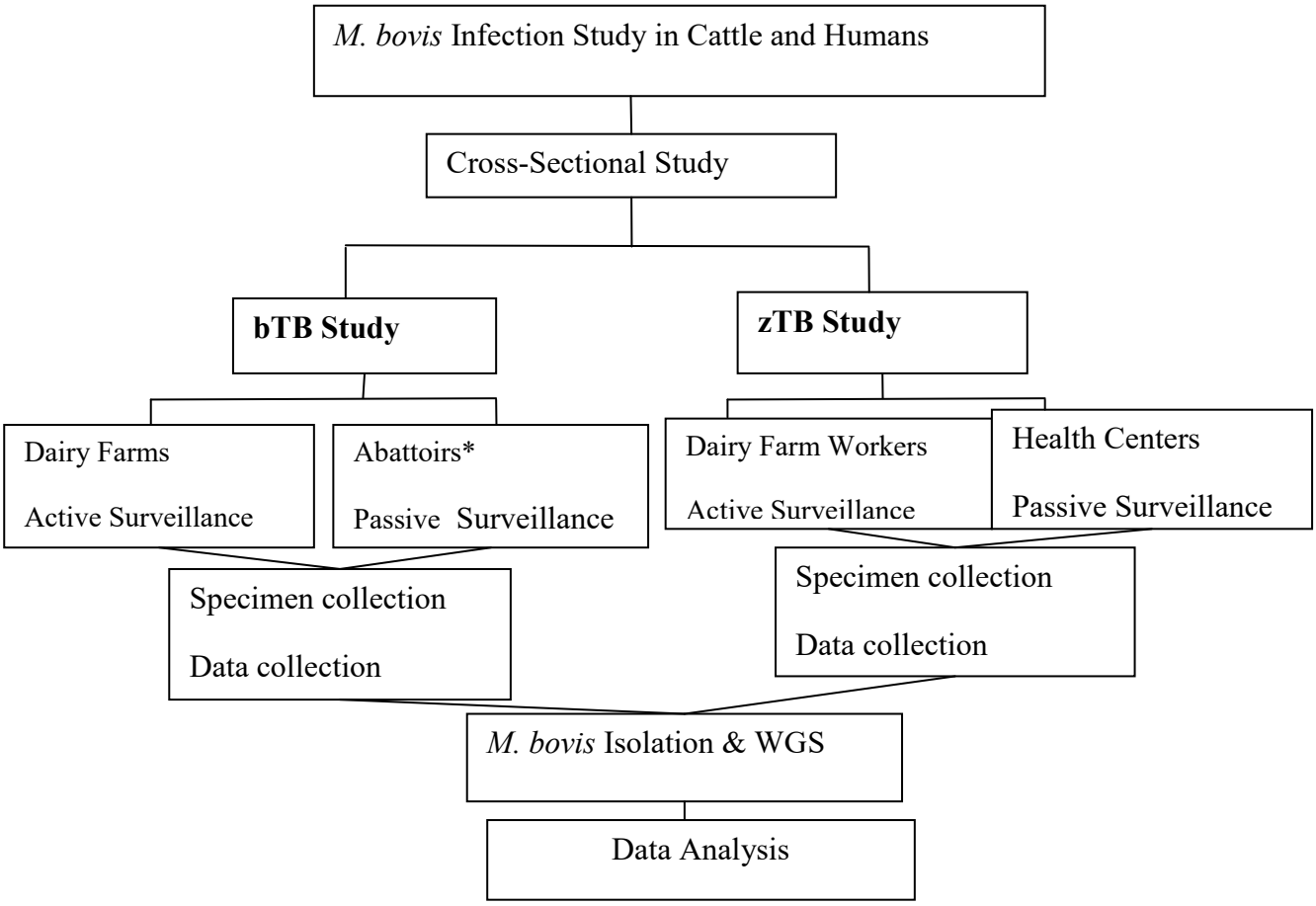


Figure 8. Flow chart for the study

* The abattoir surveillance was used as a source of sample for *M. bovis* isolation not for prevalence estimation of bTB in central Ethiopia. For estimation of bTB prevalence in central Ethiopia data from dairy farms was used.

2.2. Study Areas

For the purpose of this study central Ethiopia refers to Addis Ababa city and within 50kms radius of Addis Ababa. For the study of bTB, six study areas were selected in the urban areas of central Ethiopia, including Addis Ababa city, and Sebeta, Holeta, Sululta, Sendafa and Bishoftu towns (Fig. 9). The selection of these study areas was purposeful. Central Ethiopia, which includes the study areas, was a pioneer for the modern dairy development in Ethiopia with the first number of exotic dairy cattle arriving in the early 1950s as a donation from the United Nations [126] and this area has then over decades established itself as the most developed dairy belt in Ethiopia.

The study areas are currently the main milk suppliers for people in Addis Ababa and the surrounding peri-urban areas. Abattoir based bTB surveillance was conducted at Addis Ababa Abattoir Enterprise, a public abattoir located at Kirchos subcity and Sululta Cooperatives Abattoir located at Sululta town.

zTB study was also conducted in these same study areas and dairy farms that were used for bTB study. Dairy farm workers in bTB infected dairy farms of Addis Ababa, Bishoftu, Holeta, Sebeta, Sululta and Sendafa were screened for active cases of TB (active case) detection. Selected health centers at Addis Ababa, Bishoftu, Holeta, Sululta and Sendafa were used for passive case detection of TB. One health center refused to participate for the passive surveillance study.

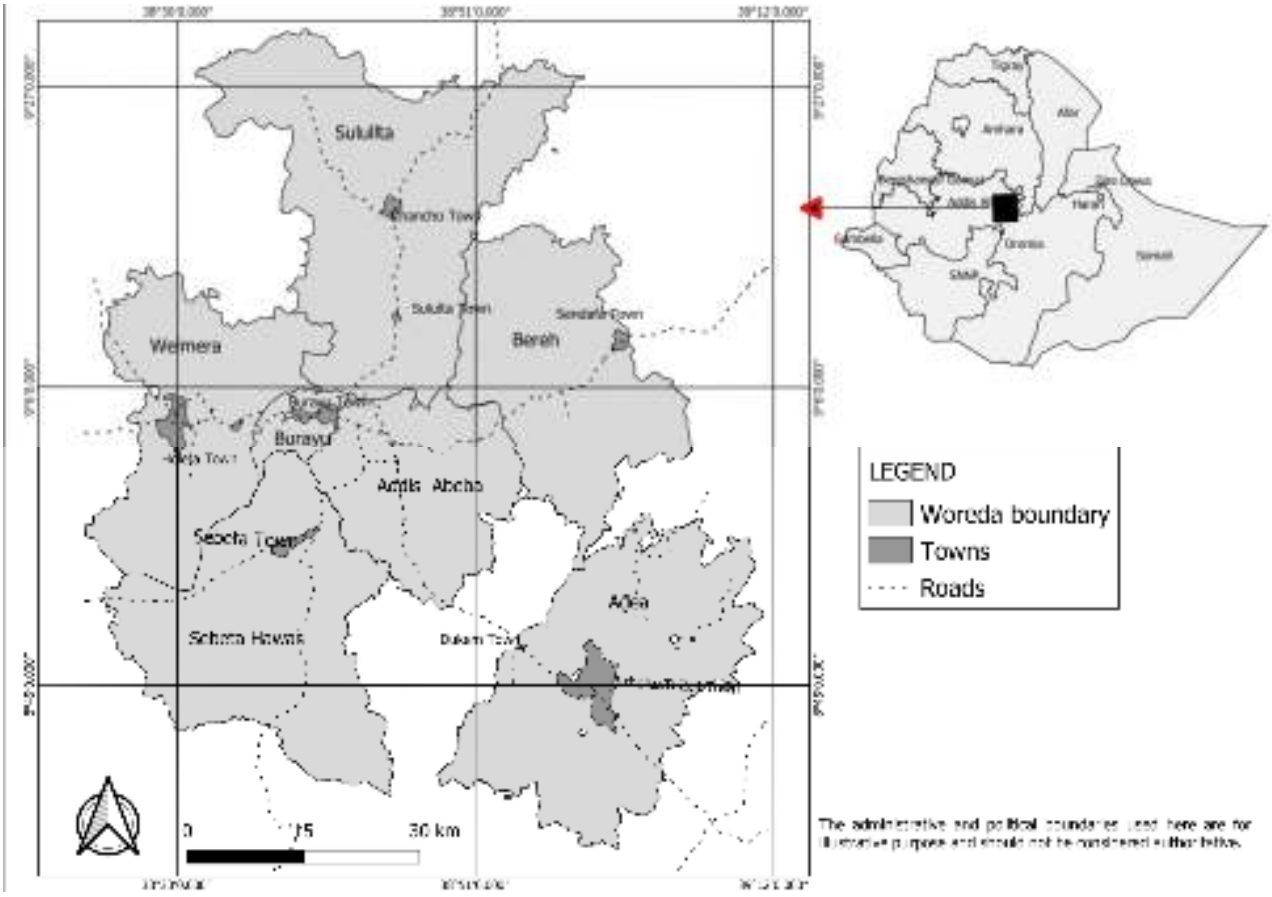


Figure 9. Map of the study areas: Addis Ababa city and Sululta, Sendafa, Holeta, Sebeta, and Bishoftu towns.

2.3. Study design

2.3.1. bTB study

2.3.1.1. Dairy farms bTB prevalence

This study was a cross sectional study where lists of herds (the study population) were established at the start of the study in collaboration with district veterinary officers in respective study sites. The term "herd" was used to describe the group of cattle that are housed on a holding at the time of data collection [277]. Herds, with the purpose of producing milk and dairy products, having five or more cattle were included and a list of 1,323 herds was established as a sampling frame. The herds were classified following the previous work of Firdessa et al. [10] with modification as small [5-20], medium [21-37], and large herds [38-168] (168 being the largest herd size in the studied herds).

Inclusion and exclusion criteria for the study herds: Herd size was the criteria used and herds with less than five animals were excluded.

Sample size

Sample size was determined following one-stage cluster sampling method taking dairy herd as a cluster [278] and every animal in the selected cluster was tested.

$$g = \frac{1.96^2 \{nVC + Pexp(1 - Pexp)\}}{nd^2}$$

Where : g = number of herd to be sampled;
n = predicted average number of animals per herd (n = 13);
Pexp = expected prevalence (Pexp = 0.3 from previous study[10]
d = desired absolute precision (d = 0.05);
VC = between - herd variance (VC = 0.233) [279]

When we fitted these numbers to the formula which assumes large population, it gave us 383 farms. It was reduced to 298, because it was adjusted for small population using the formula:

$$g_{adj} = \frac{G * g}{G + g}, [278]$$

Where : G = total number of herds

g = the calculated sample size for large herds

$$g_{adj} = \frac{1323 * 383}{1323 + 383} \approx 298$$

Hence, we tested 299 herds out of 1323 registered herds in the study sites and selection of each herd was random. All animals in the 299 herds (5,675 animals) were tested excluding animals less than 6 weeks of age and cows that were ≥8 months pregnant. By herd stratification, 212 small, 49 medium and 38 large herds were tested.

Farm data collection

Data was collected by trained research assistants through face to face interview with pre-tested structured questionnaire to capture animal and herd-level information. General information including herd structure, farm antecedents, farm management/husbandry, housing/ventilation, animal health (veterinary services) and animal bio-security were recorded. Specific information related to potential risk factors for bTB were recorded including animals age, sex, breed, physiology (pregnancy/stages of lactation/body condition), herd size, cattle sourcing (cattle movements in and out of the herd), bTB history on farm, contacts /interactions with neighboring herd/other domestic animals/wild animals etc. (Annex 1: Questionnaire). GPS data was collected for each herd for mapping bTB prevalence in the study areas.

2.3.1.2. Abattoir based bTB survey

This study was carried out at two abattoirs: Addis Ababa Abattoir Enterprise, the largest abattoir in central Ethiopia and Sululta Cooperatives Abattoir and these abattoirs were selected based on their number of daily slaughter and proximity to the laboratories for immediate transportation of specimens. All crossbred cattle (and zebu cattle if originated from dairy farms located in the study area) brought to the abattoir for slaughter were examined twice weekly (ante-and postmortem) for bTB lesion detection. Ante-mortem examination: at the lairage-animals waiting room, animals get their abattoir ID and in this room, the animal's ID, sex, breed, origin and

clinical signs (if any) were recorded in a format prepared for this purpose (Annex 2: Abattoir SOP). With the help of the assigned meat inspectors in the respective abattoirs, animals were tracked by their ID all the way to the slaughter hall where they got finally slaughtered, skinned and eviscerated. This ID is maintained throughout the abattoirs operation until the inspected and certified carcass/meat is finally delivered to the customer (butcher shops). Postmortem examination was carried out following standard procedures [280] where firm, hard nodule, whitish or yellowish-white in color was considered as tuberculous lesion; depending on the age of the lesion it can assume different characteristics (mucoid/purulent, caseous (cheesy) or calcified lesion). A detailed procedure for postmortem examination of visceral organs and lymph nodes is presented in Annex 2 (Abattoir SOP). Visceral organs including lung lobes, thoracic membrane, heart, spleen, liver, intestines, kidneys, abdominal membrane, mammary gland (cows) and whole carcass were examined. Similarly the lymph nodes examined include parotid, mandibular, retropharyngeal, tracheal (apical), bronchial, mediastinal and carcass lymph nodes (axillary, pre-scapular, pre-femoral, popliteal) and were examined following the standard procedure. When a bTB-like lesion was detected, sample was taken and transported to National Animal Health Diagnostic and Investigation Center (NAHDIC) BSL 3 Zoonotic Laboratory with ice-box for isolation of *M. bovis*. Refrigerators were deployed to these abattoirs for sample storage whenever there was difficulty for immediate transport of samples for different reasons.

2.3.2. Zoonotic TB study

Study Design: The zTB was also a cross-sectional study and was conducted at bTB infected dairy farms and health centers.

Definition: A DFW was defined as an individual (employee, family member, or owner) of a selected dairy farm and was invited to participate in the active case detection of TB and those who provided informed consent were enrolled in this study (Fig. 10).

Study Population

All DFWs in bTB infected dairy farms were invited to participate for the active case detection of zTB study and those who provided informed consent were enrolled in this study. The recruitment flow chart is presented in Fig. 10. Among the 299 dairy farms which were screened for bTB, larger dairy farms (those having > 20 cattle) and positive for bTB (at least one reactor animal in

the herd) were purposively selected as bTB thrives in intensification for the zTB study. For the passive zTB surveillance, patients who presented at selected health centers with complaints of symptoms related to TB (such as cough for two or more weeks, unintentional weight loss, lymph nodes swelling in armpits etc as described in Ethiopian National Algorithm, MoH [281]) in the study areas were considered as a study population. Individuals suspected of tuberculous lymphadenitis (TBLN) or pulmonary TB were asked for informed consent and were recruited into this study. The Ethiopian National Algorithm, MoH [281] was used for screening of DFWS who provided written consent (by clinician) for symptoms of TB (pulmonary and TB lymphadenitis (TBLN)) (Annex 10). In both active and passive zTB surveillances-FNA and sputum samples were collected and transported in icebox to Armauer Hansen Research Institute (AHRI) TB laboratory for isolation of *M. bovis*.

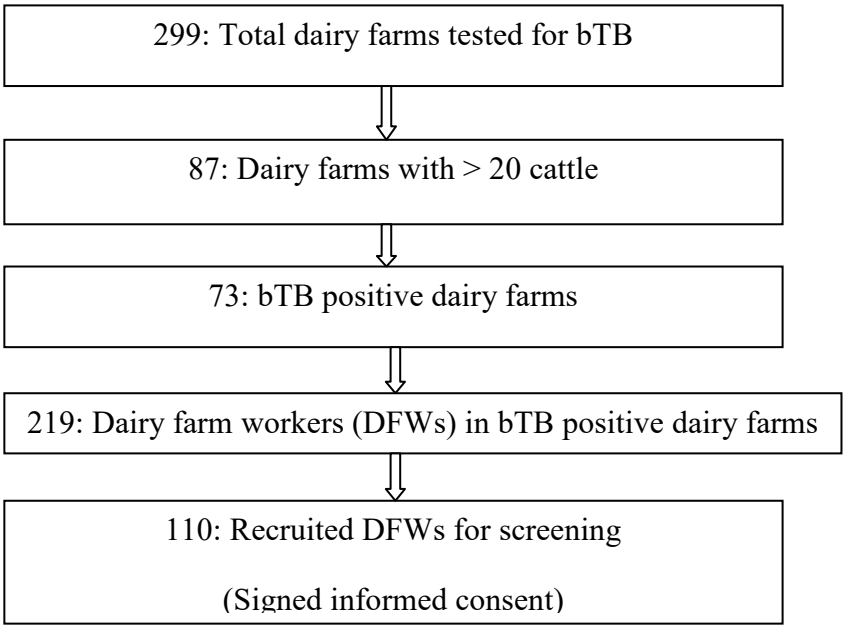


Figure 10. Flow chart of recruitment of participants for active zTB surveillance

Socio-demographic and clinical data collection

Data on demographics (age, sex, education, marital status etc.) and risk factors (occupation, habit of feeding, level of education etc.) and clinical factors (coughing, chest pain, weight loss etc.) were collected through pre-tested and standardized questionnaires (Annex 3: Questionnaire for zTB). Also data on knowledge and practices related to zTB were also collected.

2.3.3. Evaluation of TaqMan RT-PCR assay for direct detection of *Mycobacterium bovis* in clinical specimens

2.3.3.1. Study design

In this study TaqMan Real-Time PCR assay (developed by Applied Biosystems) was evaluated for the direct detection of MTBC*, M. bovis* and *M. tuberculosis* in clinical samples collected from human patients suspected of TB and cattle positive for bTB using tuberculin skin test. Analytic sensitivity of the assay was determined from tenfold serial dilution of pure genomic DNA (gDNA) of *M. bovis.* To assess the inhibitory effect of substances in the sample, analytical sensitivity was also determined from tenfold serial dilution of known negative specimens sourced from ANHRI (sputum and FNA) and NAHDIC (milk) spiked with known concentration of *M. bovis* BCG. The analytical specificity of the TaqMan Real-Time PCR assay was assessed by testing the genomic DNAs purified from *M. tuberculosis H37Rv*, *M. bovis BCG*, *M. bovis* (clinical isolates) and other non-mycobateria pathogens.

Clinical sputum and FNA samples (as described in section 2.3.3.2.) were collected from patients suspected of pulmonary TB and TBLN patients presenting at selected health centers in Addis Ababa and four surrounding towns (Holeta, Sululta, Sendafa and Bishoftu). Also sputum and FNA samples were collected from listed sites plus Sebeta town from dairy farm workers suspected of pulmonary TB and TBLN in the active case detection surveillance of TB in bovine tuberculosis (bTB) infected dairy farms that took place between January and October 2020. Raw milk sample was collected from tuberculin skin test positive cows randomly (simple random sampling) selected among the 299 farms. Negative sputum and raw milk samples were also collected from the apparently healthy individuals in the community and cows, respectively and later confirmed as negative via culture for the specificity analysis of the assay.

The processed decontaminated samples were directly tested using TaqMan Real-Time PCR assay and at the same time in parallel cultured on to egg-based LJ medium (Löwenstein-Jensen TB Medium Base, Sigma Aldrich) supplemented with 0.75% glycerol or 0.4% pyruvate, two media that favors growth of *M. tuberculosis* and *M. bovis*, respectively. The performance was evaluated by comparing results to direct smear microscopy and culture. The target genes were IS*1081*, RD4 and RD9 which are used for detection of MTBC*, M. bovis* and *M. tuberculosis,* respectively.

2.3.3.2. Diagnostic sensitivity and specificity determination

An important consideration in conducting diagnostic test evaluation is to plan and justify a sufficient sample size. The number of clinical specimens needed for this study was determined following requirements for minimum sample size for sensitivity and specificity analysis [282] taking estimated prevalence of 5% [25], 95% confidence level, 80% power and a 5% level of precision and 400 clinical specimens were required for the evaluation and in total 440 (273 milk, 36 FNA and 131 Sputum) specimens were tested. Diagnostic sensitivity and specificity of TaqMan real time PCR assay was determined/calculated from clinical samples assay results taking culture as a gold standard test for microbiological TB diagnosis. Also positive and negative predictive values (PPV and NPV) and area under the curve (AUC)/Receiver Operating Characteristic Curve (ROC Curves) were determined.

2.3.3.3. Analytical sensitivity and specificity determination

Analytical sensitivity was assessed following the previous work of Sevilla et al. [283]. Ten-fold serial dilutions were prepared ranging from a 10ng/µl stock of *M. bovis* (Kindly provided by Dr Stefan Berg, APHA, UK) to 10^{-7} dilution. This dilution series of known template concentrations (from undiluted to 10^{-7} dilution) was used to construct a standard curve for assessing the reaction efficiency. The log of each known concentration in the dilution series (x-axis) was plotted against the Ct value for that concentration (y-axis). DNA standards were run in triplicate on the same plates. Triplicate no template control (TNC) wells were included on each plate. We determined the amplification efficiency, Limit of Detection (LOD) and R^2 of the TaqMan Real-Time PCR assay.

The minimum detectable number of genome copies (limit of detection: LOD) or its equivalent minimum detectable number of *M. bovis* by the assay was calculated by the formula (Equation 1)

[283]. There are six copies of IS*1081* and a single copy of RD4 in the genome of *M. bovis* [88, 284].

Equation 1: $$\text{Number of copies/reaction} = \frac{\text{Amount of DNA (ng)} \cdot \text{Avogadro's constant}}{\text{Length of DNA (bp)} \cdot \text{Conversion Factor} \cdot \text{Average Mass of 1bp of dsDNA}}$$

Where:

- Amount of DNA – The amount of DNA in the tube, measured in nanograms (ng).
- Length of DNA template – The number of base pairs (bp) of the DNA template: Genome length values for *M. bovis* BCG: 4,255,552 bp
- Avogadro's constant – This number (6.023×10^{23}) represents the number of molecules in 1 mole.
- Conversion factor – The conversion factor (1×10^{9}) is required to convert the value to ng.
- The average mass of 1 bp of dsDNA – The average mass of 1 bp of dsDNA is 660 g/mole.

Analytical sensitivity was also determined from tenfold serial dilution of known negative specimens sourced from ANHRI (sputum and FNA) and NAHDIC (milk) spiked with known concentration of *M. bovis* BCG (Manufacturer: Green Signal Pvt Ltd; Lot No: TN00002462; Batch No:GSV-158/18) and serially diluted tenfold up to $2x10^{1}$ CFU/ml concentration (Table 13). Briefly, a lyophilized *M. bovis* BCG vial was diluted with 1ml of the diluent provided together with the vaccine and this suspension after thorough mixing is estimated to have $2x10^{6}$CFU/ml concentration according to the manufacturer product description. The serial tenfold dilution of *M. bovis* BCG stock is described in Table 13. Simply 100µL of *M. bovis* BCG was transferred to 900 µL of specimen confirmed to be negative for MTBC and this same amount was continued until the last dilution. For every dilution series one further tube was spiked with sterile water and served as a negative control. The purpose of spiking negative specimens was to assess the background effect of specimens in interfering the PCR reaction and this was judged based on PCR efficiency results.

The assay LOD for the spike experiment was defined as the lowest number of CFU which, when spiked into the specimen, would result in the detection of *M. bovis* BCG for RT-PCR test.

Table 13. Experimental protocol for evaluation of qPCR for direct detection of *M. bovis* from Human and cattle specimens

Strain	Serial dilutions*				Total
Sputum Spiked with test strain *M. bovis* BCG (CFU/ml)	10^4(3[a])	10^3(3)	10^2(3)	10^1(3)	12
FNA Spiked with test strain *M. bovis* BCG (CFU/ml)	10^4(3)	10^3(3)	10^2(3)	10^1(3)	12
Milk Spiked with test strain *M. bovis* BCG (CFU/ml)	10^4(3)	10^3(3)	10^2(3)	10^1(3)	12
Water Spiked with test strain *M. bovis* BCG (CFU/ml)	10^4(3)	10^3(3)	10^2(3)	10^1(3)	12
Sputum alone spiked with sterile water (negative control) (one/dilution)	n=1	n=1	n=1	n=1	4
FNA alone spiked with sterile water (negative control) (one/dilution)	n=1	n=1	n=1	n=1	4
Milk alone spiked with sterile water (negative control) (one/dilution)	n=1	n=1	n=1	n=1	4
Water alone (negative control)	n=1	n=1	n=1	n=1	4
Total					96

[a] : number 3 in the bracket for the respective dilution indicate-the experiment replicated three times.

[*] Every dilution is multiplied by 2 as the original conc is $2x10^6$CFU/ml

The analytical specificity of the TaqMan Real-Time PCR assay was assessed by testing the genomic DNAs purified from *M. tuberculosis H37Rv*, *M. bovis BCG*, *M. bovis* (clinical isolates) and other non-mycobateria pathogens (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Haemophilusinfluenzae*). These pathogens were selected based on their ability to cause a related/similar condition (respiratory symptoms and abscess formation) with that of *M. bovis*. These are not the only bacteria that needs to be evaluated but this study was limited to these species only due to lack of availability.

The detailed laboratory procedure of this assay including primer and probe sequences is presented in laboratory methods and sample flow section below.

2.3.4. Field and Laboratory Methods and Sample Flow

For *M. bovis* isolation, tuberculous lesions and raw milk were collected. Likewise from humans, sputum from pulmonary TB suspected patients and FNA from TB lymphadenitis were collected. The laboratory tests used to analyze these specimens include Ziehl Neelsen staining, culture, TaqMan Real-Time PCR, deletion typing, spoligotyping and WGS. Samples were processed in three different institutions of which one was overseas: National Animal Health Diagnostic and Investigation Center (NAHDIC) based at Sebeta, Ethiopia; Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia and Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). The sample flow chart (Fig. 11) and description of each laboratory methods used is presented.

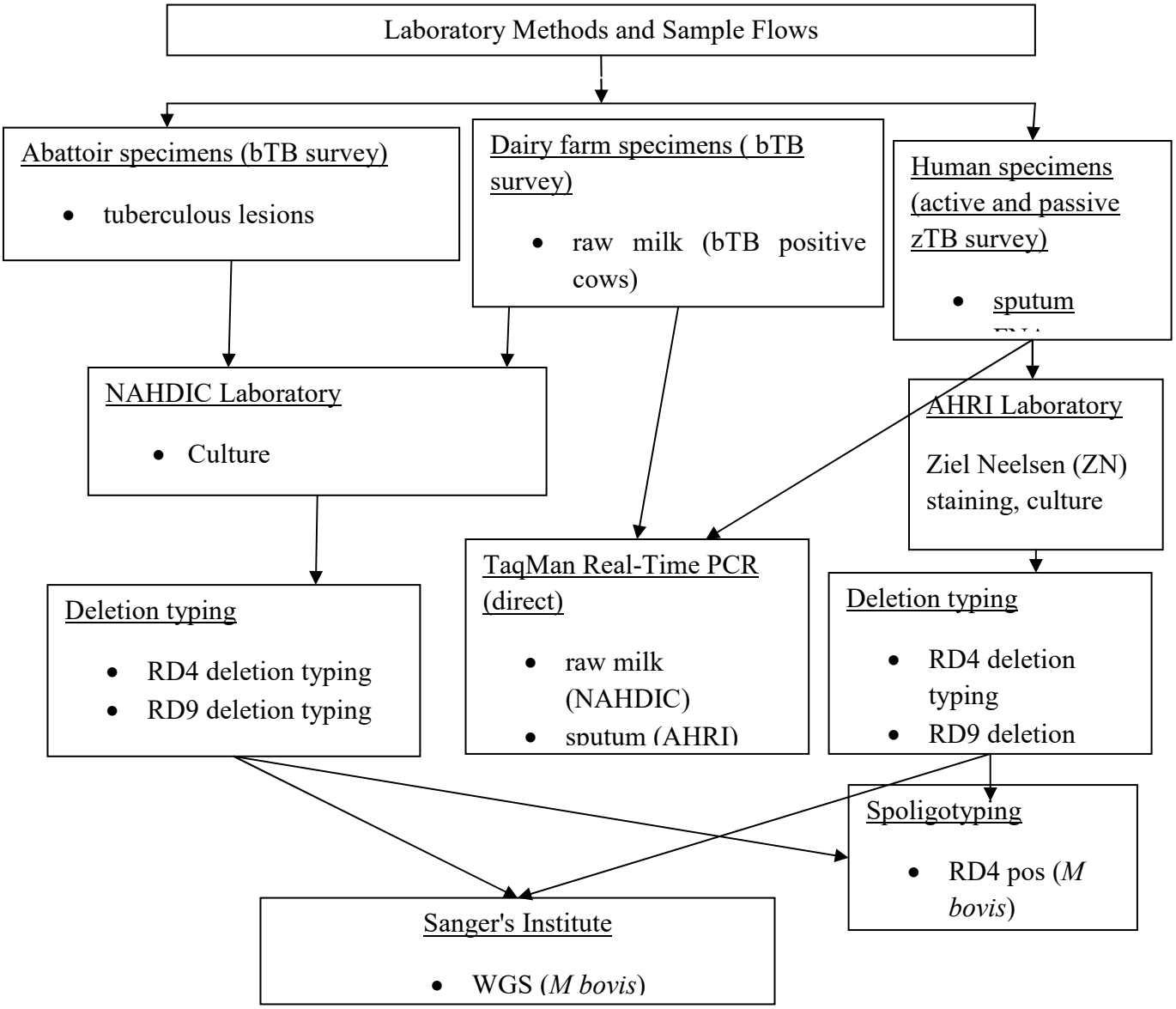


Figure 11. The sample flow chart

2.3.4. 1. Single Intradermal Cervical Comparative Tuberculin (SICCT) test

The procedure for SICCT test was adapted from OIE Terrestrial Manual, 2009 and the supplier of Tuberculin PPD was Prionics, Lelystad, The Netherlands. The injection site used was at the border of the anterior and middle thirds of left side (for consistency) of the neck. Two sites were used, one for bovine PPD (lower site) and the other for avian PPD (upper site). The upper site was 10 cm below the crest and the lower site was 12.5 cm from the upper site, on a line drawn

parallel with the line of the shoulder. The selected site of injection was shaved to an adequately sized area for identification of the injection sites and cleansed. Before injection, a fold of skin at each of the intended injection sites and within the clipped area was taken between the forefinger and thumb and measured to the nearest millimeter using the same digital caliper (0-150mm range) throughout the survey. Then 0.1 ml of Bovine Tuberculin PPD and 0.1ml of Avian Tuberculin PPD was injected intradermal in the lower and upper site, respectively. A correct injection was confirmed by palpating a small pea-like swelling at each injection site. The two injection sites were re-measured after 72 hours by the same person who measured the skin thickness before the injection. For the interpretation, the SICCT was considered positive if a reaction to bovine tuberculin PPD which is more than 4mm greater than the reaction to avian tuberculin PPD; inconclusive if a reaction to bovine tuberculin PPD which is from 1 to 4mm greater than the reaction to avian tuberculin PPD; and negative if a reaction to bovine tuberculin PPD which is equal to less than the reaction to avian tuberculin PPD.

2.3.4. 2. Isolation of *Mycobacterium bovis* from tuberculous lesions

Tuberculous lesions were collected from lymph nodes and visceral organs of cattle brought for slaughter at the two abattoirs and culled bTB positive cattle from dairy farms. Whenever multiple lesions encountered, more than one specimen per cattle was taken. Sampled lymph nodes include parotid, mandibular, retropharyngeal, tracheal, bronchial, mediastinal, mesenteric, pre-scapular, supra-mammary, inguinal, popliteal, and portal and organs such as lung, liver and spleen. Cross-contamination of specimens during collection and transportation was avoided by using different sets of sterile instruments and containers for each respective samples collected.

Samples were transported at 4^{o}C to the Biosafety Level 3 (BSL 3) laboratory of NAHDIC and processed for culturing, either immediately upon arrival or after being stored at -80^{o}C. Sample processing and culturing for mycobacteria was conducted following standard procedures described elsewhere [201, 285]. The detail procedure is described in Annex 4: SOP *Myobacterium* culture-Tissue. Briefly; the tissue was ground with sterile mortar and pestle and the homogenate was decontaminated by adding of equal volume (5ml) of 4% NaOH for 15min and neutralized by adding sterile PBS to 50ml mark (four times (four fold)) [286]. The suspension was centrifuged at 3,000 rpm for 15 min. The sediment after re-suspended in 2ml PBS was cultured in duplicate on to slants of egg based L-J medium (Loewenstein-Jensen TB

Medium Base, Sigma Aldrich) supplemented with 0.75% glycerol and 0.4% pyruvate, respectively, and incubated at 37^{o}C. The slants were observed daily for growing of colonies during the first week, then on a weekly basis. Slants with no growth at week 8 were considered as negative.

2.3.4. 3. Isolation of *Mycobacterium bovis* from raw milk

Thirty to fifty ml of raw milk was collected and transported at 4^{o}C and cultured immediately or if delayed stored at -80^{o}C. Milk culture was performed by modifying the methods described by Zumarraga et al. [213] and Medeiros et al. [287]. Before opening the sampling bottle, the outside surface was disinfected with 70% alcohol to avoid contamination and centrifuged at 4,000 x g for 15 minutes. After centrifugation of the raw milk, the pellet was re-suspended in 10 ml 0.75% (w/v) hexadecylpyridiumchloride (HPC) (Sigma) for 5 h at room temperature [287] for decontamination. The decontaminated milk was then centrifuged (4,000 x g, 15 min) and the pellet suspended in 2 ml PBS and 500 µl of it was inoculated in duplicate on to L-J slants supplemented with 0.75% glycerol and 0.5% pyruvate, respectively. Like the tuberculous lesions, milk cultures were also considered negative when no colony growth was detected after eight weeks of incubation (Annex 5).

2.3.4. 4. Microscopic examination and isolation of *Mycobacterium bovis* from Sputum and FNA

FNA was collected by a pathologist aseptically from enlarged cervical lymph nodes with a 21-gauge needle attached to a 10 ml syringe and then rinsed into a tube with 1 ml sterile PBS solution and used for microscopy and culture. Sputum - about 10ml of sputum was collected in 50 mL sterile, plastic, screw-capped centrifuge tubes and splited in to two (5ml each) one was stored for future use and the other used for decontamination. When it is less than 10 mL, the volume was brought up to 10 mL with sterile saline. Decontamination, Ziehl Neelsen staining and culture producers are described in Annex 6. Briefly 5ml of the sputum was retained as a back up and the remaining was decontaminated with 2 % N-acetyl-L-cysteine sodium hydroxide and centrifuged (3000xg) and the sediment was re-suspended with 2 ml sterile phosphate buffer. Smear was prepared by using a disposable loop from the sediment and at the same time two slants from each (pyruvate and glycerol) of L-J media were inoculated with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface. Then it was incubated

at 37°C until growth is observed or discarded as negative after eight weeks. Suspected colonies were confirmed by deletion typing. FNA samples were not decontaminated and and 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the sample was directly inoculated on to L-J media.

2.3.4. 5. RD4 Deletion typing

Deletion analysis of "Region of Difference 4" (RD4) by PCR was used to confirm *Mycobacterium* colonies, which were positive for Acid Fast Bacilli (AFB) by Ziehl-Neelsen staining, as *M. bovis*. The analysis was carried out as previously described [10, 285] (Annex 7). Briefly, three to four bacterial colonies were scrapped, heat-killed at 80°C for 1hr in waterbath and subjected to DNA extraction (QIAamp DNA Mini Kit). Following DNA extraction, PCR and gel electrophoresis were run and result was read by comparing the size of the PCR products with the DNA ladder and the positive controls where a PCR product with a size of 446bp was interpreted as RD4 being deleted and hence the typed strain being confirmed as *M. bovis*.

2.3.4. 6. Spoligotyping

Spoligotyping was performed following the method described by Kamerbeek et al. [288] (Annex 8). Spoligotyping membranes were a gift from the Animal and Plant Health Agency, United Kingdom. Briefly, the direct-repeat (DR) region was amplified with the primers DRa and DRb and amplified spacers were hybridised to 43-spacer oligonucleotides covalently bound to a membrane by incubating at 60°C for 1 hr. Hybridized DNA was detected by incubating the membrane in 40 ml of ECL detection liquid for 1 min and visualized by exposure of a light sensitive film for 20 min. The spoligotypes were identified by recording the presence or absence of signals on developed autorads. *M. tuberculosis* (H37Rv) and *M. bovis* were included as controls. The results were submitted to the *M. bovis* spoligotype database https://www.mbovis.org/ (accessed Nov 20, 2020) and SB numbers obtained based on the gained patterns. In addition to the conventional spoligotyping, the binary spoligotype data from WGS was also used for the spoligotyping.

2.3.4. 7. Whole genome sequencing (WGS) of *Mycobacterium bovis* isolates/ SNP analysis

WGS was performed at the Wellcome Trust Sanger Institute (Hinxton, Cambridge) using the Illumina HiSeq 2500, MiSeq,NextSeq 500, and HiSeqX platforms to produce paired-end reads of between 50 and150 base-pairs in length. Sequence reads were classified using Kraken v0.10.6 [289] and the abundance of the classification was refined to a single level using Bracken v1.0

[290]. Samples with less than 70% of all reads assigned to the *Mycobacterium* genus were excluded.

Sequence reads were mapped to the *M. bovis* AF2122/97 reference genome (NC0002945) using BWA v0.7.17 (Burrow-Wheeler Aligner) (minimum and maximum insert sizes of 50 and 1000, respectively) [291]. Single nucleotide polymorphisms (SNPs) were called using SAMtoolsv1.2 mpileup and BCFtoolsv1.2 (minimum base call quality of 50 and minimum root squared mapping quality of 30) as previously described [291, 292]. Samples with reads mapping to less than 90% of the AF2122/97 reference were excluded. Genomic regions consisting of GC-rich sequences such as PPE proteins and PE-PGRS repeats were masked in the resulting alignment using previously published coordinates [293]. Variable sites were extracted from the masked alignment using snp-sites v2.5.1 [294].

2.3.4. 8. TaqMan Real-Time PCR assay

Before DNA extraction, the sample (sputum, FNA and milk) was heat inactivated in water bath set at 80^{o}C for an hour. DNA was extracted following QIAGEN Quick Start Protocol (DNeasy®Blood & Tissue Kit, Cat No. 69506, Germany). Finally eluted in 100µl elution buffer and genomic DNA was stored at -20^{o}C until tested.

Primers and probes synthesis (Table 14) was done by Eurofins Genomics, Germany. Each Real-Time PCR assay was performed in a 25 µl final reaction volume. The thermal cycling conditions for all reactions were as follows: initial incubation of 50°C for 2 min followed by a 95°C incubation for 10 minutes. Then 40 cycles of the following profile: denaturing at 95°C for 15 seconds and anneal/extend at 58°C for 60 seconds. Then the assay was performed on a Rotar-Gene 3000 real-time PCR machine (GR CORREBETT RESEARCH, Australia).The assay targeted IS*1081*, RD4 and RD9. All primer and probe sequences are shown in Table 14.

IS*1081* loci and RD9 locus duplex TaqMan Real-Time PCR assay: Each reaction mixture consisted of 12.5µl Environmental Master Mix 2.0 (ABI; Prod code: 4396838), 1.25µl of 10µM IS*1081*_Fw and 1.25µl of 10µM IS*1081*_Rev primers, 0.25ul of 10µM IS1081_Probe, 5µl of purified genomic DNA, and 2µl of nuclease free water (Qiagen, Germany) that was also used for re-suspension of the primers. The corresponding composition for the detection of the *RD9* locus

in a single reaction was similar. The oligonucleotide primers: RD9_Fw and RD9_Rev primers and RD9_Probe used are published by King et al. [295].

RD4 locus TaqMan Real-Time PCR assay: The final reaction volume 25 µl was similar with the duplex PCR above but with increase in primer volume; 2.5µl of 10µM RD4_Fw and 2.5µl of 10µM RD4_Rev primers, and 0.5µl of 10µM RD4_Probe published by King et al. [295].

Quality control: Duplicate *M tuberculosis* H37Rv reference strain, *M. bovis* AF2221/97 and negative control with distilled water were included for each real-time PCR run.

Table 14. Primers and probes

Target, primer and probe	Sequence (5'-3')
IS1081	
F	GATCCTTCGAAACGACCA
R	CGGTGTCGATAAGATGAGA
P	CGAAGGAAATGACGCAATGACCTC
RD4	
F	TGTGAATTCATACAAGCCGTAGTCG
R	CCCGTAGCGTTACTGAGAAATTGC
P	AGCGCAACACTCTTGGAGTGGCCTAC
RD9	
F	TGTGAATTCATACAAGCCGTAGTCG
R	AGCGCAACACTCTTGGAGTGGCCTAC
P	AGCGCAACACTCTTGGAGTGGCCTAC

F, Forward primer; R, Reverse primer; P, Probe

Interpretation of TaqMan real-time PCR. A positive result was set from the serial dilution of a known concentration of gDNA of *M. bovis* and Ct values <38 were considered as positive and negative if not.

2. 3. 5. Eligibility Criteria

The inclusion criteria:

i) bTB study:

- All dairy cattle above six weeks of age and < 8 months pregnancy. All crossbreed cattle brought for slaughter at abattoir were included. It was assumed that crossbreed cattle originate from the peri-urban region of central Ethiopia.

ii) zTB study

- All age and sex presented at selected health centers in the study areas suspected/complained of lymphadenitis and pulmonary TB cases were included.
- All dairy farm workers in bTB infected dairy farms

The exclusion criteria:

i) bTB study: Herds with less than 5 cattle were excluded

ii) zTB study: patients already started taking anti-TB drugs were excluded.

2. 3.6. Study variables

Independent variables

Independent variables include risk factors (age, breed, herd size etc..) listed in the statistical analysis section

Dependent variables: bTB status (positive or negative) based on tuberculin skin test

2. 3. 7. Ethical Considerations

This study was first reviewed and approved by the Department of Ethical Review Committee (DERRC) (MF/MICRO/273/2011) and then by College of Health Sciences Institutional Review Board (IRB) (Protocol number: 073/19/DMIP/Date Oct-02-2019). Written Informed Consent was obtained from the participants and the procedure was following the procedure reviewed by the IRB and the form is presented in Annex 9. For the cattle study Ethical clearance was obtained from National Animal Health Diagnostic and Investigation Center (NAHDIC) Animal Research Scientific and Ethics Review Committee (ARSERC)(ARSERC/EC/002/31/07/2019)

2. 3. 8. Statistical analysis

Data from questionnaires and the tuberculin skin test were curated and coded. All the statistical analysis was performed using the R statistical language [296] and RStudio [297]. Based on the SICCT test, the animal level and herd level bTB prevalence for Addis Ababa city and surrounding five study areas was described and 95% confidence interval calculated. The Kruskal–Wallis test was used for comparison of variability in within herd bTB prevalence (%) among studied dairy herds. Direct method of standardization (adjustment) [278] was employed to adjust for the effect of having a higher representation of larger farms in the crude overall bTB prevalence result. Our dataset was hierarchal in nature *i.e*. individual animals were clustered within herds and herds were clustered within study areas. To account for this clustering and deal with variation in prevalence between study areas and in particular between herds, a Generalized Linear Mixed Model (GLMM) [298] was used which allowed us to treat herd and study areas as random effects with a binary response as an outcome variable (bTB reactor or not reactor). Animals with reading difference between 1-4 mm were treated as negatives. We used the glmer () function in the lme4 package [299]. The statistical unit of analysis was the individual animal. We performed a univariable screen to select variables for inclusion in the multivariable model. All variables with a p-value of < 0.20 and those with a high biological relevance (breed, sex, stages of lactation and age) were considered as candidate variables for the model building. These candidate explanatory variables were investigated further for collinearity requiring that all selected variables for the multivariable model have a variance inflation factor (VIF) of < 5 [156]. Statistical significance was set at the 5% level.

For binary data a binomial response (more specifically, the Bernoulli distribution) was used [300].

To specify the model, we define the binary response variable:

$$Y_i = \begin{cases} 1 & \text{If the animal is positive for bTB,} \\ 0 & \text{Otherwise.} \end{cases}$$

$Y_i \sim \text{Bin}(P_i)$

The probability P_i of the i^{th} animal being bTB positive is:

$$\log\left(\frac{P_i}{1-P}\right) = \beta_o + \beta X_i + \mu_{herd(i)} + \gamma_{area(i)},$$

Where:

β_o is the intercept

β is a parameter of fixed effects,

X_i are explanatory variables values for the i^{th} animal,

$\mu_{herd(i)}$ is the random effect of the herd (which contains animal i),

$\gamma_{area(i)}$ is the random effect of the study area (which contains animal i),

All screened predictors were initially included in the global model, including biologically plausible two-way interactions. Breed was considered as potential confounder for herd size. As some confounding is invariably present, and the important issue is how large the confounding effect is, not whether or not it is present [298]. We specified a difference of 20 % change in the odds ratio as an indication of confounding [298]. The removal of breed from the final model changed the logit of herd size by 13.2% (7.7.-6.8)/6.8) for medium herds and by 19.2% (9.9-8.8)/8.3) for large herds, thus no strong confounding effect was found between the two factors.

For model fitting in addition to the global model, a set of models were proposed to identify potential risk factors that most affect the outcome variable of interest i.e. bTB status. We used the Akaike information criterion (AIC) for comparing and selecting between models. As described by Burnham and Anderson [301], the AIC approach is first to calculate an AIC value for each model proposed and to examine the differences between the AIC values of competing models to the model with minimum value of AIC (often termed as the best model). To put this mathematically: $\Delta AIC = AICi - minAIC$; where AIC*i* is the competing model and minAIC is the model with the minimum AIC value. We used this ΔAIC value to rank and identify candidate models. A threshold was set for identifying candidate models; where models with ΔAIC < 3 and Akaike weights (w > 0.05) [156] were set as candidate models. A model with highest Akaike weights value (often interpreted as the probability that model is the best model) was used for selecting the best model. In our data we identified that the interaction effect between herd and

breed was biasing estimates of other variables (skewing the estimate for the herd size variable) due to the small number of zebu cattle in the medium herd level category. Dropping this interaction – results in the global model having both the lowest AIC and highest Akaike weight and explained the data well and subsequently selected for reporting.

In silico spoligotyping was performed using SpoTyping v2.1 [302] and the binary spoligotype representations were queried against the *M. bovis* spoligotype database (www.mbovis.org) to extract spoligotypes named in the format of SBXXXX. Clonal complexes were assigned to samples using RD-analyzer v1.0 [303] with samples not identified as belonging to previously described *M. bovis* clonal complexes [European 1 (Eu1), European 2 (Eu2), African 1 (Af1), and African 2 (Af2)] designated as 'Other' [13, 111, 173, 174]. Further assignment to clonal complex was based on the phylogenetic lineages recently identified by Loiseau et al. [274].

Maximum likelihood phylogenetic trees were constructed using IQ-tree v1.6.5 [290] (constant sites added to alignment, extended model selection and 1000 bootstraps) and the resulting trees were annotated and rooted in iTOL [304]. Pairwise SNP distances for all genomes were calculated using pairsnp (https://github.com/gtonkinhill/pairsnp).

CHAPTER THREE

3. RESULTS

3.1. Bovine TB Study

3.1.1. Prevalence of bTB in the study cattle population

This study investigated 299 dairy herds (212 small, 49 medium, and 38 large farms) for bTB using the SICCT test in the urban and peri-urban areas of central Ethiopia. In addition, descriptive data on these herds were collected (Annex 11). In total 5,675 cattle from 299 herds were tested by using the SICCT test. Overall there were 1,776 reactors (31.3% crude animal prevalence- not adjusted for herd size; 95% CI: 30-33%) in 180 herds (60.9% crude herd prevalence; 95% CI: 55.2 - 66.2%), with each positive herd having at least one reactor (Table 15). Sebeta had the highest prevalence (42% at animal level with 95% CI: 38-46% and 74%at herd level with 95% CI: 55-87%) among all six regions whereas Holeta had the lowest prevalence (17% at animal level with 95% CI: 14-20% and 27% at herd level with 95% CI: 13-46%). There was significant association between study areas and positivity of animals to tuberculin test (χ^2 = 143.18, df = 5, p-value <0.001).

Table 15. Animal and herd level bTB prevalence for 299 dairy herds in the six study areas

Level	Addis Ababa	Sebeta	Holeta	Sululta	Sendafa	Bishoftu	Total
Animal level:							
% Prev. (95%CI)	32.8(31-35)	42.2(38-46)	16.8(14-20)	41.9(38-46)	25.5(22-30)	25.5(23-28)	31.3(30-33)
Positives	797	250	90	257	134	248	1776
Total number tested	2432	593	537	614	525	974	5675
Herd level:							
% Prev.(95%CI)	63 (55-70)	74 (55-87)	30 (13-46)	60(39-78)	54(33-74)	73.3(50-85)	60.9(54-66)
Positives	100	23	9	15	13	22	182
Total number tested	159	31	30	25	24	30	299

3.1.2. Herd-size specific prevalence of bTB

The bTB prevalence was stratified on herd size based on the study population. The results showed a different prevalence between herd sizes with a significant increase in prevalence with herd size group (Table 16).

Table 16. Prevalence of bTB stratified by herd-size for the study population

	Study population				
	Herd size group	**Herds sampled**	**Population**	**bTB positives**	**Prevalence % (95% CI)**
Animal Level	Small herds (>4 to ≤20)	212	2058	373	18.1 (16.5-19.4)
	Medium herds (>20 to ≤37)	49	1233	402	32.6 (30-35.3)
	Large herds (>37 to ≤168)	38	2384	1001	42.0 (40-43.9)
	Total	299	5675	1776	**31.3 (**30-33)
Herd Level	Small herds (>4 to ≤20)	212	212	108	50.9**(**44.3-57.6)
	Medium herds (>20 to ≤37)	49	49	41	83.7**(**71-91.5)
	Large herds (>37 to ≤168)	38	38	33	86.8 **(**72.7-94.2)
	Total	299	299	182	**60.9 (**55.2-66.2)

Using GPS data for individual farms, bTB prevalence maps were created for the six study areas, each visualizing the bTB burden for large, medium and small herds (Table 16, Fig 12).

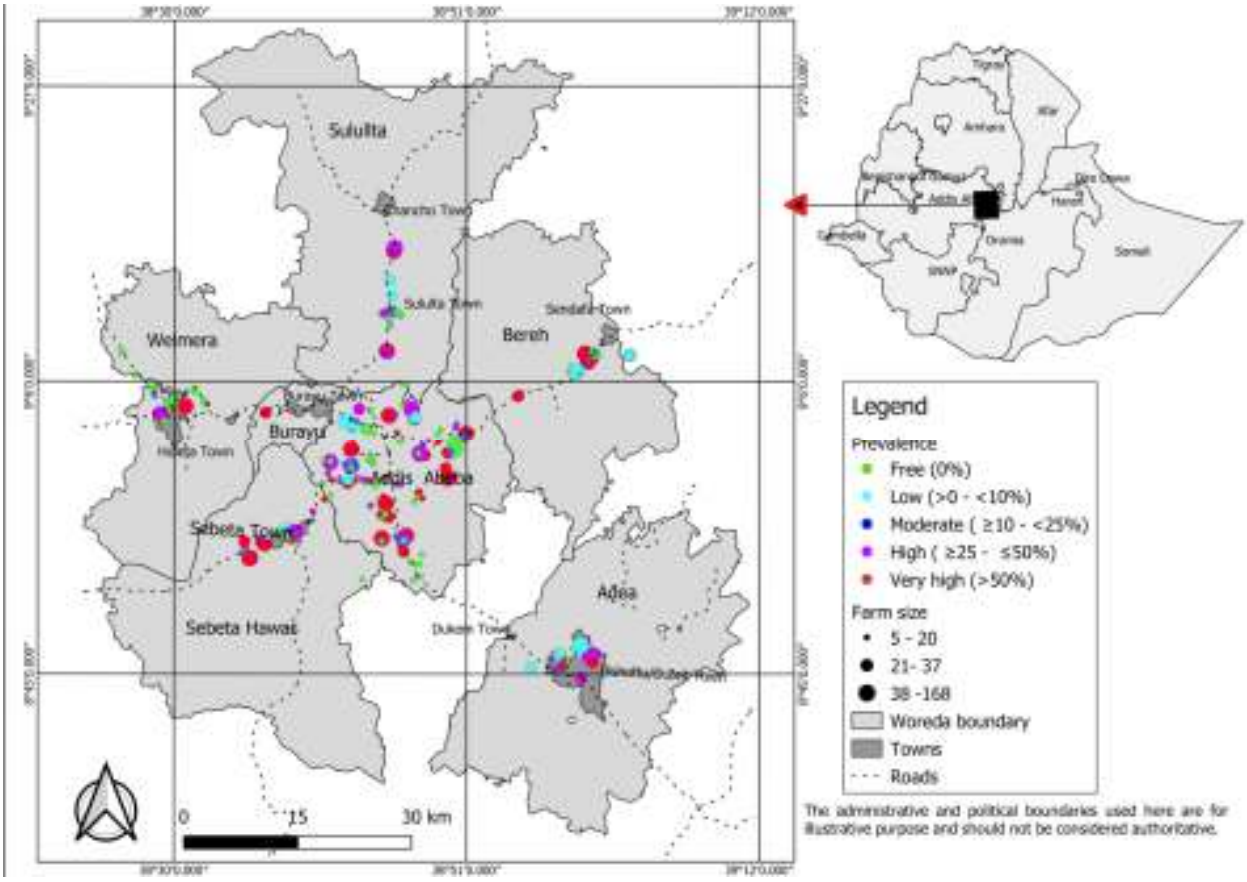


Figure 12. Map showing the geographical locations and the sizes of bTB-positive and negative herds and within-herd prevalence in central Ethiopia.

3.1.3. Risk factor analysis of bTB

3.1.3.1. Univariable analysis of potential risk factors for bTB

Sixteen potential risk factors, based on knowledge and understanding of the husbandry system and biological relevance were considered and screened by univariable analysis. Twelve variables with p-value of < 0.20 and with OR > 1 were selected for multivariable analysis (Table 17).

Table 17. Univariable analysis of potential risk factors for cattle tuberculin reactors

Risk factors	Level	Proportion % (bTBpositives/total examined)	OR (95% CI)	P value
Herd size	>4 to ≤20	18.1 (373/2058)	ref	
	>20 to ≤37	32.6 (402/1233)	2.2 (1.8-2.6)	<0.001
	>37 to ≤168	42 (1001/2384)	3.3 (2.8-3.8)	<0.001
Age (yrs)	>0.1 to ≤2	21.3 (422/1980)	ref	
	>2 to ≤4	33.1 (470/ 1420)	1.8 (1.5-2.1)	<0.001
	>4 to ≤6	34.3(376 /1095)	1.9 (1.6-2.3)	<0.001
	>6 to ≤8	39.7(224/564)	2.4 (1.9-3)	<0.001
	>8 to ≤10	41.6 (82/19)	2.6 (1.9-3.6)	<0.001
Source	On farm bred	30 (1431/4757)	ref	
	Purchased	37.5 (344/916)	1.4 (1.2-1.6)	<0.001
Breed	Zebu	7.8 (19/244)	ref	
	Cross and exotic	32.3 (1757/5431)	5.7 (3.6-9.4)	<0.001
Sex	Male	18 (78/433)	ref	
	Female	32.4 (1698/5242)	2.2 (1.7-2.8)	<0.001
Farm age (yrs)	>4to ≤20	25.4 (695/2736)	ref	
	>20 to ≤35	36.6 (715/1951)	1.7 (1.4-1.9)	<0.001
	>35 to ≤68	30 (213/708)	1.3 (1-1.5)	0.01
bTB history at farm	No	33.4 (538/1607)	ref	
	Yes	40.8 (381/932)	1.4 (1.1-1.6)	<0.001
Contact with other domestic animals	No	31.5 (254 /806)	ref	
	Yes	32.5 (702/2161)	1.04 (0.8-1.2)	0.64
Stocking density (no. cattle/m^2)	Less	28.6 (1314/4601)	ref	
	Satisfactory	35.4 (34/96)	0.7 (0.5-1.1)	0.14

	High	39.8 (300/753)	1.2 (0.8-1.9)	0.4
Ventilation	Very good	28.6 (608/2127)	ref	
	Satisfactory	29.7 (506/1706)	1 (0.9-1.2)	0.46
	Poor	34.9 (548/1572)	1.3 (1.2-1.5)	< 0.001
Viral disease outbreak	Yes	30.6 (851/2784)	ref	
	No	31.2 (867/2728)	0.9 (0.8-1.1)	0.35
Biosecurity measures	Present	26.4 (384/1457)	ref	
	Absent	32.8 (1349/4109)	1.4 (1.1-1.6)	< 0.001
Neighbor herd	No	21.5 (106/494)	ref	
	Yes	31.4 (1527/4857)	1.7 (1.3-2.1)	<0.001
House type	Cubicle	21.4 (281/1313)	ref	
	Loose	34.5 (1329/3856)	1.9 (1.6-2.2)	<0.001
	Free movement	27.2 (94/345)	1.4 (1-1.8)	0.02
Regular de-worming	No	35.3 (428/1212)	ref	
	Yes	29.2 (1239/4247)	0.8 (0.7-9.9)	<0.001
Stages of lactation (months)	>0 to ≤2	34.7 (137/395)	ref	
	>2 to ≤4	36.2 (179/494)	1.1(0.8-1.4)	0.63
	>4 to ≤8	39.2 (304/776)	1.2(0.6-1.6)	0.13

Total number of examined animals (3rd column in Table 4) used for analysis of respective risk factor may differ from the overall number of animals tested (N= 5,675) due to missing values.

Contact with other domestic animals, stages of lactation, viral disease outbreak, and regular de-worming did not fulfill the stated criteria and were excluded from analysis based on the criteria described above (OR and p-value). A full description of the measured risk factors is provided in Annex 13.

3.1.3.2. Multivariable analysis of potential risk factors for bTB using GLMM with herd and area as random effect

Based on their high OR, absence of collinearity and statistical significance (p-value <0.2), twelve variables (Table 18) were considered in the final multivariable model. The final model thus consisted of four variables: herd size, age, bTB history at farm, and breed as significant risk factors for bTB. Animals from large herds had 8.3 times the odds of being a bTB tuberculin reactor compared to animals living in small herds. There was also a strong effect of age, with animals 8-10 years of age having 8.9 times the odds of being reactors compared to the youngest category (Table 18). Cattle from farms with history of bTB were 5.2 times to be reacotos compared to cattle from farms with no history of bTB. Cross and exotic (pure) breed cattle had 2.5 times the odds of being a bTB tuberculin reactor compared to Zebu breeds (Table 18).

Table 18. GLMM multivariable analysis of potential risk factors for bTB positive cattle using herd and area as random effect

Risk factor	Level	OR (95% CI)	P value
Herd size	>4 to ≤20	ref	
	>20 to ≤37	6.8 (2.6-17.9)	0.001
	>37 to ≤168	8.3 (2.2-31.5)	0.008
Age (yrs)	>0.1 to ≤2	ref	
	>2 to ≤4	2.7.1 (2.1-3.6)	<0.001
	>4 to≤ 6	3.5 (2.6-4.8)	<0.001
	>6 to ≤8	5 (3.5-7.2)	<0.001
	>8 to ≤10	8.9 (5-15.6)	<0.001
bTB history at farm*	No	ref	
	Yes	5.2 (2.1-12.9)	0.003
Breed	Zebu	ref	
	Cross and exotic	2.5 (1.2-4.5)	0.032

* Data based on records of the farm (if tested for bovine TB during the last three years of the study date)

3.2. ZoonoticTB study

3.2.1. Dairy Farm workers demographic and clinical characteristics

All DFWs (n=219) in the 73 bTB infected dairy farms were invited to participate for the active case detection of TB and those who provided informed consent (n=110) were enrolled in this study. Among the 110 DFWs who were screened for human TB, 41 had at least one of the six constitutional symptoms shown in Table 19 (i.e. coughing sputum, coughing blood, chest pain, weight loss, fever, and night sweats) that are typical for TB. None of the DFWs had signs of all six constitutional symptoms, but one patient had 5/6 symptoms, seven patients had 4/6 symptoms, 14 patients had 3/6 symptoms, 11 patients had 2/6, while eight patients had 1/6 of the constitutional symptoms. Three DFWs had swollen nodes at their neck, a symptom typical for TB lymphadenitis, while two DFWs had a history of TB. Patients with fever or night sweats only were excluded as TB suspects.

Table 19. Demographic and clinical characteristics for TB suspected dairy farm workers

	Characteristic	Category	Frequency	%
Demographic	sex	Female	8	19.5
		Male	33	80.5
	age	≤20 years	8	19.5
		>20 - ≤45 years	21	51.2
		>45 years	12	29.3
	Education	Illiterate	15	36.5
		Primary	14	34.1
		Secondary	9	21.9
		College/University	3	7.3
	Marital status	single	18	43.9
		married	21	51.2
		separated	0	0
		divorced	1	2.4
		widowed	1	2.4
Clinical	Currently coughing up sputum	Yes	20	48.8
	Currently coughing up blood	Yes	1	2.4
	Chest pain	Yes	12	29.3
	Weight loss	Yes	18	43.9
	Fever	Yes	23	56.1
	Night sweats	Yes	30	73.1
	Presence of swollen nodes at neck	Yes	3	7.3
	BCG Vaccination	Yes	9	21.9
	History of TB	Yes	2	4.9

3.2.2. Assessment of knowledge and practices related to zTB

The majority (61%) of the 41 TB suspected DFWs did not know about cattle TB (Table 20). Raw milk consumption was practiced by more than two thirds of the DFWs with symptoms (68.2%, Table 18). The practice of raw meat consumption among DFWs with symptoms was much higher (85.4%, Table 20) as compared to raw milk. More than two thirds of DFWs with symptoms did not think TB could be transmitted via raw or undercooked meat.

Table 20. Knowledge and practices on risk factors for zTB transmission among TB suspected dairy farm workers

Practice/knowledge/risk factor	Category	Frequency	%
Consumption of raw milk	Yes	28	68.2
	No	13	31.7
Consumption of *Ergo* (traditional fermented milk)	Yes	31	75.6
	No	10	24.4
Do you think drinking raw milk or ergo can transfer TB from animals to humans?	Yes	21	51.2
	No	20	48.8
What is your main source of meat?	home slaughter	3	7.3
	butchery	16	39.0
	communal slaughter ('*Qircha*')	9	22.0
	More than one source	13	31.7
Consumption of raw meat/kitfo/Dulet/Kurt	Yes	35	85.4
	No	6	14.6
Do you think eating raw meat/kitfo/Dulet/Kurt can transfer TB from animals to humans?	Yes	12	29.3
	No	29	70.7
Do you have close contact with cattle?	Yes	34	82.9
	No	7	17.1
Were there coughing cattle in your herd?	Yes	21	51.2
	No	20	48.8
Do you know about cattle TB?	Yes	16	39.0
	No	25	61.0
Occupation	a. Farm owner	3	7.3
	b. Veterinarian	1	2.4
	c. Guard/shepherd	10	24.4
	d. Farm worker (other than milking)	9	21.9
	e. Farm manager	1	2.4
	f. Family member	2	5.0
	g. Milker	15	36.6

3.2.3. *Mycobacterium bovis* isolation from human specimens

A total of 167 specimens (sputum=131; FNA=36) were collected from 161 TB suspected individuals for the isolation of *M. bovis* (Addis Ababa (39), Sebeta (4), Holeta (18), Sululta (17), Sendafa (32), Bishoftu (57)). Forty four (44) specimens were obtained from dairy farm workers and 123 were from health facilities. Six individuals provided double samples (sputum and FNA). Of these processed specimens three *M. bovis* isolates were detected in three patients (1.8%, n=161); one was from sputum culture and additional two were from culture negative FNA samples using RT-PCR. The *M. bovis* isolate obtained from culture was sequenced and the genotype was spoligotype SB1476, a previously reported spoligotype from cattle in Ethiopia (Fig. 15). Also from these processed specimens 41 *M. tuberculosis* were isolated. From DFWs specimens no *M. bovis* was detected and one *M. tuberculosis* was detected by RT-PCR.

3.2.4. Potential sources of infection for human TB caused by *M. bovis*

3.2.4.1. *Mycobacterium bovis* isolation from cattle tuberculous lesions and raw milk

A total of 827 cattle from abattoirs and dairy farms were examined during postmortem exercise, 76 of them (9.2%) had tuberculous lesion. Out of this, a total of 137 specimens (more than one specimen per animal was taken when necessary) were collected and cultured for the isolation of *M. bovis* (Table 21). Seven specimens were not used due to contamination. From tuberculin skin test positive milking cows, 490 composite milk samples were collected and cultured for isolation of *M. bovis* (Table 21). Sixty two *M. bovis* isolates (47.7 %, n=130; seven samples excluded for contamination) were obtained out of a total 137 tissue samples cultured. The number of cattle with the tuberculous lesion that yield these 62 *M. bovis* isolates were 42 (55.3%, n=76) (18 were with >one isolate) and seven had mixed *M. bovis* infections. Similarly out of 975 milking cows which were tuberculin skin test positive (37.8%, n=2582), 490 raw milk samples were collected and of these 11(2.2%) yield *M. bovis* isolates showing evidence that raw milk is not safe and can be a source of infection for human TB due to *M. bovis*. All isolates were confirmed to be *M. bovis* using RD4 deletion typing.

Table 21. *Mycobacterium bovis* isolates from cattle tuberculous tissue lesions and raw milk

Sample		Abattoir/Farm location						
		Sululta	Addis Ababa	Bishoftu	Holeta	Sebeta	Sendafa	Total
Tisuue TB lesion	Number of animals examined pm* (a)	451	350	11	15	-	-	827
	Number of animals with tuberculous lesion(b) (%=b/a*100)	29 (6.4)	27 (7.7)	9 (81.8)	11 (73.3)	-	-	76 (9.2)
	Number of animals with *M.bovis* isolated (c)(%=c/b*100)	16 (55.2)	10 (37)	6 (66.7)	10 (90.9)	-	-	42 (55.3)
Raw milk	Number of raw milk sample collected from bTB positive cows (d)	40	44	100	73	120	113	490
	Number of *M.bovis* isolated (e) (%=e/d*100)	0	0	2 (2)	2 (2.7)	7 (5.8)	0	11 (2.2)

*pm: post mortem; letters in bracket (a-e) are placed for easy understanding of how the % in the table is calculated.

3.2.4.2. Proportion of *Mycobacteriumbovis* isolates by lymph nodes

Most of the isolates (58%; 36/62) were obtained from lymph nodes of the head and the thoracic region and mediastinal lymph node yield the highest isolate 22.6% (n=14) (Fig. 13).

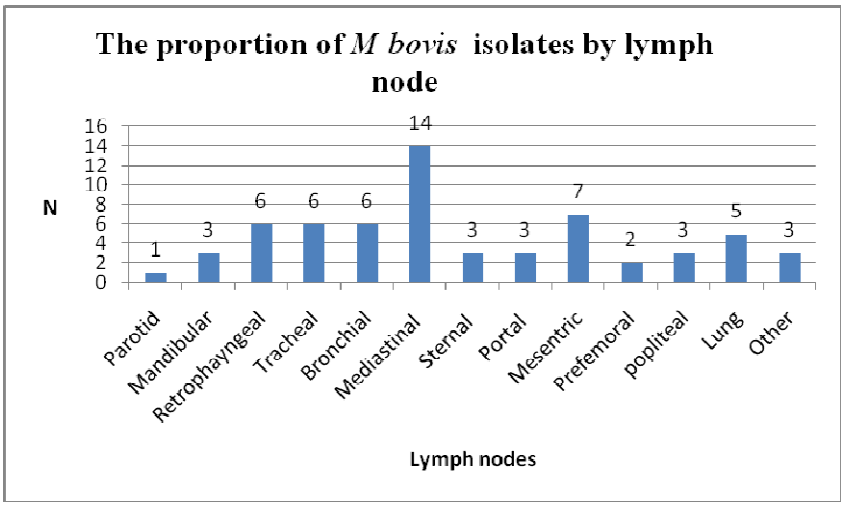


Figure 13. Frequency of *M. bovis* isolation by lymph nodes and other tissues

3.3. Genetic diversity of *Mycobacterium bovis* isolates that originated from human and cattle specimens

3.3.1. Spoligotyping

Out of the total 73 *M. bovis* isolates obtained from cattle, spoligotype patterns were determined for 70 isolates (59 from tissue lesions and 11 from milk). Of the three *M. bovis* detected in humans, one was spoligotyped (two were not sucessful). All the isolates were run for conventional and *in silico* (WGS based) spoligotyping (Table 22). Disagreement between the two typing methods was seen only for six isolates in which case conventional typing method was used for reporting. Combining the results of the two typing methods revealed ten spoligotypes, one being a human isolate. SB1176 was distributed across all sites except Sendafa from where we collected milk samples only (Table 22). In addition to two slaughter houses where much of the isolates were obtained, 22 isolates originated from and spoligotyped from eight dairy farms. Of these farms, two farms were identified with more than one spoligotype. SB1176 was the most prevalent type in most of the farms though diversity was seen between farms. Of the three from human, one *M. bovis* isolate was spoligotyped and it was SB1476 which was previously reported from cattle in Ethiopia suggesting possible zoonotic transmission.

Table 22. Geographic distribution of spoligotypes

Spoligo Number			Study site						Total (%)
	Clonal complex	Host	Sululta	Addis Ababa	Bishoftu	Holeta	Sebeta	Sendafa	
SB1176	Af2	Cattle	14	5	7(†1)	1(1)	4(4)	0	31 (44.3)
SB0133	Af2	Cattle	6	4	0	0	1	0	11 (15.7)
SB0912	Af2	Cattle	1	0	0	9	0	0	10 (14.3)
SB0134	Eu3	Cattle	3	4	0	0	0	0	7 (10.0)
SB1521	Af2	Cattle	1	0	0	2	1(1)	0	4 (5.7)
SB1878	Af2	Cattle	2	0	1(1)	1(1)	0	0	4 (5.7)
SB2521	Af2	Cattle	0	1	0	0	0	0	1 (1.4)
SB2693	Af2	Cattle	0	0	0	0	1(1)	0	1(1.4)
Unkown	Af2	Cattle	0	0	0	0	1(1)	0	1(1.4)
SB1476	Unkown8	Human	0	0	1	0	0	0	na*

*: na: not applicable-single isolate, †Numbers in bracket represent isolates from milk, e.g. '7(1)' equals seven isolates of which one is from milk and the remaining were from tissue; human spoligotype(sputum)

In cattle spoligotype SB1176 was the most prevalent type (n=31, 44.3%) followed by SB0133 (n=11, 15.7%) and the least were SB2521 and SB2693, a single isolate (Fig.14).

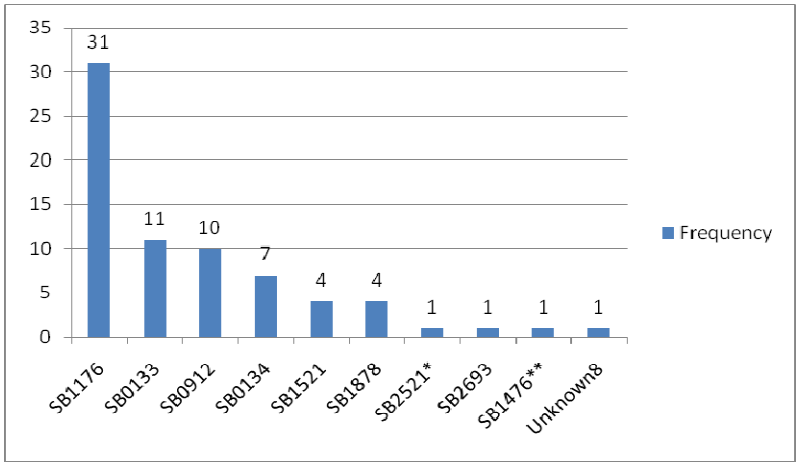


Figure 14. Frequencies of spoligotype patterns for *M. bovis* in cattle and humans; the asterisks (*) denote first report from Ethiopia, (**) denote human spoligotype.

3.3.2. Whole genome sequencing (WGS)

3.3.2.1. Phylogenetic relationships of *Mycobacterium bovis* genomes

A total of 55 *M. bovis* isolates were successfully sequenced and passed QC; these included 54 from cattle (21-- farm and 33 -- abattoir) and a single isolate from human TB patient. Three different clonal complexes were observed in the dataset. The phylogenetic tree of 55 genomes, rooted with the Eu1 reference AF2122/97, shows a distinct phylogenetic structure with the three clonal complexes clearly segregating in the phylogeny (Fig. 15): African 2 (Af2; n=47), European 3 (Eu3; n=7) and Unknown8 (n=1). Taking a common node (Most Recent Ancestor-MRCA), there were two cluster groups or sublineages originating from African 2 complex (Fig 15) showing the genetic diversity within the complex. However, for Eu3 unlike Af2 where we saw high genetic diversity (different spoligotypes), only one spoligotype i.e. SB0134 was seen (high clonality) and this spoligotype is genetically distant compared to other spoligotypes reported in this study. Isolates from Holeta showed high clonality and segregation-not reported from other regions of this study (Fig 15).

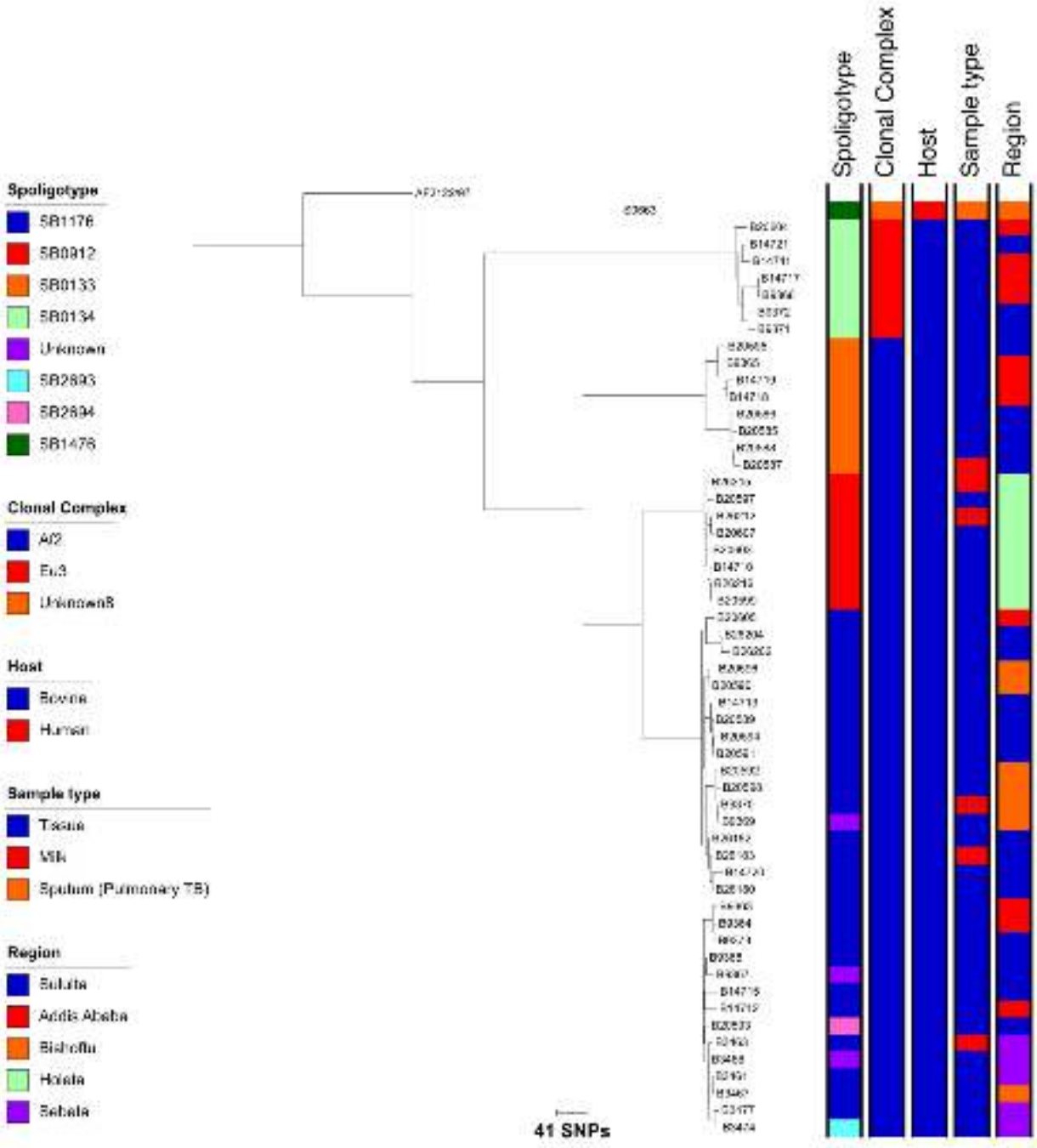


Figure 15. Maximum-likelihood phylogenetic tree of 55 genomes rooted using *M.bovis* AF2122/97

3.3.2.2. Genetic diversity of *Mycobacterium bovis* isolates based on single nucleotide polymorphisms (SNPs)

The distribution of all pairwise SNP distances of 191 median (range: 2– 696) within the dataset was multimodal reflecting the population structure of the dataset (Fig. 16A). Categorizing the pairwise SNP distances by clonal complex showed that there was considerably more diversity within Af2 compared to Eu3 with the maximum pairwise SNP distance within Af2 being 396 SNPs (median 180 SNPs), compared to Eu3 47 SNPs (median 38 SNPs) (Fig. 16 B). The within-host pairwise SNP diversity had a median of nine SNPs (range 2–18 SNPs) whilst the between-host pairwise SNP diversity had a median of 192 SNPs (range 2–696 SNPs; Fig. 16C). The maximum within-host pairwise SNP distance was 647 SNPs.

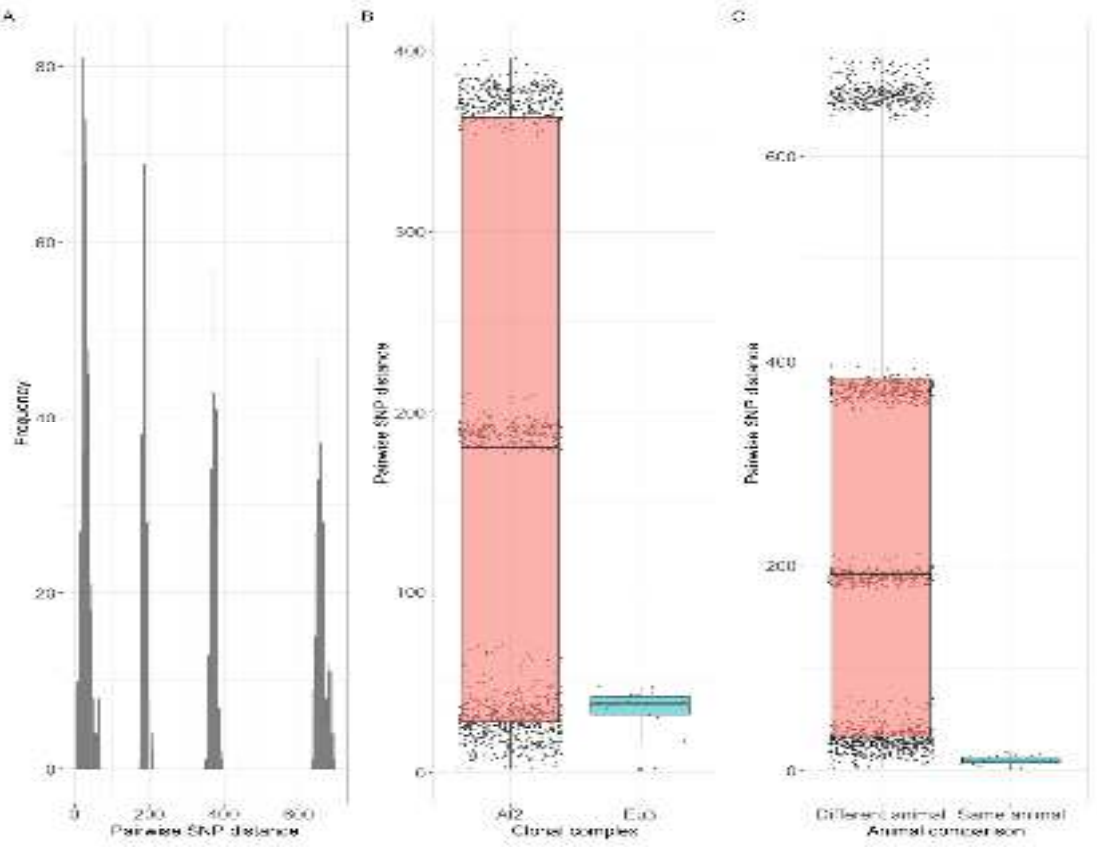


Figure 16. (A) Histogram of all pairwise SNP distances. (B) Boxplot of all pairwise SNP distances separated by clonal complex. (C) Boxplot of all pairwise SNP distances separated by within- and between-animal

3.4. Evaluation of the performance of a TaqMan Real-Time PCR assay

3.4.1. Analytical sensitivity in purified genomic DNA

The limit of detection (LOD) and amplification efficiency (%) of IS*1081* and RD4 target genes in the TaqMan RT-PCR assay were estimated. Analytical sensitivity was estimated on ten-fold serial dilutions done in triplicate from a 10ng/µl stock of gDNA of *M. bovis* (Table 23). The lowest concentration of DNA detected by the Real-Time PCR for RD4 target was $10*10^{-6}$ng/µl (10 fg/ul) for RD4 and $10*10^{-7}$ng/µl for IS*1081*(1fg/ul). Fitting this value to the formula for calculating the number of copies/reaction (Formula 1) provide 10 templates for RD4 and 6 templates for IS*1081*. This means the assay can detect upto 10 *M.bovis* using RD4 and a single *M. bovis* using IS1081 as it has 6 copies of IS*1081*. For IS*1081* amplification Ct values were recorded upto 10^{-7} dilution unlike RD4 and this could be due to the presence of more copies of IS*1081* or due to non specific reaction (Fig. 18).

Table 23. Ten-fold serial dilutions done in triplicate from a stock of gDNA of *M. bovis*

Dilution series (10ng/µL gDNA *M bovis*)	Targets	*R-1	R-2	R-3	Average Ct Value	**NTC
10^0 (original/undiluted)	IS*1081*	15.25	15.14	15.12	15.17	***UD
	RD4	18.06	17.91	17.67	17.88	UD
10^-1	IS*1081*	17.66	17.55	17.57	17.59	UD
	RD4	20.98	20.86	20.74	20.86	UD
10^-2	IS*1081*	20.64	20.65	20.65	20.65	UD
	RD4	24.23	24.14	24.13	24.16	UD
10^-3	IS*1081*	24.01	23.94	23.97	23.97	UD
	RD4	27.58	27.47	27.49	27.51	UD
10^-4	IS*1081*	28.24	28.25	28.22	28.23	UD
	RD4	32.11	31.86	31.98	31.98	UD
10^-5	IS*1081*	32.08	30.9	32.01	31.66	UD
	RD4	36.29	36.1	36.3	36.23	UD
10^-6	IS*1081*	33.05	33.01	33.25	33.1	UD
	RD4	36.37	38.84	36	36.40	UD
10^-7	IS*1081*	35.17	36.63	36.54	36.11	UD
	RD4	UD	UD	UD	UD	UD

*R-1, R-2, R-3: Replicates where 1-3 stands for the first, second and third replicates; **NTC: negative template controls i.e. Qiagen H_2O; ***UD: Undetected.

A standard curve was constructed for IS*1081* and RD4 (Fig.17 and 19) to assess the PCR efficiency (from the slope) and how well the dilution series was performed (linearity: R2). The reaction efficiency for IS*1081* and RD4 respectively was 101.8% and 97.6%.

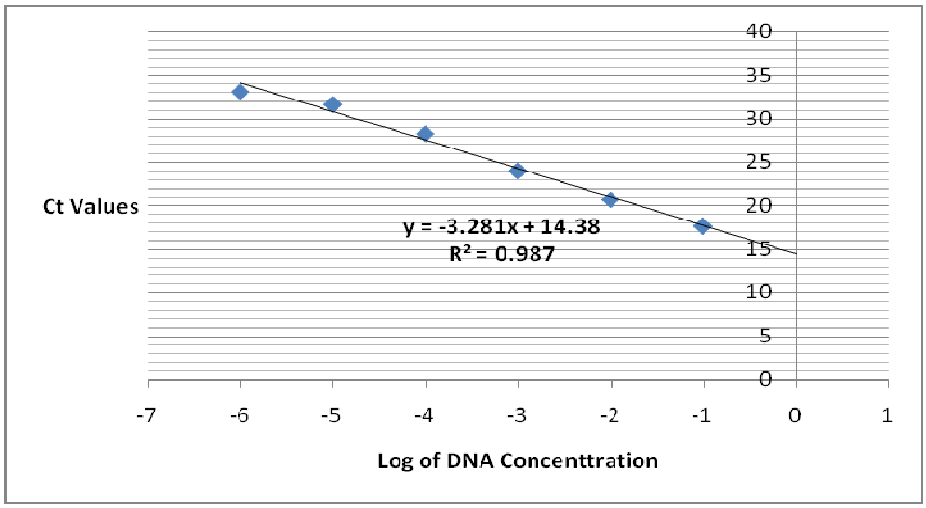


Figure 17. Standard curve generated by graphing the log of the DNA concentration used vs. the CT value for *IS1081*.

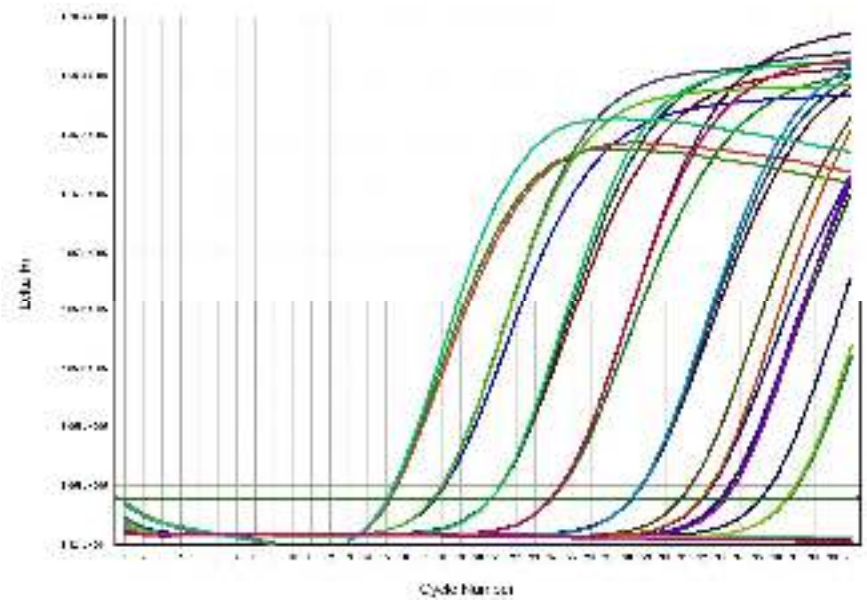


Figure 18. Amplification plot for *IS1081* for a 10-fold dilution series (from undiluted 10ng gDNA/ul to 10^{-7} dilution) left to right respectively

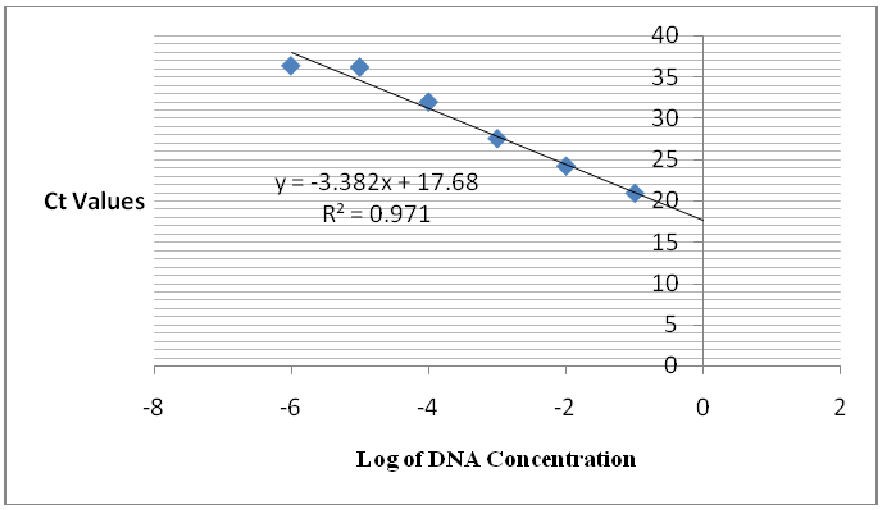


Figure 19. Standard curve generated for RD4 by graphing the log of the DNA concentration used vs the Ct value.

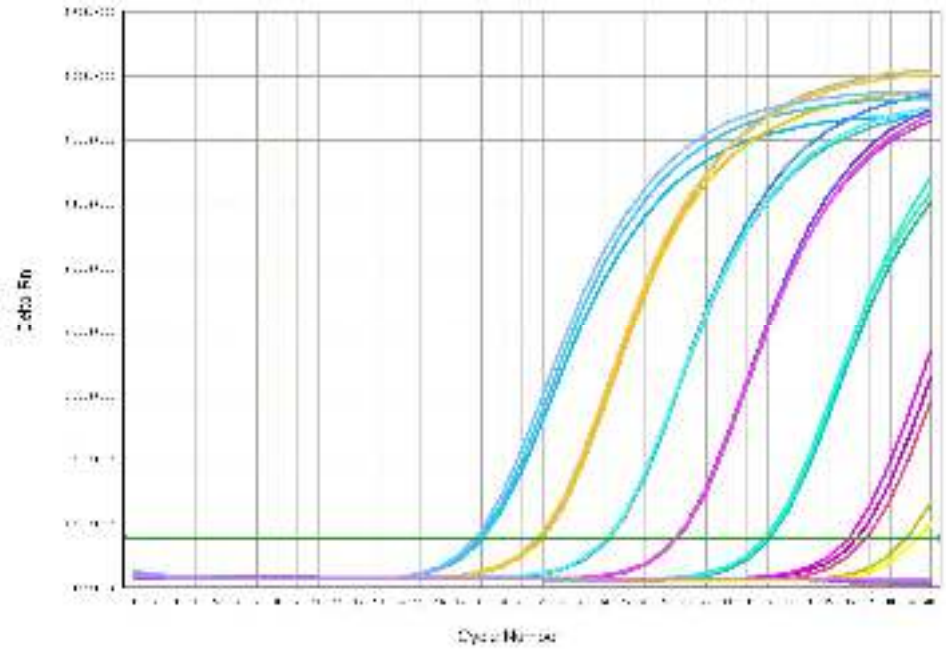


Figure 20. Amplification plot for for RD4 a 10-fold dilution series (from undiluted ($10x10^{6}$) to 10^{-7} dilution) left to right, respectively.

3.4.2. Analytical sensitivity in spiked samples

The analytical LODs and amplification efficiency of the assay was also tested by spiking serial dilutions of quantified CFU of *M. bovis* BCG into negative sputum, FNA and milk samples in order to explore the effects of the matrix in these samples (Table 24). The TaqMan RT-PCR assay using IS*1081* and RD4 targets, each detected the presence of *M. bovis* BCG correctly 100% of the time that a sample was tested, down to dilutions of 2x10 CFU/ml in all samples. The amplification efficiency was within a range of 90.3% -147.2% for IS*1081* and 98.4% - 108.6% for RD4 gene in all samples (Table 24). Higher amplification efficiency (147.2%) was recorded for milk sample.

Table 24. Limits of detection for *M. bovis* BCG by amplification of RD4 and IS1081 with RT-PCR in spiked samples

Sample type	Target & average Ct value	2x10^4 CFU/ml	2x10^3 CFU/ml	2x10^2 CFU/ml	2x10^1 CFU/ml	Efficiency (%)	R^2
Sputum	IS1081 (average Ct Values)	12.67	15.99	18.53	22	112.1	0.997
	RD4 (average Ct Values)	22.6	25.3	28	31.6	101.8	0.987
FNA	IS1081(average Ct Values)	14.01	19.07	22.6	23.69	99.8	0.931
	RD4 (average Ct Values)	20.73	23.46	26.24	31.01	98.4	0.978
Milk	IS1081 (average Ct Values)	12.95	17.92	18.94	21.09	147.2	0.908
	RD4 (average Ct Values)	22.1	22.2	27.5	29.7	104.1	0.992
Water	IS1081 (average Ct Values)	12.58	15.17	17.41	23.76	90.3	0.933
	RD4 (average Ct Values)	17.3	21.48	24.9	26.6	108.6	0.968

3.4.3. Analytical specificity

The analytical specificity of the assay was tested on four different non-Mycobacteria species (Gram-positive and -negative bacteria) in triplicate samples to assess the cross-reactivity of the assay primers and probes. No signals were generated from the two detection probes targeting the IS*1081* and RD4 genes for all the replicates tested (Table 25). Nontuberculous mycobacteria (NTM) that should have been tested for the cross-reactivity of the assay were not included in this study due to difficulty in sourcing such isolates.

Assay inclusivity: The assay capacity to detect a variety of *M. bovis* strains was examined. DNAs from three different clinical *M. bovis* isolates, *M. bovis* BCG, and *M. tuberculosis* H37Rv were tested in three replicates. The presence of *M. bovis* was detected in all replicates (Table 25), indicating the assay inclusivity. However, this has to be evaluated in a reasonable large sample size and representative spoligotypes prevalent in Ethiopia.

Table 25. Evaluation of TaqMan real-time PCR specificity with mycobacteria and non-mycobacteria isolates.

Bacteria	Source	Test specificity for	
		IS*1081*	RD4
Inclusivity testing			
M bovis BCG	NAHDIC	+	+
M bovis (clinical isolates)	NAHDIC	+	+
M tuberculosis H37Rv	AHRI	+	na
Exclusivity testing			
M tuberculosis H37Rv	AHRI	na	-
S aureus	AHRI	-	-
Str. pneumonae	AHRI	-	-
Hae. influenzae	AHRI	-	-
Kl. pneumonae	AHRI	-	-

na: Not applicable

3.4.4. Evaluation of a TaqMan Real-Time PCR assay in clinical samples

The TaqMan RT-PCR assay was evaluated in detecting MTBC, *M. tuberculosis* and *M .bovis* in clinical specimens. This study assayed specimens from human (sputum=131; FNA=36) and cattle (milk=273) using the TaqMan RT-PCR assay with primers IS*1081*, RD9 and RD4 and culture as a gold standard (Table 26). The TaqMan RT-PCR assay was compared to the gold standard-culture by the proportion of the true positive ('diagnostic sensitivity') and true negative ('diagnostic specificity') samples that it identifies. According to the gold standard, TaqMan RT-PCR correctly identified 30 of the 31 sputum positive samples using IS*1081*, so the estimated diagnostic sensitivity of test was 30/31×100 %= 96.8 % (95% CI, 81.5, 99.8). Ninety four of the 100 negative samples were correctly identified, so the estimated diagnostic specificity of test was 94/100×100 %=94 % (95%CI, 86.9, 97.5). The respective values for the remaining primers and specimens are presented in Table 26.

Table 26. Comparison of real-time PCR results with microscopy and culture for detecting *Mycobacterium tuberculosis complex*

Specimens		Culture (*M.bovis*)	Culture (*M tb*)	IS1081(Ct value <38)	RD9 (Ct value <38)	RD4 (Ct value <38)
Sputum	total	131	131	131	131	131
	positive	1	30	61	33	1
	negative	130	101	70	98	130
	Sensitivity(95%CI)	gold standard	gold standard	96.8(81.5, 99.8)	93.3(76.5, 98.8)	100(54.6, 100)
	Specificity(95%CI)	gold standard	gold standard	94(86.9, 97.5)	96(89.5, 98.7)	100(96.4, 100)
	PPV			83.3(30/36)	90.9(30/33)	100(1/1)
	NPV			98.9(94/95)	98(96/98)	100(130/130)
FNA	total	36	36	36	36	36
	positive	0	11	27	20	2
	negative	36	25	9	16	34
	Sensitivity(95%CI)	gold standard	gold standard	100(67.8, 100)	100(67.8, 100)	100(19.8, 100)
	Specificity (95%CI)	gold standard	gold standard	44(25, 64.7)	56(35.3, 74.9)	100(87.3, 100)
	PPV			44(11/25)	52.4(11/21)	
	NPV			100(11/11)	100(15/15)	
Milk	total	490	490	273	ND	273
	positive	11	0	186		11
	negative	479	490	87		262
	Sensitivity(95%CI)	gold standard	gold standard	54.5(24.6, 81.9)		9.1(0.5, 42.9)
	Specificity(95%CI)	gold standard	gold standard	50.6(42.7, 58.5)		90.5(86.1, 93.6)
	TPV			32.3 (6/186)		
	NPV			94.3 (82/87)		

Sensitivity (%)=(a/a+c)*100; specificity (%)=(d/b+d)*100; positive predictive value (PPV) (%)=(a/a+b)*100; negative predictive value(NPV) (%)=(d/c+d)*100; a:True positive; b: False positive; c:False negative; d:True negative

3.4.5. Evaluation of a TaqMan Real-Time PCR assay in samples from apparently healthy individuals and cows

The assay performance was evaluated in 31 sputum samples collected from individuals with no evidence or complaints of pulmonary TB (e.g. cough, fever etc...) to assess its specificity. The specimens were all negative for culture (on L-J media). Five specimens become positive for IS*1081* with diagnostic specificty of 83.9% (26/31) and the similar diagnostic specificity value for RD9 and RD4 was 100% (31/31) as all samples were negative(Table 27). A similar assay was run in 72 milk samples collected from double tuberculin skin test negative cows and with no history and clinical signs of bTB such as coughing, emaciation etc.The result showed no growth in LJ, all negative for RD4 typing but five samples became positive for IS*1081* giving it a diagnostic specificty of 93.1 % (67/72) and the similar value for RD4 was 100% (72/72). RD9 was not run for all milk samples (Table 27).

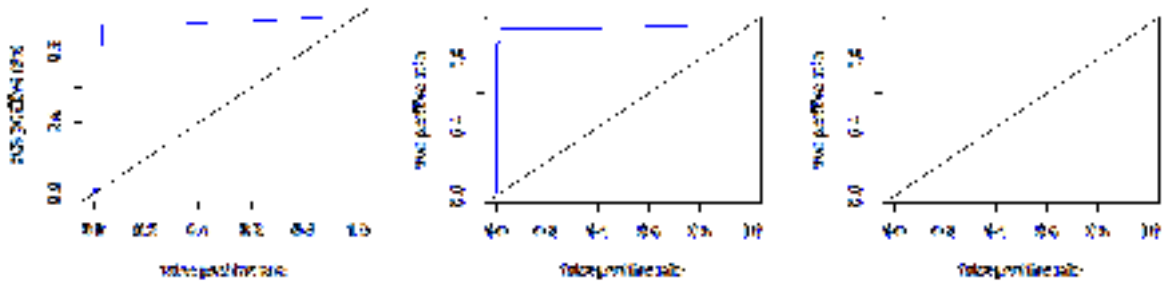
Table 27. Evaluation of a TaqMan Real-Time PCR assay in known negative samples

Specimens	Number	Culture (-)[a] (*M.bovis*)	Culture (-) (*M tb*)	IS1081(-)[b]	RD9 (-)	RD4 (-)
Sputum (known negative samples)	31	31	31	26	31	31
Specificity(95%CI)		Gold standard	Gold standard	83.9(65.5-93.9)	100(86.3-100)	100(86.3-100)
Milk (known negative samples)	72	72	72	67	ND	100
Specificity(95%CI)		Gold standard	Gold standard	93.1(83.9-97.4)	ND	100(86.3-100)

[a:] culture negative; [b] : Ct value greater than or equal to 38 was considered as negative; ND: Not done

3.4.6. Receiver Operating Characteristic Curve and Area Under The Curve (AUC)

ROC analysis was used (Fig. 21) to find the cutoff point-the point where maximum sensitivity and specificity (Youden index) is obtained. AUC- an effective way to summarize the overall diagnostic accuracy of the test was also calculated. The intrepretation was: AUC of 0.5 suggests no discrimination (i.e., ability to diagnose patients with and without the disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding. Sputum and FNA data was used to caculate cuttoff point and AUC and the cuttoff point for all targets was Ct value of 40 and the corresponding AUC for RD9, IS1081 and RD4, respectively was 95.2%, 98% and 98% (Fig 21).



(A) (B) (C)

Figure 21. ROC curves of RD9 (A), IS101 (B) and RD4(C) of TaqMan RT-PCR assay

CHAPTER FOUR

4.0 DISCUSSION

This study was set out to perform the largest bTB prevalence study so far in dairy farms in central Ethiopia to get a comprehensive understanding of the scope of the burden of the disease and identify potential risk factors contributing to the transmission of bTB within the study area. Also this study aimed to examine the genetic diversity of *M. bovis* obtained from cattle and human specimens. This study also assess the prevalence of zTB and potential risk factors in central Ethiopia. Another important feature of this study was evaluation of the performance of RT-PCR as an alternative diagnostic method for direct detection of *M. bovis* in human and cattle specimens.

4.1. bTB study

This study recorded an overall crude animal prevalence of 31.3% (n=1,776) (herd size adjusted: 24.5%) and a 60.9% (n=180) crude prevalence at herd level (herd size adjusted: 54.4%). However, there was variation between the six study areas: relatively low prevalence was recorded in Holeta and this could be related to earlier work to control for bTB in selected infected government herds in that area, which at the time were supplying heifers to surrounding farmers [140]. In this survey we also noted significant variation of within-herd bTB prevalence (P-value < 0.05) among the studied dairy herds, which ranged from 0 to 100% and with a mean for all herds of 31.5% ± 30.7 SD. This variability could be due to differences in transmission due to husbandry and other risk factors listed in univariable logistic regression analysis of this thesis or as reviewed by Broughan et al. [158]. By herd stratification, large herds recorded the highest within-herd prevalence (mean: 40.6%) and a larger proportion (65.8%) had a within-herd prevalence greater than the population average. Such high herd prevalence could be due to an increased risk of within-herd transmission in farms with larger herd size [305]. This finding is relevant for control measures such as limited test and removal which could be economically viable in the lower prevalence herds.

Risk factors influence transmission and can be categorised at regional, herd, and animal level [153] and vary across regions for several reasons, such as difference in farm management practices [154]. Analysis of this can be useful to develop a strategy for risk-based surveillance

and control for bTB. The present study has identified several risk factors for bTB. Animals from large herds had 8.3 times the odds of being tuberculin reactor compared to those from small herds. Herd size is the most frequently reported risk factor for bTB in Ethiopia and elsewhere [161, 306]. The risk of infection in a herd increases with herd size and this could be due to overcrowding which increases probability of contact between animals in larger herds implying that transmission may be density dependent [158]. High density creates favorable environment for bTB as aerosol is one main route of transmission. The postmortem data collected by Firdessa and colleagues [10] support this as most animals had TB lesions in lungs and/or lung associated lymph nodes. Also, larger herds often have a larger grazing area, which may expose them to greater environmental risk factors (e.g. wildlife reservoir though not confirmed in Ethiopia) and may also expose them to more neighboring herds [161]. Although the number of large herds in Ethiopia are few (even in the central part of the country) their impact on bTB transmission is likely to be significant as many of them are highly infected and they are primary suppliers of heifers to smallholder farms as well as of milk to consumers and could therefore be most potential sources of infection. If a future bTB control program in Ethiopia would focus on these farms, such intervention could possibly be financially affordable given their small number and turning them into bTB free herds could potentially have a significant impact on the overall bTB prevalence in the Ethiopian dairy sector.

Age was also identified as another risk factor. Animals between 8 – 10 years old had the highest odds of being bTB reactors (OR: 8.9, 95% CI: 5-15.6) compared to the baseline category, which was the youngest age group. A linear increase between bTB infection and age was reviewed by Broughan et al. [158] and observed in slaughterhouse surveillances in cattle in Northern Ireland and Great Britain [162, 300]. The mean age of reactor cattle was 4.4 years (95% CI: 4.29-4.56). Longevity increases probability of exposure and it also increases the chance for development of visible TB lesions and detection in slaughterhouse surveillances. In addition, purchase of older cattle - particularly from high risk areas - could increase the risk of introducing bTB in a herd. Instead, the adoption of risk-based trading has the potential to reduce the risk of bTB spread [307].

We found also that animals from herds with history of bTB had 5.2 times odds of disease detection compared to herds with no history of bTB. In a tuberculin positive herd which did not

remove reactors after skin testing, there could be an increase in infection and hence reactor animals. Even in herds which did cull the reactors, there could be recurrent incidents attributable to persistence of infection in such herds due to failure to detect and remove all infected cattle associated with the performance of the skin test [158].

Exotic and cross bred cattle are known to be more susceptible to bTB [98,158]. Here we found 2.5 times (95% CI: 1.5-5.8) odds of being bTB reactor in these breeds compared to the indigenous zebu breed. The strategy to meet high milk demand is still geared towards improved dairy cattle as a crossbred dairy cow produces on average at least five times more milk than an indigenous zebu cow [308]. With the Ethiopian Government setting a policy to significantly increase the number of crossbred cattle, intensification is likely to increase and thereby the risk of bTB transmission [11, 165]. The final important risk factor we identified is the introduction of cattle to the herd through purchase. We found that cattle purchased from another farm were more often reactors (37.5%) compared to cattle bred at own farm (30%). Although this difference is not statistically significant, it warrants further investigation.

Overall, when comparing our study with previous surveys of dairy cattle in this established dairy belt of Ethiopia, there was no major difference in bTB animal prevalence but our study showed a slight increase in herd prevalence. Firdessa and colleagues [10] recorded in 2009/2010 a 30% (n=2,956) animal and 50% (n=88) herd level bTB prevalence while Tsegaye and colleagues [27] in 2006/2007 recorded 34.1% (n=1,132) animal and 53.6% (n=56) herd bTB prevalence, respectively, which is comparable to our corresponding figures. This consistency over time suggests that bTB has reached an endemic equilibrium in these herds. The burden of bTB in the dairy belt in central Ethiopia (31%) is much greater than for emerging dairies in regional states, estimated to range from 0.3% to 12% animal prevalence [156, 309]. At present Ethiopia has no bTB control program but if implemented should consider the central region of the country as a bTB high risk area and this report opens up for a scientific approach for future risk-based surveillance and disease intervention. A cattle trading from this region pose high risk of introducing bTB infection to new herds and underlines the significance of cattle trade regulation with pre-movement testing. The significantly lower bTB prevalence recorded in many emerging dairies in the regional states (which could be considered as low risk regions) presents an opportunity for intervention *e.g*. by trade restrictions to prevent further disease transmission from

high risk areas like central Ethiopia and introduce testing to support farmers to keep their herds free from bTB. A recent survey by Mekonnen and colleagues [309] recorded an average disease rate of 5.2% (95% CI: 4-6%) in three emerging dairies in regional states, including Hawassa (3%), Gondar (1.4%), and Mekelle (12%). An earlier report in 2014 [134] documented also lower prevalence (below 7%) in eight out of twelve emerging dairies, but ranging from 0.8% to 24% with a few hot spots in Kombolcha (24%) and Mekelle (14%), the latter confirmed by Mekonnen et al. [309]. The lower bTB rates in many of these emerging dairy regions could be due to less cattle movement from high risk regions and less intensification, as they may have emerged more recently. However, if these emerging dairy regions will intensify, and without a strategy for bTB disease control in Ethiopia, it is likely that these regions will be more affected by bTB in the future.

The present study also assessed the genetic diversity of *M. bovis* isolated from slaughtered cattle with tuberculous lesions or from raw milk (tuberculin skin test positive cows) in central Ethiopia and a single isolate from sputum. All spoligotyped 70 isolates from tuberculous lesions and milk and a single human isolate revealed ten spoligotype patterns lacking the spacers that marks *M. bovis* and SB1176 was the most prevalent type (44.3%) and with a wider distribution. In central and other parts of Ethiopia, Biffa and colleagues [142] also recorded SB1176 as a predominant spoligotype (41.2%). This relatively wider distribution of the same strain type could be related to the free movement of cattle in the region. This is not surprising though for a country with no livestock movement control regulation and policy in place for controlling prevalent bTB disease in central Ethiopia. There were also localized strains such as SB0912; the third prevalent spoligotype (14.3%) where all except one was isolated from a single farm. The second prevalent and East African dominant spoligotype (SB0133) which has been isolated from cattle in Uganda, Tanzania and Zambia [179] was also recorded in this study (15.7%). This suggests a wider distribution most possibly due to the movement of livestock and wild animals [179]. Cross border illegal livestock trade is a common practice in east Africa region. With regard to the family of the isolates, almost all (88.6%) of the isolates were of the *M. bovis* African 2 clonal complex (Af2) that also lack spacers 3-7. This complex is known to be restricted to East Africa in comparison to the African 1 clonal complex that seems confined to West Africa [13, 89]. No isolate of Af1 was identified in this study.

All spoligotypes recorded in this study were all previously reported from Ethiopia except SB2521 which was for the first time added to the global database in 2018 from Algeria (https://www.mbovis.org/database.php (accessed: August 23, 2021)). With regard to SB1476 which was isolated from human specimen, it was this study that reported for the first time in Ethiopia but has been reported previously from cattle specimens [183].

When comparing the spoligotypes in the current study with those previously published studies listed in Table 9, it was clear that the most frequent spoligotypes of Af2 and Eu3 identified in Ethiopia (SB1176, SB0133, SB0912, SB1477 and SB0134) were also represented in the present study data set with their prevalence observed at a similar frequency. Spoligotype SB1476 has also been frequently observed in cattle in previous studies [142] but this study provide the first evidence of this spoligotype in humans, possibly as a result of zoonotic transmission.

In herds where we were able to isolate *M. bovis* there was both between and within herd-diversity. Among eight herds, five shared the same spoligotype (SB1176) though located in different locations (not adjacent herds). However, this needs to be supported by epidemiological data to prove herd to herd transmission. Two herds were identified with more than one spoligotype (within herd diversity) and one herd had a unique spoligotype not detected in the other herds. The underlying reasons for this diversity might result from the presence of multiple sources of infection (introduction of the pathogen) particularly for between herd diversity and/or a higher mutation rate (within herd diversity) though *M. bovis* has slow mutation rate. Not always possible to trace the origin of cattle in the herd and these undocumented cattle could also contribute to the diversity.

From the WGS study, a total of 55 isolates (48= cattle tubercculous lesion; 6=raw milk; 1=sputum) from six study sites were successfully sequenced and three distinct clonal complexes (Af2, Eu3 and Unknown8) were observed. From the SNP-phylogenetic tree analysis there were two SNP cluster groups or sublineages which showed clustering belonging to Af2 complex. The name 'Eu3 clonal complex' was first proposed in 2020 after WGS analysis of *M. bovis* strain Mb3601 which was isolated from the lymph node of an infected bovine in Burgundy, France [310]. Eu3 clonal group was also highly prevalent in Italy [310]. Eu3 clonal complex is a newly reported complex in Ethiopia as Af2 was the only complex used to be reported [12]. While greater genetic diversity amongst SNP cluster groups in Af2 complex was observed, a less

diversity and only a single spoligotype SB0134 was seen in the SNP cluster group belonging to Eu3. In addition, there was a large difference observed in genetic diversity between clonal complexes Af2 and Eu3; this is likely due to the long-term endemic nature of Af2 in Ethiopia which has allowed for significant genetic divergence over time with the emergence of clear sub-lineages within this clonal complex observed. Conversely, the comparatively recent introduction of Eu3 into Ethiopia, has not allowed enough time for more genetic diversity to emerge.

The position of Eu3 as the sister group to Af2 also implies a likely East African origin; however, the study of French isolates suggests that the currently circulating Eu3 lineage in Ethiopia may in fact be European in origin. One possible explanation for this is that the ancestor of Eu3 was brought to Europe a few hundred years ago and that the modern Ethiopian and Eritrean genomes are descendants of that population, not of the ancestral population that may or may not still be circulating somewhere in East Africa. What is reasonably clear, given the long branches and subsequent expansion, is that there were two introductions of Eu3 sublineages, consistent with the study that first analyzed the Eritrean WGS data [311], into Ethiopia between 1958 and 1987 (which also included Eritrea during that time) with the median estimate for introduction being in the early 1970s. Given the very similar dates, it is possible that these were part of the same series of cattle imports. In terms of likely origin of these imports, France should be viewed as a proxy for the *M. bovis* diversity seen in mainland Europe [312], so the actual origin may be elsewhere in Europe. There are historical records of the first dairy cattle being imported into Ethiopia around 1950 as part of the United Nations Relief and Rehabilitation Administration (UNRRA) [313] with further subsequent imports from Kenya in 1959 [314]. Several livestock and dairy development projects that took place in the 1950–70s and funded by Sweden and the World Bank may have brought in dairy cattle of exotic breeds from overseas [315]. The other clonal complex found in Ethiopia, Unknown8, represented by a single human genome with the spoligotype SB1476, has thus far only been found in Ethiopia [144]. The position of this lineage in the global tree, and the hypothesized East African origin of *M. bovis* [89], suggests that this clonal complex may be the ancestor of Eu1, the most prevalent and geographically distributed *M. bovis* lineage known to date. However, further work would need to be done to confirm this through the collection of larger numbers of isolates with spoligotype SB1476.

A further in-depth genome analysis showed as expected a considerable genetic diversity between isolates from different animals with some isolates, from different clonal complexes, being as much as 696 SNPs apart. Categorizing the pairwise SNP distances by clonal complex showed that there was considerably more diversity within Af2 compared to Eu3 with the maximum pairwise SNP distance within Af2 being 396 SNPs (median 180 SNPs), compared to Eu3 47 SNPs (median 38 SNPs). The reason could be as explained above the endemicity of Af2 compared to the newly reported Eu3. Less expected was the range of within-host diversity observed. The majority of isolates from the same animal were up to 30 SNPs (median=10 SNPs) apart from each other; given the previously estimated mutation rate of *M. bovis* of 0.15–0.53 SNPs per genome per year [316, 317], it is likely infections are being maintained over a long period of time. Less closely related isolates from the same animal were also found showing that multiple infections by different strains was taking place amongst our samples.

4.2. zTB study

A total of 167 human specimens (sputum=131; FNA=36) were investigated and three *M.bovis* were detected (1.8%); two were from FNA and the remaining single isolate was from sputum. Two *M. bovis* isolates were detected using RT-PCR from culture negative samples. Compared to previous culture based studies in Ethiopia, this study relatively recorded a higher prevalence of *M. bovis*. Firdessa and his colleagues [10] in their large scale study covering larger parts of the country (northwest, south and central Ethiopia) reported only four (0.4%) confirmed *M. bovis* cases from humans. However, this study investigated samples from TB suspected patients visiting hospitals or health centers and did not include risk groups such as dairy farmers. In a pastoral setting-Oromia and Somali regional state Gumi et al. [117] processed 260 sputum and 32 FNA samples and found three confirmed *M. bovis* cases. The prevalence of bTB in pastoral areas such as this was at low level [318] but drinking raw milk is widely practiced which might lead to exposure to zoonotic diseases such as *M. bovis* infection. A lower isolation rate was recorded by Getahun et al. [319] who typed archived 1735 isolates obtained from pulmonary TB cases in 32 health facilities across Ethiopia during November 2011–June 2013 as part of a drug resistance survey. And this RD4 typing identified only 2 (0.13%) of 1,599 of the isolates as *M. bovis*.

However, direct moleclar testing approach showed an increased detection even much higher than the present study. Kidane and colleagues [216] run direct PCR on FNA specimens obtained from 40 patients presenting at a rural health center in South Ethiopia and 35 patients were diagnosed as positive by PCR for tuberculous lymphadenitis. Of these 35 PCR-positive cases, 29 (82.9%) were caused by *M. tuberculosis* and six (17.1%) were caused by *M. bovis,* the highest report in Ethiopia so far as far as *M. bovis* is concerned. Except this direct molecular testing study, all other previous reports in Ethiopia presented here were lower than both the global average (1.4%) that also included developed countries and African average (2.8%) of zTB [18, 274]. At the start of the study our hypothesis was: the low burden of zTB in the previous few reports in Ethiopia might be due to the existence of a parallel similar low burden of bTB in cattle unlike the present study period plus lack of risk based study. This trend was observed in England and Wales in the 1930-1940s during which bTB prevalence was estimated at 30-40% and by then zoonotic transmission suggested to account for around 5-6% of the human TB burden [19, 320].

The finding in this study suggest a low overall rate of zoonotic transmission of *M. bovis*, particularly in central Ethiopia, but needs further large scale study before excluding this as not a significant public health concern. For example, the finding of the present study was closely in agreement with a recently finalized project by AHRI in central Ethiopia where out of 300 specimens processed, there were six (2%) culture and deletion typing confirmed isolates of *M .bovis* (Ayalew et al., unpublished). The exceptionally low prevalence of zTB in Ethiopia even when comapred to Africa (2.8%) [18, 274] in previous reports now seems to do partly with lack of experiences in diagnosis of *M. bovis* as much of the expertise was on *M. tuberculosis*. One other possible theory could be that *M. bovis* infection in humans could turn to latent TB in larger proportion compared to *M. tuberculosis*. The possible reason for the disproportionate prevalence of *M. bovis* between cattle and DFW could be the difference in virulence in cattle and human. Comparison of the virulence/pathogenecity of these two pathogens in cattle and humans can further explain the latency difference in the respective hosts. Cattle are the natural hosts for *M. bovis* though it infects other animals including humans as described in the literature review section. Experimental infection of cattle with *M. tuberculosis* showed very small pathology score (0.5) compared to *M. bovis* (5.5) [271]. In Mexico Torres-Gonzalez et al. [235] examined the prevalence of latent and active tuberculosis among dairy farm workers exposed to cattle infected by *M. bovis* using tuberculin skin test (TST) and interferon-gamma release assay (IGRA). Out of

311 individuals tested, the overall prevalence of latent tuberculosis infection (LTBI) was 76.2% by TST and 58.5% by IGRA. Two subjects were diagnosed with pulmonary tuberculosis, both caused by *M. bovis*. This study has clearly showed the higher proportion was LTBI compared to only two active cases; however, this study failed to include controls (individuals not vaccinated-BCG) to exclude cross-reactivity. In Ethiopia, too it might be interesting to see the extent of LTBI due to *M. bovis* in a well designed study. Overall, the sequenced human *M .bovis* isolate in this study was the spoligotype of SB1476 which was previously reported from cattle in Ethiopia and hence suggestive of possible zoonotic transmission in central Ethiopia.

This study also assessed the knowledge and practices of DFWs related to zoonotic TB. The assessment revealed a higher proportion of DFWs (61%) which were suspected TB patients/suspected to be infected with TB, did not know about cattle TB. This figure when compared to other African countries such as Nigeria was high where in the Nigerian similar study only 40% were unaware of zoonotic TB [321]. In Eastern Ethiopia, Kemal and colleagues [164] recorded a similar finding with the present study where only 33% had the knowledge of bovine tuberculosis and only 23% of the respondents were aware of the zoonotic importance of the disease. With regard to practices of raw milk consumption which is one of the principal modes of transmission of *M. bovis* to humans; was practiced by by more than two thirds of the DFWs with symptoms (68.2%). The practice of raw meat consumption was even much higher compared to raw milk not surprising though as this practice is a common feature even in the capital city Addis Ababa. We were also able to isolate *M. bovis* from raw milk collected from cows positive for bTB in this high bTB burden region (2.2%) signifying its zoonotic potential. Optimization of the LJ medium with 0.5% pyruvate and a less harsh decontamination protocol i.e., 0.75% (w/v) HPC compared to 4% NaOH, is likely to have helped improve the isolation rate of *M. bovis* from milk. Unfortunately, in this study we did not have the opportunity to perform post-mortem on these cows shedding *M. bovis*. This could have enabled us to examine the correlation between *M. bovis* excretion in milk and the detection of tuberculous lesions especially in udder and mammary lymph nodes. Spoligotypes of *M. bovis* isolates from milk in this study (SB1176, SB1521, and SB1878) were also recorded among isolates from tissues and suggest the existence of such correlation.

4.3. RT-PCR study

Microscopy can only detect mycobacterial concentrations above ~10,000 AFB/mL of specimen and hence prone to false negative results [322]. Culture is the most time-and resource consuming test for the detection and identification of mycobacteria in clinical samples. According to Barletta et al. [322] the real time PCR cost ~5US$ per sample and provided same-day results. Not sure if this would be cheap in low income countries like Ethiopia but same-day or within hour's results is a huge benefit of the assay compared with 2–8 weeks for culture.

The real-time based test-Xpert MTB/RIF assay is endorsed by WHO in 2010 initially for diagnostic testing of individuals suspected of multidrug-resistant tuberculosis or HIV-associated tuberculosis [323] and later on in 2014 expanded its recommendation for use in all patients [324]. However, this assay has limitations as seen in the systematic review of 27 unique studies involving nearly 10 000 participants that Xpert MTB/RIF yielded a pooled sensitivity of only 68% in negative smear microscopy patients [324]. Xpert MTB/RIF Ultra (Xpert Ultra) was developed to overcome this limitation and Xpert MTB/RIF Ultra has improved sensitivity compared to that of Xpert [324].

Researches of such kind were lacking here in Ethiopia. Therefore, this study had set out a design that evaluated the performance of TaqMan RT-PCR using different markers (targets) i.e. IS*1081*, RD9 and RD4 in different specimens-sputum, FNA and milk.

This study has showed that the TaqMan assay performed well in pure gDNA, spiked specimens and clinical specimens particularly for sputum specimens. The assay has the potential of being used as a rapid TB diagnostic in place of smear microscopy. The limit of detection (LOD) using gDNA tenfold dilution series of *M. bovis* was upto 10 copies for RD4 target and a single *M. bovis* using IS*1081*. As *M. bovis* genome has 6 copies of IS*1081,* it was reasonable to be more sensitive compared to RD4. This result was similar to the findings of Sevilla et al. [283] study that their assay was able to detect DNA amounts equivalent to 1 to 10 mycobacterial cells per reaction; however, the target gene was the *devR* gene that can detect all the *M. tuberculosis* complex group. Our analytical sensitivity finding was also closely in agreement with the work of Barletta et al. [322] (on *M. tuberculosis*) where they recorded a detection limit of their PCR assay that targeted IS *6110* was 10 fg of DNA/reaction which is equivalent to 1 bacillus. In

spiked specimens too, the assay was able to detect upto the lowest dilution in the experiment i.e. 20 CFU/ml. The study of Sevilla et al. [283] is similar to the present study in that it evaluated the analytical sensitivity of Tetraplex Real-Time PCR assay using not only in serially diluted mycobacterial DNA but also in spiked negative samples with decreasing bacterial concentrations. Based on the literature search carried out, there were no studies of such kind in Ethiopia to compare with.

In addition in this study the assay did not cross react with any of the non-mycobacterial organisms' tested-*Streptococcus pneumoniae*, *Klebsiella pneumoniae, Haemophilus influenzae Staphylococcus aureus*, which are known for causing respiratory infection and also abscess formation. The assay correctly identified *M. bovis* BCG in spiked different specimens and there was no inhibition- by interfering substances that are supposed to be present in the tested specimens as seen from efficiency values (Table 24). However, higher amplification efficiency (147.2%) was recorded for milk sample in spiked specimen indicating possible inhibition.

This finding of no significant change in assay performance by substances in the specimen was in agreement with Scoleri et al. [325] study that assessed the potential for carryover of clinical sample components to influence assay performance in three different approaches. First by addition of DNA extracts from culture- and qPCR-negative bronchoalveolar lavage (BAL) and sputum samples; secondly addition of purified human DNA at a concentration that substantially exceeded the levels in respiratory clinical sample culture- and qPCR-negative bronchoalveolar lavage (BAL) and sputum samples and third, via addition of horse blood prior to DNA extraction. In each case, no significant change in assay performance was observed. However, the performance of the assay in milk needs further investigation as relatively high reaction efficiency was recorded.

With regard to the assay performance in clinical specimens, a total of 440 clinical specimens (131 sputum, 36 FNA and 273 milk) were run parallel by L-J culture and RT-PCR (IS1081, RD9 and RD4 targets). The assay performed well in sputum specimens for all targets with sensitivity ranging from 93.7% - 100% and specificity of 94%-100%. This record was higher when compared with the study of Wang et al. [326] who reported 88% sensitivity and 97% specificity for pulmonary specimens; the difference could be due to difference in target used (IS6110). A sensitivity of 98.3% and 95% specificity which was comparable to the present study was

reported by Demirci et al. [327]. In FNA specimens 100% sensitivity was recorded for all targets but the specifity was with wide ranges from 44%-100%. The reason for this could be the number of culture negative FNA specimens ran were small (n=25) and concluding of low specifity would be difficult in this regard and specificity of the assay in FNA specimens needs to be evaluated in larger negative samples in future. The sensitivity of TaqMan RT-PCR assay in milk in this study was poor. The presence of Taq polymerase inhibitors in milk was attributed to this in previous studies due to the presence of calcium in milk which might compete with the Mg ion in the PCR reaction [328]. According to Wei et al. [329] systematic review and meta-analysis of diagnostic accuracy of RT-PCR, the pooled sensitivity and specificity were 0.96 and 0.92, respectively. In this same review the area under the summary ROC curve (AUC) was 0.9791 which is comparable to the present study (AUC for RD9, IS1081 and RD4, respectively was 95.2%, 98% and 98%). In this review the studies with high-quality assessment and application of respiratory specimen were associated with better accuracy.

4.4. Limitations of the study

In this study, it was not possible to confirm positive reactor animals via postmortem examination for economic reasons. This is a major issue in low and middle-income countries where test and slaughter policy is not feasible. The sample size for the zTB study was small due to resource and time limitation; and a large-scale study is needed to determine the true burden of zTB in bTB endemic central Ethiopia. In our abattoir bTB survey for some of the animals, it was not possible to trace specifically the farm where they came from, rather the region/area they came was used and this was also a challenge in this study. An obvious limitation of the TaqMan RealTime PCR assay was its inability to validate the assay with genomic DNA of enough number of MTBC, NTM and non-mycobacteria due to difficulty in getting these isolates. This would have helped to assess the assay performance for its inclusivity and exclusivity.

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATIONS

The present study recorded high bTB prevalence in the large dairy belt in and around Addis Ababa based on the SICCT test. In addition, this study identified herd size, animal age, cattle breed, and bTB history at farm as important risk factors contributing to the high prevalence of bTB in the central parts of the country and this is important data for designing future control strategies in central Ethiopia.

This study isolated 73 *M. bovis* strains from tissues and milk sampled from dairy cattle from central Ethiopia and we recorded high genetic diversity- ten spoligotypes. The most prevalent spoligotype detected in our sample was SB1176. This study also performed the first WGS-based analysis of *M. bovis* isolates from Ethiopia and identified for the first time Eu3 clonal complex in addition to the previously reported Af2 clonal complex With regard to zTB compared to previous studies in Ethiopia, this study reported high prevalence of human TB due to *M. bovis* infection where out of the 167 specimens processed, three *M. bovis* were detected (1.8%, n=167). And the sequenced human *M .bovis* isolate was spoligotype of SB1476 which was previously reported from cattle in Ethiopia which showed the possibility zoonotic TB transmission. Furthermore, isolation of *M. bovis* from raw milk and knowledge gap and practice of consumption of raw milk among farm workers showed potential for zoonotic TB transmission.

The TaqMan RT-PCR PCR assay was found to be a specific and sensitive method particularily for sputum samples for the rapid detection of mycobacteria directly from clinical specimens. Further research is needed validate this assay by including a good number of MTBC isolates, NTM isolates and non-mycobacterial pathogens and internal control included in large sample size and different specimen types.

Based on the findings of this study the following recommendations are forwarded.

- This study revealed the high and variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia and this provides opportunities for targeted intervention (large farms) which can be economically feasible for low income countries like Ethiopia which cannot afford a wider compensation scheme.

- The detailed WGS based analysis of *M. bovis* in this work if supported with network epidemiological data is important to help better understand bTB transmission in cattle and humans in Ethiopia and can potentially inform national strategies for bTB control in Ethiopia and beyond.
- The isolation of *M. bovis* from raw milk and low level of awareness about zoonotic TB particularly among risk groups i.e. DFWs in this study, clearly showed the need for public health education and other control measures. This study used HPC instead NaOH for decontamination of milk and this method can be used for isolation of *M.bovis* from milk with further validation.

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7. ANNEXES

Annex 1. Questionnaire for collection of epidemiological data of bovine tuberculosis in central Ethiopia,

PART 0: Identification

1. Questionnaire number____ 2. Region_____ 3. District_____ 4. Kebele_____

5. Farm ID (ETHICOBOTS) _____

Date of the interview (DD/MM/YYYY; Ethiopian calendar) ____/____/_____

Interview performed by Enumerator: _____

Supervisor name: _____

Information provided is confidential and will not be disclosed/ transferred to other party without the consent of the respondents. Answering the questions depends only on your good. Please answer the questions as accurate as possible. Thank you for your collaboration in advance.

A. <u>GENERAL INFORMATION</u>

1. Farm location (GPS, coordinates in UTM): way point-----X(E)_____Y(N)_____Alt ____
2. Dairy farm name: _____
3. Name of respondent_____, Position _____ 1) manager, 2) owner, 3) Other
4. Owner's name/manager: _____Phone (fixed) _____ (Mobile) _____ E-mail:_____
5. Respondent's age:_____ Sex: 1) Male 2) Female
6. Educational level of the respondent? _____years of schooling, 0=illiterate, 99=religious
7. Respondent's religion 1) Orthodox 2) Protestant 3) Muslim 4) Other
8. Ownership of the farm; 1) Private 2) Government 3) cooperative 4) share
9. Number of farm workers living on site Male:_____ Female:_____
10. Demographic characteristics of the farm household (of the respondent)

No	Name of household member (start with respondent)	Age	Sex Code A	Education Code B	Participation in dairy farm (1=Yes, 0=no)
1					
2					
3					
4					
5					
6					
Code A	**Code B:** 0. None/Illiterate				

0. Female 1. Male	1. Adult education or 1 year of education * Give other education in years 99. Religious education

11. Total number of farm workers? hired_____; family _____Total_____

12. Demographic characteristics of farm workers (hired and family members) (Note: for institution large farmers such as universities, research, keep lists separately in paper having the following variables)

No	**Name of farm worker**	**Employment status** Code A	**Age**	**Sex** **Code B**	**Education** **Code C**	**Position** **Code D**	**Earning per month in Birr**	**months of service on the farm**
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Code A	**Code B**	**Code C**	**Code D**	
1. Hired 0. Family member	1. Male 0. Female	0. None/Illiterate 1. Adult education or 1 year of education * Give other education in years 99. Religious education	1. Manager 2. Barn worker 2. Store man 3. Guard	4. Herd keeper 5. Milker 6. Secretary 7. Other, specify

13. Herd structure:

Herd composition	**Pure HF**	**HF Z cross**	**Jersey**	**J Z cross**	**Local**	**Total**
Calf (0-1yr)						
Heifers						
Bullock/Steers (1-2 yrs)						
Cows						
Bull/Oxen						
Total						

*HF-Holstein Frisian, Z- Zebus, JZ - Jersey Zebu cross,

14. Species other than cattle in the farm:

Species	Number	Possible contact with the dairy cattle	
		Yes	No
Sheep			
Goats			
Equine*			
Dogs			
Cat			
Swine			
Poultry			
Total			
None			

*Mention the species: horse, mule or donkey in the remark column

B. FARM ANTECEDENTS

15. When did the farm start? (Month, year in E.C) --/----

16. How started this dairy farm its business?

    1) Owner bought existing farm/enterprise; 2) Owner established the farm by her/himself; 3) Owner inherited the farm; 4) other (specify) _____

17. How does the farm get replacement stock (multiple options possible)?

    1) AI, 2) Use of own bull, 3) By purchasing, 4) Use of bull from other farm, 5) Gift, 6) Government heifer/bull programme 7) Other (specify) _____

18. What was the cattle herd size two years ago? _____

19. Have you introduced cattle to your farm in the last two calendar years? A) Yes B) No

    If 'yes', how? A) Purchase B) gift C) calves (new born) D) other

20. a) Complete the following animal details if your answer to question number 19 is choice A or B

Animal source								Purpose (trading, Rearing, etc)	Date animal entered farm (MM/YY)	Contact address
Region	City/ town	sub city	District	Kebele	Farm name	Ethicobots animal ID	ID from the source			

b) Complete the table below if you have sold or given cattle to other farm (s) or someone.

Destination						Animal ID when sold/given	Purpose	Date animal sold/given (MM/YY)	Contact address
Region	City/ town	District	Sub city	Kebele	Farm name				

C. FARM MANAGEMENT /HUSBANDRY

21. Housing condition

1. What type of house is in use (answered by the interviewer)? A) loose house allowing free movement, B) loose house with tie, C) housing with tie in cubicle, C) housing without tie in cubicle, D) housing shared with people, E) other (specify)_____
2. What is the housing situation of calves? A) separate pen, B) share same barn but no close contact (at least 3m apart) with other animals, C) mixed with others
3. Do sick animals have isolation pen? A) Yes, B) No
4. How is the cow layout/arrangement in the house, A) face to face, B) tail to tail, C) one row, D) other (specify)_____
5. Size of the house/barn?
   i. Floor surface area (LxW)? _____ (m^2).
   ii. Indoor height (floor to average point of ceiling)? _____meter.
6. Wall condition? A) Solid, B) solid with few windows, C) half open, D) full open
7. How often do you dispose manure from the barn? A) daily, B) every other day, C) weekly, D) other (specify)_____
8. What method you use for manure disposal? A) Septic tank, B) Accumulated in open pit, C) Accumulated in the dairy compound, D) used as organic fertilizer/fuel (In Amharic: *kubet*)
9. How do you (interviewer) rate the ventilation situation? A) Poor (closed wall with few windows), B) Satisfactory (above half of the two sides of the wall opened/meshed), C) very good (above half of the four sides of the wall opened/meshed, and through the roof)
10. How do you (interviewer) rate the light exposure situation? A) Poor (light enters only through few windows, B) Satisfactory (morning and afternoon light enters through opened opposite side walls, C) very good (morning and evening light enters through any of the four side, through the roof)
11. How do you (interviewer) rate the overall waste drainage system? A) Poor (floor is levelled prohibiting one side flow or waste flows, and not disposed far away), B) Satisfactory (slant floor, waste easily drains, waste disposed far away), C)

Very good (slant floor, waste easily drains, wastes disposed into disposal pit or septic tank, tolerable odour)

12. How do you (interviewer) rate the overall hygiene of the farm? A) Unhygienic (soiled floor with still waste drainage, bad odour and unethical to see) B) Satisfactory (waste drained well, tolerable odour), C) Neat (waste drained well, disposed far away and thus has no bad odour, floor is clean)

22. Feed and Water

a) Do you feed colostrum to neonates? 1) Yes 0) No
If 'yes',

i) What is the time of first feeding after birth? 1) Before 6 hrs, 2) 6-12 hrs, 3) 12-24 hrs, 4) after 24 hrs

ii) What is the duration of feeding? 1) For 24 hrs, 2) for 24 hrs to 4 days, 3) for greater than 4 days

b) How do you feed calf with milk? 1) bucket/bottle feeding from bulk milk, 2) bucket/bottle feeding from dam milk, 3) suckling
c) How is the feeding condition for adult animals? 1) Zero grazing (roughage with supplement only), 2) partial grazing (roughage with limited supplement), 3) Grazing only (no supplement)
d) Feeding schedule for roughage, 1) all time, 2) three times a day 3) two times a day 4) once a day 5) Other (specify) _____
e) What type of feed do you feed to your cattle in addition to the roughage (as a supplement)? A) Brewery by product, 1) Yes 0) No, B) wheat bran, 1) Yes 0) No, C) molasses, 1) Yes 0) No, D) formulated ration from feed plant, 1) Yes 0) No, E) homemade 1) Yes 0) No, F) Others (specify)_____ 1) Yes 0) No, G) Cake 1) Yes 0) No
f) Feeding schedule for supplement, 1) three times a day, 2) two times a day 3) once a day 4) every other day, 5) Other (specify)_____
g) Amount of feed purchased during the last 12 months?

Type of feed	Quantity purchased (last 12 months)	Unit	Unit price
Molasses			
Bran			
Cake			
Hay			
Crop residue			

Brewery by product			
Mineral lick			
Salt			

h) How do you use a feeding trough? 1) separate for each animal, 2) common-one trough for all, 3) no feeding trough at all e.g. the floor is used 4) common-one trough for two or more animals

i) Do you produce improved forage? 1) Yes 0) No

If 'Yes', please fill the following information on forage production on this farm:

Type of feed	Area (sq.m)	Estimate price if it would have been purchased
Alfalfa		
Elephant grass		
Trulecern tree		
Napier grass		
Other, specify		

j) How do you use a watering trough?

1) Separate for each animal, 2) common-one trough for all, 3) others (specify) _____

k) What is your water source (s)? 1) Tap water, 2) well water, 3) Stream, 4) river, E) other (specify)_____

l) How frequently are the animals given access to water? 1) All time, 2) four times a day, 3) three times a day, 4) twice a day, 5) Once a day, 6) as necessary, 7) other specify _____

m) What is your monthly expense for water supplied to the cattle on farm? _____ Birr

n) What is your monthly expense for electricity bill related to dairy business (for office, barn,…etc) _____Birr

o) What is the cost of fuel and oil related to dairy business during the last twelve months? ___ Birr

D. FARM BIO-SECURITY

23. How is the farm enclosure (response by interviewer)? 1) Complete enclosure, 2) partial enclosure, 3) not fenced at all (free movement (in and out) and possibility for cattle to mix with other cattle herds)

24. Which service does the farm mainly use while the cows get heat? 1) AI, 2) own bull, 3) borrowed bull

25. If you borrow bulls, how often do you get the service? 1) Every time any of your cows get heat, 2) only when AI is not possible, C) Rarely

26. Do you know the bovine TB status of the herd you borrow the bull from? 1) Yes 0) No

27. Do you share vets, AI technicians or attendants with neighbouring farms? 1) Yes, 0) No

28. Do wildlife have access to the farm/herd (hyrax, genet, warthog, antelope etc)? 1) Yes 0) No

If yes, mention species of wildlife: _____

29. Do dogs and/or cats enter the farm? 1) Yes 0) No

30. How is the interaction of animals with neighbouring herds? 1) share pasture 2) share water 3) share both pasture and water, 4) possibility of direct contact through boundaries although there is no sharing of water and pasture, 5) indirect contact through sewage, manure, fomites, visitors etc., 6) no possibility of contact at all

31. Does any of the employees on farm have his/her own farm (cattle/poultry)? 1) Yes 0) No 2) I don't know

32. Does the farm have latrines/sanitation facility for the farm workers? 1) Yes 0) No

33. Has any animal in the herd had chronic cough/chronic body wastage during the last six months? 1) Yes 0) No

34. Has this farm been tested for bovine TB during the last three years? 1) Yes, 0) No

35. If 'yes',

a) When was the test done (E.C)? _____
b) Was there any positive animals identified by the test? 1) Yes 0) No
c) If 'yes', what happened afterwards to those bovine TB positive animals? 1) slaughtered, 2) sold, 3) segregated, 4) No action (animal(s) remained in the herd)

36. Do you take any measures to minimize possibility of disease transmission? A) Yes B) NO

If 'yes', which measures? (Multiple options possible)

A) No visitors allowed,

B) Only AI technicians, vets or special guests are allowed

C) Visitor is subjected to use disinfectants at the entry and exit,

D) No access for wildlife, cats and/or dogs to the farm and feed storage,

E) The farm and feed storage has no access for wildlife but for dogs and/or cats,

F) No knowledge on bio-security and thus no measure taken at all,

G) Restrictions on herd grazing/mixing with other herds

H) Practice of burying/ burning fetal membrane and dead bodies

E. IMMUNOSUPPRESSANT FACTORS

a) Do you practice regular de-worming of the herd? A) Yes, B) No
If yes, how frequent? A) Twice a year B) Once a year C) others (Specify) _____

b) How often your animals get health care /service? A) regularly, B) only when ill health situation exist in the herd, others (specify)_____

c) Do you vaccinate your cattle? A) Yes, B) No
If yes, i) How? A) Regularly, B) when there is an outbreak

ii) For which diseases? A) FMD, B) LSD, C) Anthrax, D) Blackleg, E) Pasteurellosis, F) Other (specify)____

d) How often did you experience the incidence of viral disease (FMD, LSD, BVD, etc) in the cattle during the last one calendar year? A) Just once B) Twice C) not at all

e) In your opinion (interviewer), rate the following stressors based on a 5-point scale where l is zero contribution and 5 high contribution (consider points in brackets and circle one number).

i.	Housing (based on light, ventilation, floor surface condition)	1	2	3	4	5
ii.	Stocking density (based on the standard	1	2	3	4	5
iii.	Diseases (previous exposure for viral diseases, other diseases/syndromes)	1	2	3	4	5
iv.	Feeding (based on the available feed such as	1	2	3	4	5
v.	Watering (based on availability)	1	2	3	4	5

Thank you for your time and Cooperation

Annex 2. Standard Operating Procedure (SOP) Developed for Collection of bTB Data in Abattoirs

1. Scope

This standard operating procedure applies to a study while conducting bovine tuberculosis survey at Addis Ababa Abattoir Enterprise and Sululta Cooperative Abattoir.

2. Purpose

This document clearly defines the procedures and protocols to be followed for Ante and Postmortem examination for abattoir survey of bovine tuberculosis.

3. Materials

Knives

Scalpel handle & blades

Aprons & head caps

Boots

Universal sampling bottles

Sterile forceps

Icebox and ice bags

PPE (eye goggles, masks, gloves)

4. Procedure

4.1. Ante-mortem examination (as applied to bTB survey)

The inspector shall perform the under listed activities before slaughter at the lairage.

Each animal to be slaughtered shall bear an identifying mark enabling to trace its origin.

The animals age (estimation), sex, breed, body condition score and origin shall be recorded (see annex I format).

Whether the animals are suffering from a disease which is communicable to man and to animals or whether they show symptoms or are in a general condition such as to indicate that such a disease may occur;

Advanced TB cases is associated with a typical intermittent, moist cough with the head held low and tongue extended, great loss of condition, sluggishness, fast and difficult breathing, anorexia and usually

rough coat. Enlargement of the retropharyngeal lymph nodes produces a typical snoring sound with difficulty in swallowing.

TB mastitis takes the form of induration and enlargement of the udder, especially its upper part, along with similar changes in the supramarnrnary lymph nodes

4.2. Postmortem examination (as applied to bTB survey)

The initial duty where tuberculosis is suspected is to examine those parts or organs in which tuberculosis is most likely to occur, and as the commonest portals of entry are by the respiratory or digestive tract it is essential to examine the head and its lymph nodes, the lungs and their nodes, the mesenteric nodes, and the liver and its portal nodes (see annex II (a) & (b) format). Make at least six incisions (5mm thick) while examining the lymph nodes and 3cm thick slices for lungs.

Following routine inspection where the inspector has reason to suspect that granulomatous lesions similar to those caused by M. *bovis* are detected in one of these primary sites (head, lung and mesenteric lymph nodes), the carcass lymph nodes shall be incised and examined for lesions.

For practicality only accessible carcass lymph nodes such as supramammary (female), prefemoral, superficial inguinal (Male) and prescapular will be considered.

Examine in detail specific organs (affections of specific organs) (see annex II (a) & (b) format).

Pathological scores and Description of lesions (see annex II (a) & (b) format)

Tubercle-definition

Tissue reaction to the invading and multiplying bacilli takes the form of a proliferation of epithelioid and giant cells with necrosis and caseation or calcification and the formation of connective tissue creating a tumour-like granulomatous nodule or *'tubercle'.* Typical tubercles are not always formed in tuberculous infection - a fibrinous or fibrinopurulent exudate may be the only evidence in rapidly fatal situations, e.g. tuberculous meningitis, and in immunocompromised animals.

A typical *tubercle*

It is firm, hard nodule, whitish or yellowish-white in colour and about 1-2 cm in diameter. This can become larger owing to the combination of two or more adjacent tubercles. If body resistance is high, the tubercle becomes reduced in size and encapsulated by fibrous tissue.

Depending on the age of the lesion it can assume different characteristics (see table below).

Tubercle/lesion type	Description	Age/stage of the disease
Mucoid/purulent	Sticky, containing mucous-like matter/ Containing or consisting of pus	
Caseous (cheesy)	These are large, dry, cheesy areas interspersed with small haemorrhages which in lymph nodes often take the form of radiating lines of caseation - the so called *stellate caseation* in which calcification is absent.	Such changes are indicative of complete absence of body resistance.
calcified	A grating feeling is experienced on section when calcification is present (calcium deposits).	

Pathological scoring

Scoring of lung lobes:

0 = no visible lesion

1 = no gross lesion but lesion apparent on slicing of the lobe

2 = <5 gross lesions

3 = >5 gross lesions

4 = gross coalescing lesions

Scoring of Lymph nodes:

0 = no gross lesion

1 = small lesion at one focus (1-2mm, just starting)

2 = small lesions at more than one focus

3 = extensive necrosis

Scale of lesions

Scale	Description
Focal	when one tubercle is observed
Multifocal	when two or more tubercles are observed
Extensive	when multifocal tubercles coalesce

5. Sample collection, Transportation and storage

All TB-like lesions observed (~100g) during post-mortem examination will be collected and sorted by organ per animal in sterile universal tubes (containing sterile PBS buffer) and transported at 4^{o}C (icebox) to the laboratory for further processing. If samples were to be stayed either at the abattoir or laboratory and do not processed immediately for different reasons shall be stored at -20^{o}C. Label each tube with Date, Animal ID, and Organ. This is very IMPORTANT.

6. Safety Instructions

Work using PPE

7. References

Gracey, J.F., Collins, D.S. and Huey, R.J. (1999): Meat Hygiene. Tenth edition. W.B. Saunders Company Ltd. London, UK.

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Part I. Ante-Mortem examination form

Name of the abattoir_____
Region_____Town_____Farm ID (if known)_____Date_____

Abattoir type: Municipal ☐ Export ☐ Backyard ☐

Animal ID (Given by farm)	Animal ID (Given by Abattoir & ETHCOBOTS)	Origin Region /Zone /district/Farm name	Breed	Age (yr)	BCS (1-5)	Sex	Clinical signs observed (if any)

If culled reason for culling_____

Name _____Position_____Title: Dr ☐ AHA ☐ AH technician ☐ Signature _____

Part II. Post-Mortem Examination Form

(a): Inspection of visceral organs

Name of the abattoir_____Region _____Town_____ Date_____

Abattoir type: Municipal ☐ Export ☐ Backyard ☐ Animal ID (Given by abattoir/ Farm) _____Animal ID (ETHICOBOTS)_____ Farm ID _____

* use separate form for each animal

Pathological scores & description of lesions	TUBERCULOSIS LESION STATUS (BASED ON INSPECTION)																
	Lung lobes							Thoracic Membrane	Heart	Spleen	Liver	Small intestine	Large Intestine	Kidneys	Abdominal Membrane	Mammary gland	Whole carcass
	Apical		cardiac		Diaphragmatic		Accessory(R)										
	L	R	L	R	L	R											
Type of lesion (m/p, cas, cal)																	
Scale of lesion (s/ml/ex)																	
Pathological score (0-4) (for lung lobes only)																	

m/p: mucoid or purulent; cas: casaeous; cal: calcified; s: single; ml: multiple; ext-extensive;0=no lesion; 1=no gross lesion but lesion on slicing of lobes; 2=<5 gross lesions; 3=>=5 gross lesions 4=gross coalescing lesions

Name of the person completing the format_____Position_____Title: Dr ☐ AHA ☐ AH technician ☐ Signature _____

Part II. Post-Mortem examination form

(b): Inspection of lymph nodes (please consider this as a continuation of table a)

Inspection of visceral organs

Name of the abattoir_____ Region_____Town_____ Date_____

Abattoir type: Municipal ☐ Export ☐ Backyard ☐ Animal ID (Given by abattoir/Farm)_____Animal ID (Research Project)_____Abattoir/Farm code___

* use separate form for each animal

m/p: mucoid or purulent; cas: casaeous; cal: calcified; s: single; ml: multiple;ext: extensive: 0=no gross lesion; 1=small lesion at one focus; 2=small lesions at more than one focus;

Pathological scores & description of lesions	Parotid		Mandibular		Retropharengial		Prescapular		Tracheal	bronchial			Mediastinal		Portal	Mesenteric	Prefemoral	Supra-mammary /superficial
	left	right	left	right	Lateral	Medial (internal)	left	right		Left	Middle (cranial)	Right	anterior	posterior				
Type of lesion (m/p, cas, cal)																		
Scale of lesion (s/ml/ext)																		
Pathological score (0-3)																		

3=extensive necrosis

Name of the person completing the format _____Position_____Title: Dr ☐ AHA ☐ AH technician ☐Signature _____

Abbreviations

PLN	Parotid lymph node
MnLN	Mandibular lymph node
RLN	Retropharyngeal lymph node
PrLN	Pre-scapular lymph node
ALN	Apical lymph node (Tracheal lymph node)
BLN	Bronchial lymph node
MdLN	Mediastinal lymph node
PoLN	Portal or hepatic lymph node
MsLN	Mesentric lymph node
PfLN	Pre-femoral lymph node
SmLN	Supramammary lymph node
SiLN	Superficial inguinal lymph node
Tm	Thoracic membrane
Am	Abdominal membrane
Mg	Mammary gland
SI	Small intestine
LI	Large intestine
A	Absent (for some lymph nodes-right bronchial)
N	Normal
C	Carcass
Ex	Exotic breed
Cr	Cross breed
Z	Zebu breed

Sample labeling

E.g. lesion found at retropharyngeal lymph node

Date: 24/7/2017

Animal ID: 55(1C0001); 1=Adddis Abeba; C=Carcass

Place: Kara

Breed: Ex

Tissue: RLN

Annex 3. Questionnaire for zTB data collection

Interviewer Name/ID:_____

Date of the interview (DD/MM/YYYY; Ethiopian calendar) ____/____/_____

Part I. Socio-Demographic Characteristics

Name_____Patient Code:_____Phone no._____

Address:
Region_____Zone/subcity_____Woreda/town_____Kebele_____

Farm Name_____Farm Code_____Date_____

S/N	Question	Response (circle the answer)
HAC1	Sex	a. Male b. Female
HAC2	Age	----------Years
HAC3	Educational status	a. Illiterate (Unable to read and write) b. Primary c. Secondary d. College/University
HAC4	Monthly income	a. ≤1000 Eth. birr b. 1001-2000 Eth. birr c. 2001-3000 Eth. birr d. ≥ 3001 Eth. birr
HAC5	Marital status	a. single

		b. married
		c. separated
		d. divorced
		e. widowed

PART-II. Data on Potential Risk factors

S/N	Questions	Response (circle the answer)
HAC6	Consumption of raw milk	1. Yes, if yes how often a. regularly (daily) b. twice per week c. once per week d. only once in life time e. other (mention)_____ 2. No
HAC7	Consumption of Ergo	1. Yes, if yes how often a. twice per week b. once per week c. twice per month d. only once in life time e. other(mention)_____ 2. No
HAC8	Do you think drinking raw milk or ergo can transfer TB from animals to humans?	1. Yes 2. No
HAC9	What is your main source of meat?	1. home slaughter 2.buchery

		3. communal slaughter (Qircha) 4. all
HAC10	Consumption of raw meat/kitfo/Dulet/Kurt	1. Yes, if yes how often a. twice per week b. once per week c. twice per month d. only once in life time e. other(mention)_____ 2. No
HAC11	Do you think drinking raw meat/kitfo/Dulet/Kurt can transfer TB from animals to humans?	1. Yes 2. No
HAC12	Your occupation	a. farm owner/dairy or other animal farmer b. veterinarian c. guard/shepherd d. farm worker (other than milking) e. farm manager f. family member g. milker
HAC13	Do you have close contact with cattle?	1. Yes 2. No
HAC14	Were there coughing cattle in your herd?	1. Yes 2. No
HAC15	Do you know about cattle TB?	1. Yes 2. No

PART-III. Clinical information

S/N	Signs & symptoms	Response (circle the answer)
HAC16	Currently coughing up sputum or phlegm	1. Yes 2. No
HAC17	Currently coughing up blood	1. Yes 2. No
HAC18	Currently have chest pain	1. Yes 2. No
HAC19	Weight loss (in the last month)	1. Yes 2. No
HAC20	Fever	1. Yes 2. No
HAC21	Night sweats	1. Yes 2. No
HAC22	Loss of appetite	1. Yes 2. No
HAC23	Weakness	1. Yes 2. No
HAC24	Swelling around your neck(excluding goiter, trauma etc...)	1. Yes 2. No
HAC25	If yes to question to HAC24, feel pain on your swelling	1. Yes 2. No
	Characteristic of swelling (physical examination)	
HAC26	Duration of neck swelling	a. 0-4 weeks b. >4-12 weeks

		c. >12-52 weeks d. >52 weeks
HAC27	Perceived increase rate	a. slow b. moderate c. fast
HAC28	Pain swelling	a. painless b. painful
HAC29	Location of nodes	a. unilateral b. bilateral
HAC30	Tenderness of node	a. non tender b. tender
HAC31	Number of nodes	a. single b. few(2-4) c. multiple(>=5)
HAC32	Mobility of node	a. non mobile b. mobile
HAC33	Type	a. soft
		b. discrete
		c. matted
		d. hard
		e. firm
		f. drainage sinus

Past Medical History of Tuberculosis

S/N	Signs & symptoms	Response (circle the answer)
HAC34	BCG vaccination	1. Yes 2. No 3. Do not know
HAC35	History of TB	1. Yes 2. No 3. Do not know
HAC36	If yes when?	1. before 6 month
		1. before 1 year
		1. before 2 years
		1. before 5 years
		1. Other (mention)_____
HAC37	Have you been treated with anti-TB drugs ?	1. Yes 2. No
HAC38	If yes to Q HAC36 for how long did you take the treatment ?	_____/months/weeks/
HAC39	If yes for Q HAC36 what was your treatment outcome?	a. cured b. completed c. default d. failure e. relapse f. I do not know

Thank you for your participation!

Annex 4. SOP for culturing of Mycobacteria culture (From tissues)

Tissue homogenization

1. Take samples from the freezer (Field samples) - Frozen samples must be completely defrosted prior to processing: samples should be kept in class 1 BSC and left to defrost over night.

2. Make sure that the samples have proper labels and/or description

3. Divide into two equal portions one for culturing and the other stored at -80°C to allow re-testing if necessary

4. Take approximately 50g of tissue (Biffa et al., 2010). Remove excess fat (Habarugira et al., 2014). Cut the tissue into pieces using a sterile scalpel blade and ground with sterile mortar and pestle by adding 10ml PBS

5. Then transfer the aliquot to 50ml sterile (blue cap) bottle using sterile Pasteur pipette

6. Homogenize further by vortexing.

7. Label one centrifuge tube per sample

8. Process only five samples at a time. To minimize cross contamination, group samples by their geographical origin. Do the homogenization one sample at a time.

9. Decontamination (modified Petroff's Method) and inoculation

10. Transfer 5ml of the homogenate into the labeled centrifuge tube (preferably 50ml centrifuge tube).

11. Add equal volume (5ml) of 4% NaOH and shake using vortex for 15min

12. Fill the 50ml tube to its full mark (add 4x buffered) with phosphate buffer.

13. Centrifuge at 3000 rpm for 15 min

14. Decant the supernatant carefully into a discard can containing 5% phenol or other mycobacterial disinfectant.

15. Add 2ml of buffered peptone to the sediment and mix by vortexing

Inoculation procedures

16. Take two from glycerol and two from pyruvate LJ medium tubes for each sample inoculation, and label both with the farm and animal ID and date of inoculation.

17. Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted.

18. A common fault in inoculation is the use of too small an inoculum. Either loops (wire or disposable) or pipettes can be used for primary cultivation, although plastic Pasteur pipettes are recommended. Each slope should be inoculated with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface.

Incubation and follow up

19. All cultures should be incubated at 37°C until growth is observed or discarded as negative after eight weeks.

20. Inoculated media should preferably be incubated in a slanted position for at least 72 hours with loosened caps to ensure even distribution of inoculum and to allow circulation of CO^2 for the initiation of growth. Caps should be tightened after one week in order to minimize evaporation and drying of media.

Reading of cultures

21. Examine the media within five to seven days to detect rapidly growing mycobacteria which may be mistaken for *M. tuberculosis/M bovis*, and weekly thereafter for up to eight weeks.

22. Typical colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigmented (cream coloured) and slow- growers, ie. only appearing three weeks after inoculation. With doubtful cultures or when less experienced staff read cultures, the acid fastness should be confirmed by Ziehl-Neelsen (ZN) staining. A very small amount of growth is removed from the culture using a loop and gently rubbed into one drop of sterile saline on a slide. At this point the ease with which the organisms emulsify in the liquid should be noted: Tubercle bacilli do not form smooth suspensions, unlike some other mycobacteria. The smear is allowed to dry, fixed by heat and stained by the ZN method.

For preliminary identification of tubercle bacilli the following characteristics apply:

- Tubercle bacilli do not grow in primary culture in less than one week and usually take three to four weeks to give visible growth
- The colonies are buff coloured (never yellow) and rough, having the appearance of bread crumbs or cauliflower
- They do not emulsify in the saline used for making smears but give a granular suspension
- Microscopically they are frequently arranged in serpentine cords of varying length or show district linear clumping. Individual cells are between 3μm and 4μm in length

23. It is useful to label containers with cultures with the dates necessary for examination and to place containers in the incubator in chronological order. Should contaminated cultures be found during the examination, those where the surface has been completely contaminated or where medium has been liquefied or discoloured should be sterilised and discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from the egg (indicated by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded. Cultures with partial contamination should be retained until the eighth week. Late contamination does not exclude the presence of *M. tuberculosis*; it is therefore advisable to prepare a smear from the surface of the medium. Should microscopy indicate the presence of acid-fast bacilli, an attempt could be made to re-decontaminate and re-inoculate the culture.

References

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Annex 5. Milk Culture protocol for *M bovis*

1. Before opening the sampling bottle the outside surface shall be disinfected with 70% alcohol to avoid contamination.

2. Take 30-50ml (*keep half of it for future use*) of milk and centrifuge at 4000 rpm for 15 min. The cell layer obtained will be washed twice with sterile PBS to remove milk fat from the supernatant.

3. The pellets formed after centrifugation will be re-suspended in 10 ml 0.75% (w/v) HPC (Sigma) and incubated for 5 h at room temperature (21°C).

4. Following incubation, the samples will be centrifuged (4000 x g, 15 min) and the pellet suspended in 2 ml PBS.

5. Make AFB stain

6. Take one from glycerol and two from pyruvate (0.5%) LJ medium tubes for each sample inoculation, and label both with the ID and date of inoculation.

7. Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted.

7. Inoculate 500 µl to each tube.

8. All cultures should be incubated at 37°C until growth is observed or discarded as negative after eight weeks.

9. Inoculated media should preferably be incubated in a slanted position for at least 3 days hours to ensure even distribution of inoculum. Thereafter, if incubator space is needed, bottles could be placed upright. Tops should be tightened to minimize evaporation and drying of media

10. All cultures should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are examined weekly

Quality control

Both positive and negative control has to be included in the procedure

References

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Annex 6. Protocol for *M. bovis* isolation from human specimens

The collection of human samples will be following the previous work of Ameni (2006) and Berg *et al*.(5) .

Specimen collection

FNA material will be collected aseptically from enlarged cervical lymph nodes with a 21-gauge needle attached to a 10 ml syringe and will be rinsed into a tube with 1 ml PBS solution and used for culture.

Sputum (one early morning specimen) Collect at least 10ml sputum in 50 mL sterile, plastic, screw-capped centrifuge tubes and split in to two (5ml each) one will be stored for future use and the other used for decontamination. If less than 10 mL, , the volume can be brought up to 10 mL with sterile saline

FNA samples will be stored at 4°C at the field sites and during transportation to the TB laboratory of the Armauer Hansen Research Institute (AHRI) in Addis Ababa.

Samples will be properly identified and labeled and if not processed will be stored at -20^{o}C or -80^{o}C.

Decontamination

Transfer 5ml of the purulent part to another falcon tube and proceed this allocate for the subsequent steps

Transfer 2 to 5ml of FNA/tuberculous lesion homogenate to falcon tube and proceed this allocate for the subsequent steps

Mix 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%)

Add 0.25gm of NALC in to the NAOH-sodium citrate solution and mix it well

Add NaOH-NALC-sodium citrate solution in a volume (5ml) equal to the quantity of specimen. Tighten the cap.

Start timer for 15 min

Vortex the tube at a slow speed for about 20 seconds or until homogeneous, invert several times to cover all surfaces inside the tube

Retrieve safety centrifuge cups from centrifuge and set centrifuge to sample processing speed

Pour PBS into specimen tube up to 50 ml mark

Place centrifuge safety cups in BSC. Remove safety cap and place tubes in cup - balance tubes

Centrifuge tubes at 3000 g, 4ºC for 15 minutes

Remove safety cap inside BSC. Lift up insert and check tubes for leakage

Carefully pour the supernatant into the liquid discard container in ONE smooth motion (no rock back!). Always watch the pellet to ensure it does not dislodge. If pellet starts to move, stop pouring off, place tube in rack and remove supernatant with a transfer pipette

Re-suspend the sediment with pre-measured 2 ml phosphate buffer. Vortex to mix (1-2 seconds)

Make smear by using a disposable loop from the sediment and at the same time inoculate the slants

Ziehl-Neelsen Staining

Place the slides with smear upwards on the staining rack over a sink about finger-width apart

Add carbol-fuchsin staining solutions over the smears

Prepare the torch by dipping its cotton wool end in burning spirit and light it

Heat all slides keeping the torch a little below them until steam arises

Leave the heated stain on the slide a minimum of 5 minute. do not let staining solution dry on the slides

Tilt each slide using forceps to drain off the staining solution

Rinse the slides well with clean water from a beaker.

Pour decolourising solution over the smears covering them completely

Allow to act for 3 minutes

Tilt each slide with forceps to drain off the acid

Gently rinse each slide again with clean water. Do not splash adjacent slides.

If needed, repeat until all macroscopically visible stain has been washed away.

Flood smear with methylene blue solution for 1 minute.

Gently rinse each slide with water. Do not splash adjacent slides

Tilt each slide with forceps to drain off excess water

Clean back of slide with moist paper

Using forceps place it on draining rack and allow to air dry. Always keep smears out of direct sunlight.

Examine slides after it has dried under oil immersion

Culture

Remove condensed moisture at the bottom of culture medium slants before inoculation

Take two slants from each (pyruvate and glycerol) and inoculate with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface.

Incubated at 37°C until growth is observed or discarded as negative after eight weeks.

Inoculated media should preferably be incubated in a slanted position for at least 72 hours and should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants.

cultures examined weekly for eight weeks and if no growth occurs considered as negative

Annex 7. Large sequence polymorphism (Deletion) typing

Colonies were scraped off or specimens were put in 200µL distilled water and heat-killed at 80°C for one hour

DNA extraction following manufacturers instruction

prepare PCR mix(2µlH_2O, 12.5µl master mix, Primers – 2ul from each of 3 primers

Add 5µl of DNA template (sample) to each tube. Do not forget to include a negative control.

Place the tubes in a PCR-machine for amplification, and perform the cycling as indicated in the under table

96 ^{o} C	for	15 min	1 cycle
96 ^{o} C	for	30 min	35 cycle
55 ^{o} C	for	1 min	
72 ^{o} C	for	30 sec	
72 ^{o} C	for	10 min	1 cycle
4 ^{o} C	Hold		

run the gel electrophoresis and read in GelDoc

M. bovis and M. tuberculosis reference strains as positive control and Qaigen H_2O a negative control

If RD4 is absent (deleted): RD4 Ff and RD4 Fr will amplify a product size of 446 bp, thus it is *M. bovis*.

Annex 8. Spoligotyping

Spoligotyping was performed at AHRI following the previous work of Biffa *et al*. (84).

After extraction of DNA amplification of the spacer DNA was carried out in a thermal cycler using 30 cycles of DNA denaturation for 1 min at 96°C; annealing for 1 min at 55°C and elongation for 30 sec at 72°C.

The presence of amplified products was visualized by electrophoresis at 100 V for 30 min in a 2% agarose gel stained with ethidium bromide (0.5 μg/ml).

The amplified spacers was then hybridized with the known oligonucleotide spacers ligated to the membrane. Hybridization was performed by incubating the membrane at 60°C for 1 hr. Hybridized DNA was detected by incubating the membrane in 40 ml of ECL detection liquid for 1 min and visualized by exposure of a light sensitive film for 20 min.

The membrane was analyzed by recording the presence or absence of signals at the sites of the individual probes. *M. tuberculosis* (H37Rv) and *M. bovis* BCG, were included as controls.

Annex 9. Informed Consent Forms

A-Informed Consent Form for Adults

Part I: Information Sheet

Introduction

Hello! Madam/Sir (Local language)

My name is Gizat Almaw and I am a PhD student at Addis Abeba University, College of Health Science, Department of Microbiology, Immunology and Parasitology. I am doing a research on zoonotic tuberculosis in Addis Abeba and surrounding cities. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research. This consent form may contain words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them of me or of another researcher

Purpose of the research

Cattle TB is increasing in Addis Abeba and surrounding cities. We want to know if this increase of TB in cattle is also affecting people in this area. We believe that you can help us by telling us about your occupation, consumption behavior of milk and meat and your contact with cattle. We want to learn about your previous history of TB diagnosis. Understanding of these might help us to learn more about TB transmitted from cattle to humans and will help TB control.

Participant selection

You are being invited to take part in this research and can contribute much to our understanding and knowledge of cattle TB transmission to human. Your are chosen randomly using lottery method.

Voluntary participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. If you choose not to participate nothing will happen to you.

Procedures

This research will involve:

a) your participation to provide interview that will take about half an hour.

b) you will be asked to provide sputum or FNA when required for laboratory investigation.

Duration

a)The interview will be held only once and will take about an half hour.

b) Specimen s(sputum or FNA) will be taken only once and will take an hour.

Risks

We are asking you to share with us some personal information such as monthly income, and you may feel uncomfortable talking about it. You do not have to answer any question if you don't wish to do so, and that is also fine. FNA is safe and requires very little time to perform but may have some pain.

Benefits

There will be no direct benefit to you, but your participation is likely to help us find out more about zoonotic TB and its control strategy in your area and other cities. If your result is positive for TB, you will be referred to the nearest health facility to be treated as per the National treatment guideline.

Reimbursements

You will not be provided any incentive to take part in the research. However, we will reimburse your travel expense which will be conducted when found necessary and will be only once during the study from your location/health center to St Paul or Black Lion hospital.

Confidentiality

We will not be sharing information about you to anyone outside of the research team. The information that we collect from this research project will be kept private. Your name will not appear on this questionnaire and will have a number on it instead of your name. Only the researchers will know what your number is. It will not be shared with or given to anyone.

Sharing the results

This research will be published in scientific journals and thesis and nothing will be attributed to you by name.

Who to Contact

If you wish to ask questions later, you may contact any of the following:

Gizat Almaw (PI), AAU, CHS, DIMP, Tikur Anbessa, e-mail: gizatalm@yahoo.com; Mob. 0960-080833

CHS IRB [Telephone 011 896 1396; email: chs.irb@aau.edu.et; P.o.Box 9086]

Part II: Consent Form

The above has been read to me. I have had the opportunity to ask questions about it and any questions I have been asked have been answered to my satisfaction. I consent voluntarily to be a participant in this study.

Print name of participant_____

Signature of participant _____

Date (day/month/year) _____

If illiterate [1]

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ Thumb print of participant

Signature of witness _____

Date (day/month/year)_____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. your participation to provide in interview that will take about half an hour.

2. you will be asked to provide sputum or FNA for laboratory investigation.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Name of researcher/person taking the consent_____

Signature of researcher /person taking the consent_____

Date (day/month/year) _____

B- Assent for Participants 12 to 17 Years Old

Part I: Information sheet

Introduction

Hello! Boy/Girl (Local language)

My name is Gizat Almaw and I am a PhD student at Addis Abeba University, College of Health Science, Department of Microbiology, Immunology and Parasitology. I am doing a research on zoonotic tuberculosis in Addis Abeba and surrounding cities. I am going to give you information and invite you to be part of a research study. You can choose whether or not you want to participate. We have discussed this research with your parent(s)/guardian and they know that we are also asking you for your agreement. If you are going to participate in the research, your parent(s)/guardian also have to agree. But if you do not wish to take part in the research, you do not have to, even if your parents have agreed. You may discuss anything in this form with your parents or friends or anyone else you feel comfortable talking to. You can decide whether to participate or not after you have talked it over. You do not have to decide immediately. There may be some words you don't understand or things that you want me to explain more about because you are interested or concerned. Please ask me to stop at anytime and I will take time to explain.

Purpose

Cattle TB is increasing in Addis Abeba and surrounding cities. We want to know if this increase of TB in cattle is also affecting people in this area. We believe that you can help us by telling us about your consumption behavior of milk and milk products, and your contact with cattle. We want to learn about your previous history of TB diagnosis. Understanding of these might help us to learn more about TB transmitted from cattle to children and will help TB control.

Choice of participants

You are being invited to take part in this research because children are the ones who usually drink milk and love animals, we feel that your participation can contribute much to our understanding and knowledge of cattle TB transmission to children. Your are chosen randomly using lottery method.

Participation is voluntary

You don't have to be in this research if you don't want to be. Its up to you. If you decide not to be in the research, it is okay and nothing changes. Even if you say "yes" now, you can change your mind later and it is still okay.

Procedures

This research will involve:

a) interview that will take about half an hour.

b) you will be asked to provide sputum or FNA when required for laboratory investigation.

I have checked with the child and they understand the procedures _____(initial))

Risks

FNA procedure is safe and requires very little time to perform but may have some pain which will not have effect on your future health.

Discomforts

There are a few other things that I want you to know.

The procedure during FNA sampling might have minimal trauma, but it will not stay long and you will not miss school.

I have checked with the child and they understand the risks and discomforts ____(initial)

Benefits

Nothing really good might happen to you. This researrch might help us to learrn more about zoonotic TB and its control strategy to prevent children not get affected. If your result is positive for TB, you will be referred to the nearest health facility to be treated as per the National treatment guideline.I have checked with the child and they understand the benefits_____ (initial)

Reimbursements

You will travel when required and will be only once during the study period from your location/health center to St Paul or Black Lion hospital. And the expense will be covered.

Confidentiality

We will not tell other people that you are in this research and we won't share information about you to anyone who does not work in the research study. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is and we will lock that information up with a lock and key.

Sharing the Findings

When we are finished the research, I will sit down with you and your parents and I will tell you about what we learnt.

Right to Refuse or Withdraw

You do not have to be in this research. No one will be disappointed with you if you say no. Its your choice. You can think about it and tell us later if you want. You can say "yes" now and change your mind later and it will still be okay.

Who to Contact

You can ask me questions now or later. You can ask the nurse questions. I have written a number and address where you can reach us or, if you are nearby, you can come and see us. If you want to talk to someone else that you know like your teacher or doctor or auntie, that's okay too.

Gizat Almaw (PI), AAU, CHS, DIMP, Tikur Anbessa, e-mail: gizatalm@yahoo.com; Mob. 0960-080833

CHS IRB [Telephone 011 896 1396; email: chs.irb@aau.edu.et; P.o.Box 9086]

PART II: Assent Form

I understand the research is to know whether cattle TB is also affecting people in Addis Abeba and surrounding cities. I understand that I will provide sputum and FNA when required which will be have some pain.

The information read to me . I have had my questions answered and know that I can ask questions later if I have them.

I agree to take part in the research.

Only if child assents:

Name of child _____

Signature of child: _____

Date (day/month/year):_____

If illiterate:

A literate witness must sign. Participants who are illiterate should include their thumb print as well.

I have witnessed the accurate reading of the assent form to the child, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness (not a parent)_____ AND Thumb print of participant

Signature of witness _____

Date (day/month/year)_____

I have accurately read or witnessed the accurate reading of the assent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given assent freely.

Name of researcher_____

Signature of researcher_____

Date (day/month/year)_____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the child understands that the following will be done:

1. your participation to provide ininterview that will take about half an hour.

2. you will be asked to provide sputum or FNA for laboratory investigation.

I confirm that the child was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this assent form has been provided to the participant.

Name of researcher/person taking the assent_____

Signature of Researcher /person taking the assent _____

Date (day/month/year) _____

Copy provided to the participant _____(initialed by researcher/assistant)

Parent/Guardian has signed an informed consent ___Yes ___No _____(initialed by researcher/assistant)

C- Informed Consent Form For Parents/Guardians

Part I: Information Sheet

Introduction

Hello! Madam/Sir (Local language)

My name is Gizat Almaw and I am a PhD student at Addis Abeba University, College of Health Science, Department of Microbiology, Immunology and Parasitology. I am doing a research on zoonotic tuberculosis in Addiss Abeba and surrounding cities. This study might help to know more about zoonotic tuberculosis and help teenagers become and stay healthier. In our research we will talk to many teenagers, both girls and boys, and ask them a number of questions. Whenever researchers study children, we talk to the parents and ask them for their permission. After you have heard more about the study, and if you agree, then the next thing I will do is ask your daughter/son for their agreement as well. Both of you have to agree independently before I can begin. You do not have to decide today whether or not you agree to have your child participate in this research. Before you decide, you can talk to anyone you feel comfortable with. There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them of me or of another researcher.

Purpose

Cattle TB is increasing in Addis Abeba and surrounding cities. We want to know if this TB in cattle is also affecting people including teenagers in this area. We believe that you can help us by telling us about your consumption behavair of milk and milk products, and your contact with cattle. We want to learn about your previous history of TB diagnosis if any. Understanding of these might help us to learn more about TB transmitted from cattle to teenagers and will help TB control.

Selection of Participants

We want to talk to teenagers about their milk drinking behavior and their relationship with cattle. We would like to ask your daughter/son to participate because she/he is a teenager and lives in this region. Your daughter/son are chosen randomly using lottery method.

Voluntary Participation

You do not have to agree that your daughter/son can talk to us. You can choose to say no and nothing will happen to you. You can ask as many questions as you like and we take the time to answer them. You don't have to decide today. You can think about it and tell me what you decide later.

Procedure

Your daughter/son will participate :

a) interview that will take about half hour.

b) daughter/son will be asked to provide sputum or when required FNA for laboratory investigation.

Duration

We are asking your child to participate in an interview which will take about half hour of her/his time. We can do this outside of school/work hours. Your daughter/son will be asked to provide sputum or FNA for laboratory investigation. This also takes about an hour.

Risks and Discomforts

Your son/daughter will be asked to provide FNA sampling. The procedure during FNA sampling might have minimal trauma, but it will not stay long and you will not miss school.

Benefits

There will be no immediate and direct benefit to your child or to you, but your child's participation in this research might help us to learn more about zoonotic TB and its control strategy to prevent teenage not get affected. If your result is positive for TB, you will be referred to the nearest health facility to be treated as per the National treatment guideline..

Reimbursements

Your daughter/son will not be provided with any payment to take part in the research. However, she/he will be reimbursed for the travel expense which will be only once during the study period from his/her location to St. Paul or Black Lion hospital..

Confidentiality

We will not be sharing information about your son or daughter outside of the research team. The information that we collect from this research project will be kept confidential. Any information about your child will have a number on it instead of his/her name. Only the researchers will know what his/her number is.

Sharing of Research Findings

Nothing that your child will tell us will be shared with anybody outside the research team, and nothing will be attributed to him/her by name. A written report will also be given to the participants which they can share with their families. We will also publish the results in order that other interested people may learn from our research.

Right to refuse or withdraw

You may choose not to have your child participate in this study and your child does not have to take part in this research if she/he does not wish to do so. Your child may stop participating in the discussion/interview at any time that you or she/he wish without either of you losing any of your rights here.

Who to Contact

If you wish to ask questions later, you may contact any of the following:

Gizat Almaw (PI), AAU, CHS, DIMP, Tikur Anbessa, e-mail: gizatalm@yahoo.com; Mob. 0960-080833

CHS IRB [Telephone 011 896 1396; email: chs.irb@aau.edu.et; P.o.Box 9086]

PART II Consent Form

I have been asked to give consent for my daughter/son to participate in this research study which will involve her in interview and sputum and FNA speciemen provision. I information has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily for my child to participate as a participant in this study.

Name of parent or guardian _____

Signature of parent or guardian_____

Date (day/month/year) _____

If illiterate

I have witnessed the accurate reading of the consent form to the parent of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ AND Thumb print of participant

Signature of witness _____

Date (day/month/year)_____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the parent of the potential participant, and to the best of my ability made sure that the person understands that the following will be done:

1. your participation to provide ininterview that will take about half an hour.

2. you will be asked to provide sputum or FNA for laboratory investigation.

I confirm that the parent was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the parent or guardian of the participant ____

Name of Researcher/person taking the consent_____

D- Animal Owner Informed Consent

Part I: Information Sheet

As the owner or duly authorized agent for the owner you have been asked to have your animal participate in a research study. Your informed consent is required prior to this use.

Project Title:	Genetic Diversity and Alternative Diagnostic Method of MycobacteriuM.bovis Isolated from Human and Animal Specimens and Growth Characteristics in Acidic Conditions
PI Name	Gizat Almaw
Faculty/School:	AAU, CHS, DIMP, Tikur Anbessa, e-mail: gizatalm@yahoo.com; Mob. 0960-080833
Contact Details:	AAU, CHS, DIMP, Tikur Anbessa, e-mail: gizatalm@yahoo.com; Mob. 0960-080833 CHS IRB [Telephone 011 896 1396; email: chs.irb@aau.edu.et; P.o.Box 9086]
Location where animal(s) participation/research study occurs	Addis Abebba, Sebeta, Holeta, Sandafa, Sululta, Bishoftu
Aims and benefits of the Research Study:	To know the status of bovine tuberculosis and characterize the causative agent *MycobacteriuM.bovis*
Duration of animal(s) participation	Three dayys
Description of animal(s)procedures to be carried out	Tuberculin skin test: The skin will be measured with digital caliper before PPD injection intradermally and then will be measured after 72 hours to evaluate the difference. If the cow became reactor milk 50ml milk will be collected.
Possible discomfort, risks and complications and steps taken to minimise risks:	There will be swelling at the neck at the injection site which will later disappear and have no further health complication on the animal. There would be minimal stress due to the reaction(test).
Possible benefits to the animal(s):	No direct benefit to the animal but the TB status of the animal will be known for the owner to take measures

Voluntary participation

The participation of your animal is voluntary. If you do not wish to participate you do not have to provide any reason for your decision. Refusal to participate or withdrawal will in no way affect the care to which animal participants are otherwise entitled.

Risks

No known risks due to this test bu unforeseen risks might arise such as abortion during struggling for restraint of nervous cows. Maximum techniques will be in place such as use of bull holder and crash to avoid this risk.

Financial Implications

There will be no cost to you for the participation of your animal in the research study. You will not be charged for any of the procedures performed solely for the study's purposes.

Knowledge Transfer/Publication of Research Findings

The results of the test will be communicated to the owner only during the reading day (3rd day). Our finding will be published in research journals to communicate the scientific community, however, the identity of the farms and the animals will be coded and will not be disclosed. This is to avoid commercial implications.

Confidentiality

Owner and animal confidentiality will be maintained. No identification of individuals will be made when reporting or publishing the data arising from this study.

Part II. Consent Form

I, ...*(please print name)* certify I am the owner (or duly authorised representative of the owner) of the above animal(s) and that the animal(s) are free of any lien or claim by any other person or persons.

I acknowledge that I have read the attached Information sheet for the research project entitled:……..and have had the participation of my animal(s) in the research study fully explained to me by the research investigator:……*(please print name)*

I have had the opportunity to ask questions and discuss any aspects of the participation with the research investigator.

I understand that the participation of my animal(s) is voluntary, and I may withdraw my animal(s) for any reason at any time. My consent is freely given.

I understand that some risk always exists when animal handling and animal procedures are performed. I understand that the research investigator(s) will inform me of any new risks that may be identified or any material changes in the way the study will be conducted.

I understand that all private data pertaining to me and my animal(s) will be treated in strict confidence.

I am aware that I should retain a copy of this Consent Form and attached Information sheet.

Consenting Owner/Authorised Agent

Name:	Proof of ownership shown
Signature:	Date:
Contact Address:	ContactTelephone:

Translation of consent forms in to local languages(Amharic & Affan Oromo, respectively)

ሀ. የ ምር ምር ተሳትፎ ፈቃድ ማጠየ ቂያ ቅጽ (ለ አ ዋ ቂ ወች) (Informed Consent Form for Adults)

ክ ፍ ል 1፡ የ ጥ ና ቱ መ ረ ጃ ገ ጽ (Part I: Information Sheet)

መ ግ ቢ ያ (Introduction)

ሰ ላ ም!

ስ ሜ ግ ዛ ት አ ል ማ ው እ ባ ላ ለ ሁ፡ ፡ በ አ ዲ ስ አ በ ባ ዩ ኒ ቨ ር ሲ ቲ የ ጤ ና ሳ ይ ን ስ ኮ ሌ ጅ በ ማ ይ ክ ሮ ባ ዮ ሎ ጂ ዘ ር ፍ የ 3ኛ ዲ ግ ሪ ተ ማ ሪ ነ ኝ፡ ፡ በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች ከ ከ ብ ቶ ች ወ ደ ሰ ው ተ ላ ላ ፊ በ ሆ ነ የ ቲ ቪ በ ሽ ታ ላ ይ ም ር ም ሬ ን በ ሞ ስ ራ ት ላ ይ እ ገ ና ለ ሁ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ስ ለ ስ ራ የ ዝ ር ዝ ር መ ረ ጃ እ ነ ግ ር ሃ ለ ሁ፡ ፡ ግ ል ጽ ያ ል ነ ሆ ነ ነ ገ ር ካ ለ በ ማ ና ቸ ው ም ጊ ዜ ል ታ ስ ቆ ሜ ኝ ት ች ላ ለ ህ፡ ፡ ጉ ዳ ዩ ግ ል ጽ ከ ሆ ነ ል ህ /ሽ ተ ሳ ታ ፊ እ ን ድ ት ሆ ን እ ጋ ብ ዝ ሃ ለ ሁ፡ ፡ ዛ ሬ መ ወ ሰ ን ላ ይ ኖ ር ብ ህ ይ ች ላ ል፡ ፡ ማ ማ ከ ር የ ም ት ፈ ል ገ ው ን ሰ ው ሁ ሉ ል ታ ማ ክ ር ት ች ላ ለ ህ፡ ፡

የ ጥ ና ቱ አ ላ ማ(purpose)

ይ ህ ጥ ና ት በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች የ ሚ ካ ሄ ድ ጥ ና ት ነ ው ፡ በ ጥ ና ቱ በ ተ ጠ ቀ ሰ ው አ ካ ባ ቢ እ የ ተ ስ ፋ ፋ ያ ለ ው የ ከ ብ ቶ ች ቲ ቪ ወ ደ ሰ ዎ ች ሊ ተ ላ ለ ፍ መ ቻ ሉ ን ና ሊ ተ ላ ለ ፍ የ ሚ ች ል ባ ቸ ው ን መ ን ገ ዶ ች /ዕ ድ ሎ ች ማ የ ት ይ ሆ ና ል፡ ፡ የ አ ን ተ ተ ሳ ት ፎ ይ ህ ን ለ ማ ወ ቅ ይ ረ ዳ ና ል፡ ፡

የ ተ ሳ ታ ፊ ወ ች አ መ ራ ረ ጥ ሁ ኔ ታ(Selection of participants)

፡ በ ዚ ህ ጥ ና ት ተ ሳ ት ፎ እ ን ድ ታ ደ ር ግ ተ ጋ ብ ዘ ሃ ል፡ ፡ ለ መ መ ረ ጥ የ ተ ለ የ መ መ ዘ ኛ የ ለ ም፡ ፡ ተ ሳ ታ ፊ ሊ ሆ ኑ ከ ሚ ች ሉ ት ው ስ ጥ በ ዕ ጣ ነ ው የ ሚ መ ረ ጡ ት፡ ፡

በ ነ ጻ ፈ ቃ ድ ስ ለ መ ሳ ተ ፍ (Voluntary Participation)

ተ ሳ ት ፎ ህ መ ሉ በ መ ሉ በ ነ ጻ ፈ ቀ ድ ላ ይ የ ተ መ ሰ ረ ተ ነ ው ፡ ላ ለ መ ሳ ተ ፍ በ መ ወ ሰ ን ህ የ ሚ መ ጣ ብ ህ ም ን ም ነ ገ ር የ ለ ም፡ ፡

የ ጥ ና ቱ ሂ ደ ቶ ች (procedure)

በ ዚ ህ ጥ ና ት ው ስ ጥ ተ ሳ ታ ፊ ወ ች የ ሚ ሳ ተ ፉ ት፡

1ኛ፡ ቃ ለ መ ጠ ይ ቅ ይ ሰ ጣ ሉ፡ ፡

2ኛ፡ ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና አ ስ ፈ ላ ጊ ሆ ኖ ሲ ገ ኝ ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና መ ና ይ ሰ ጣ ሉ፡

ተ ሳ ታ ፊ ወ ች የ ሚ ያ ደ ር ጉ ት ቆ ይ ታ (ጊ ዜ) (Duration)

ቃ ለ -መ ጠ ይ ቁ ግ ማ ሽ ሰ ዓ ት ያ ህ ል ጊ ዜ ይ ወ ስ ዳ ል፡ ፡ ና መ ኖ በ ግ ም ት አ ን ድ ሰ ዓ ት ሊ ወ ስ ድ ይ ች ላ ል፡ ፡

ስ ጋ ቶ ች (Risks)

ይ ህ ጥ ና ት ከ መ ጠ ይ ቆ ች ው ስ ጥ አ ን ዱ የ ወ ር ገ ቢ ን ማ ወ ቅ ነ ው ፡ ይ ህ የ ግ ል ጉ ዳ ይ ም ኞ ች የ ማ ይ ሰ ጥ ህ

ከሆነ አለመመለስ ትችላለህ፡ ፡ ከአንገት ከሚገኝ ዕጢና ሞኖ ሲወሰድ ሞጠነኛ ህመም ሊኖረውይችላል፡ ፡ ነገር ግን በጤና ላይ የሚያስከትለውችግር የለም፡ ፡

ጥቅሞች (Benefits)

በጥናቱ መሳተፍ የሚያስገኝውቀጥተኛ ጥቅምየለም፡ ፡ ነገር ግን የእርሶ ተሳትፎ ስለበሽታውየተሻለ እንድናውቅና ለቁጥጥር የሚረዳ ሃሳብ እንድናመነ ጭይረዳናል፡ ፡ የላቦራቶሪ ምርመራ ውጤት በሽታው መኖሩን የሚያረጋግጥከሆነ በአቅራቢያ ወደሚገኙውጤና ጣቢያ ሪፈር ተደርገህ በአገር አቀፍ በተዘጋጀውየቲቪ ህክምና መመሪያ መሰረት ህክምናውን የምታገኝ ይሆናል፡ ፡

ወጪመተካትን በተመለከተ (Reimbursements)

በጥናቱ በመሳተፈወ ምንምአይነት ማበረታቻአይከፈለወትም፡ ፡ ነገር ግን ካሉበት ቦታ/ጤና ጣቢያ ሆስፒታል ለመምጣት ትራንስፖርት መጠቀምካለበወት ለዚህ የሚሆን ገንዘብይከፈለወታል፡ ፡ ሆስፒታሎቹ ጥቁር አንበሳ ወይምቅዱስ ጳውሎስ ናቸው፡ ፡ የምትመጣውአንድጊዜ ብቻ ይሆናል፡ ፡

ምስጢር አጠባብቅን በተመለከተ (Confidentiality)

ማንኛውምአይነት መረጃ ከጥናት ቡድኑ አባለት ውጪሌላ ወገን እንዲያውቀውአይሆንም፡ ፡ ስምወትም ቃለ-መጠይቁ ላይ አይጻፍም፡ ፡ በምትኩ ሌላ የምስጢር ቁጥር ይሰጠዋል፡ ፡ ስለሆነ ምየሰጡት መረጃ ምስጢራዊነቱየተጠበቀነው፡ ፡

የ ውጤት አገላለጽ ሁኔታ (Sharing of Research Findings)

ይህ የምርምር ስራ በምርምር መጽሄቶች ላይ የሚታተምይሆናል፡ ፡ ነገር ግን የተሳታፊወችን መረጃ በሚገልጽ መልኩ ሳይሆን መረጃውተተንትኖ የሚታተምይሆናል፡ ፡

መረጃ ቢያስፈልገወ (Who to Contact)

አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ የምርምር ገምጋሚና አጽዳቂ ቦርድ፡ ስ.ቁ .0118961396፤ e-mail:chs.irb@aau.edu.et; P.o.Box: 9086

ግዛት አልማው፣ አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፤ gizatalm@yahoo.com; Mob: 0960-080833

ክፍል 2፡ የፈቃደኝነት ቅጽ (PART II: Certificate of Consent)

ከፍ ብሎ በክፍል 1የተዘረዘረውመረጃ ተነቦልኛል፡ ፡ ያልተረዳኋቸውን ጉዳዮች ጠይቄ በበቂ ሁኔታ ማብራሪያ ተሰጥቶኛል፡ ፡ በዚህ ምርምር ውስጥ በራሴ ነጻ ፈቃድ ለመሳተፍ ፈቅጃለሁ፡ ፡

የተሳታፊ ስም----------

ፊርማ----------

ቀን ----------

ማን በ ብና መጻ ፍ ለ ማይ ችሉ ፤

ፈ ቃ ዳ ች ውን ለ ሰ ጡት ተ ሳ ታ ፊ የ ኮ ን ሰ ን ት ቅ ጹ ሲ ነ በ ብ ላ ች ው ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ተ ሳ ታ ፊ ው ም ያ ል ገ ባ ች ውን ጥ ያ ቄ ሲ ጠ ይ ቁ ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ሰ ለ ሆ ነ ም ተ ሳ ታ ፊ ው ፈ ቃ ዳ ች ውን ያ ለ ም ን ም ተ ጽ ዕ ኖ ሲ ሰ ጡ አ ይ ቻ ለ ሁ ፡ ፡

የ ዕ ማ ኝ ስ ም---------- የ ተ ሳ ታ ፊ ው የ አ ሻ ራ ፊ ር ማ----------

ፊ ር ማ----------

ቀ ን ----------

የ ተ መ ራ ማ ሪ ው አ ስ ተ ያ የ ት

ለ ተ ሳ ታ ፊ ው ከ ፍ ብ ሎ በ ጥ ና ቱ መ ረ ጃ ገ ጽ የ ተ ዘ ረ ዘ ሩ ት ን መ ረ ጃ ወ ች በ ማ ን በ ብ ተ ሳ ታ ፊ ው በ ት ክ ክ ል እ ን ዲ ረ ዱ እ ና እ ን ዲ ገ ነ ዘ ቡ አ ድ ር ጌ አ ለ ሁ ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ያ ሉ ት ን ም በ ት ክ ክ ል እ ን ዲ ገ ነ ዘ ቡ አ ስ ች ያ ለ ሁ ፡ ፡

1. ጥ ና ቱ ቃ ለ መ ጠ ይ ቅ ያ ለ ው መ ሆ ኑ ን

2. ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና ሜ ኖ ይ ሰ ጣ ሉ ፡

ስ ለ ሆ ነ ም ተ ሳ ታ ፊ ው የ ፈ ለ ጉ ት ን ጥ ያ ቄ እ ን ዲ ጠ ይ ቁ ና አ ጥ ጋ ቢ ም ላ ሽ እ ን ደ ተ ሰ ጣ ቸ ው አ ረ ጋ ግ ጣ ለ ሁ ፡ ፡

የ ተ መ ራ ማ ሪ ው ፤ ስ ም----------

ፊ ር ማ----------

ቀ ን ----------

[1] አ ን ድ ቅ ጂ ለ ተ ሳ ታ ፊ ው ተ ሰ ጥ ቷ ል ፡ ፡

<u>ለ.የ ምር ምር ተሳትፎ ፈቃድ ማጠየ ቂያ ቅጽ(ለልጆች)</u> (Informed Assent Form for Children)

ክፍል 1፡ የ ጥና ቱ መረ ጃ ገ ጽ (Part I: Information Sheet)

መግ ቢ ያ (Introduction)

ሰ ላ ም!

ስ ሜ ግ ዛ ት አ ል ማ ው እ ባ ላ ለ ሁ፡ ፡ በ አ ዲ ስ አ በ ባ ዩ ኒ ቨ ር ሲ ቲ የ ጤ ና ሳ ይ ን ስ ኮ ሌ ጅ በ ማ ይ ክ ሮ ባ ዮ ሎ ጂ ዘ ር ፍ የ 3ኛ ዲ ግ ሪ ተ ማ ሪ ነ ኝ ፡ ፡ በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች ከ ከ ብ ቶ ች ወ ደ ሰ ው ተ ላ ላ ፊ በ ሆ ነ የ ቲ ቪ በ ሽ ታ ላ ይ ምር ምሬ ን በ መ ስ ራ ት ላ ይ እ ገ ና ለ ሁ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ስ ለ ስ ራ የ ዝ ር ዝ ር መ ረ ጃ እ ነ ግ ር ሃ ለ ሁ፡ ፡ ግ ል ጽ ያ ል ነ ሆ ነ ነ ገ ር ካ ለ በ ማ ና ቸ ው ም ጊ ዜ ል ታ ስ ቆ መ ኝ ት ች ላ ለ ህ ፡ ፡ ጉ ዳ ዩ ግ ል ጽ ከ ሆ ነ ል ህ ተ ሳ ታ ፊ እ ን ድ ት ሆ ን እ ጋ ብ ዝ ሃ ለ ሁ፡ ፡ ዛ ሬ መ ወ ሰ ን ላ ይ ኖ ር ብ ህ ይ ች ላ ል ፡ ፡ ማ ማ ከ ር የ ም ት ፈ ል ገ ው ን ሰ ው ሁ ሉ ል ታ ማ ክ ር ት ች ላ ለ ህ ፡ ፡

የ ጥ ና ቱ አ ላ ማ (Purpose)

ይ ህ ጥ ና ት በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች የ ሚ ካ ሄ ድ ጥ ና ት ነ ው ፡ ፡ በ ጥ ና ቱ በ ተ ጠ ቀ ሰ ው አ ካ ባ ቢ እ የ ተ ስ ፋ ፋ ያ ለ ው የ ከ ብ ቶ ች ቲ ቪ ወ ደ ሰ ወ ች ሊ ተ ላ ለ ፍ መ ቻ ሉ ን ና ሊ ተ ላ ለ ፍ የ ሚ ች ል ባ ቸ ው ን መ ን ገ ዶ ች/ዕ ድ ሎ ች ማ የ ት ይ ሆ ና ል ፡ ፡ የ አ ን ተ ተ ሳ ት ፎ ይ ህ ን ለ ማ ወ ቅ ይ ረ ዳ ና ል ፡ ፡

የ ተ ሳ ታ ፊ ወ ች አ መ ራ ረ ጥ ሁ ኔ ታ (Choice of participants) አ ን ተ የ ተ መ ረ ጥ ኽ ው ል ጆ ች ወ ተ ት አ ዘ ው ት ረ ው ስ ለ ሚ ጠ ቀ ሙ ና እ ን ስ ሳ ት ን ም ስ ለ ሚ ወ ዱ ለ በ ሽ ታ ው እ ን ደ ማ ኛ ው ም ሊ ታ ለ ጡ ይ ች ላ ሉ ከ ሚ ል እ ም ነ ት ነ ው ፡ ፡ ለ መ መ ረ ጥ የ ተ ለ የ መ መ ዘ ኛ የ ለ ም፡ ፡ ተ ሳ ታ ፊ ሊ ሆ ኑ ከ ሚ ች ሉ ት ው ስ ጥ በ ዕ ጣ ነ ው የ ሚ መ ረ ጡ ት፡ ፡

በ ነ ጻ ፈ ቃ ድ ስ ለ መ ሳ ተ ፍ (Participation is voluntary)

ተ ሳ ት ፎ ህ መ ሉ በ መ ሉ በ ነ ጻ ፈ ቀ ድ ላ ይ የ ተ መ ሰ ረ ተ ነ ው ፡ ፡ ላ ለ መ ሳ ተ ፍ በ መ ወ ሰ ን ህ የ ሚ መ ጣ ብ ህ ም ን ም ነ ገ ር የ ለ ም፡ ፡

የ ጥ ና ቱ ሂ ደ ቶ ች (Procedures)

በ ዚ ህ ጥ ና ት ው ስ ጥ ተ ሳ ታ ፊ ወ ች የ ሚ ሳ ተ ፋ ት፡

1ኛ፡ ቃ ለ መ ጠ ይ ቅ ይ ሰ ጣ ሉ፡ ፡

2ኛ፡ ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና አ ስ ፈ ላ ጊ ሆ ኖ ሲ ገ ኝ ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና ሙ ኖ ይ ሰ ጣ ሉ፡

ል ጁ የ ጥ ና ቱ ሁ ኔ ታ መ ረ ዳ ቱ ን አ ረ ጋ ግ ቻ ለ ሁ፡ ፡ ----------(የ ፊ ር ማ ም ል ክ ት)

ተ ሳ ታ ፊ ወ ች የ ሚ ያ ደ ር ጉ ት ቆ ይ ታ (ጊ ዜ) (Duration)

ቃ ለ -መ ጠ ይ ቁ ግ ማ ሽ ሰ ዓ ት ያ ህ ል ጊ ዜ ይ ወ ስ ዳ ል ፡ ፡ ና ሙ ኖ በ ግ ም ት አ ን ድ ሰ ዓ ት ሊ ወ ስ ድ ይ ች ላ ል ፡ ፡

ስ ጋ ቶ ች ና ም ቾ ት ማ ጣ ት ሁ ኔ ታ ዎ ች (Risks/Discomforts)

ከአንገት ከሚገኝ ዕጢና ሙኖ ሲወሰድ ሙጠነኛ ህመም ሊኖረውይችላል፡ ፡ ነገር ግን በጤና ላይ የሚያስከትለውችግር የለም፡ ፡

ልጁ የጥናቱ ሁኔታ ሙረዳቱን አረጋግቻለሁ፡ ፡ ----------(የፊርማምልክት)

ጥቅሞች (Benefits)

በጥናቱ ሙሳተፍ የሚያስገኝውቀጥተኛ ጥቅምየለም፡ ፡ ነገር ግን የአንተ ተሳትፎ ስለበሽታውየተሻለ እንድናውቅና ለቁጥጥር የሚረዳ ሃሳብእንድናሙነ ጭይረዳናል፡ ፡ የላቦራቶሪ ምርሙራ ውጤት በሽታው ሙኖሩን የሚያረጋግጥከሆነ በአቅራቢያ ወደሚገኝውጤና ጣቢያ ሪፈር ተደርገህ በአገር አቀፍ በተዘጋጀውየቲቪ ህክምና መመሪያ በሚያዘውሙሰረት ህክምናውን የምታገኝ ይሆናል፡ ፡

ወጪሙተካትን በተመለከተ (Reimbursements)

በጥናቱ በሙሳተፍህ ምንምአይነት ማበረታቻአይከፈለወትም፡ ፡ ነገር ግን ካሉበት ቦታ/ጤና ጣቢያ ሆስፒታል ለመምጣት ትራንስፖርት ሙጠቀምካለበወት ለዚህ የሚሆን ገንዘብይከፈለወታል፡ ፡ ሆስፒታሎቹ ጥቁር አንበሳ ወይምቅዱስ ጳውሎስ ናቸው ፡ የምትሙጣውአንድ ጊዜ ብቻ ይሆናል፡ ፡

ምስጢር አጠባብቅን በተመለከተ (Confidentiality)

ማንኛውምአይነት ሙረጃ ከጥናት ቡድኑ አባለት ውጪሌላ ወገን እንዲያውቀውአይሆንም፡ ፡ ስምወትም ቃለ-ሙጠይቁ ላይ አይጻፍም፡ ፡ በምትኩ ሌላ የምስጢር ቁጥር ይሰጠዋል፡ ፡ ስለሆነ ምየሰጡት ሙረጃ ምስጢራዊነቱ የተጠበቀነው ፡

የውጤት አገላለጽ ሁኔታ (Sharing the Findings)

ይህ የምርምር ስራ በምርምር መጽሄቶች ላይ የሚታተምይሆናል፡ ፡ ነገር ግን የተሳታፊወችን ሙረጃ በሚልጽ ሙልኩሳይሆን ሙረጃውተተንትኖ የሚታተምይሆናል፡ ፡

በጥናቱ ያለመሳተፍን ወይምጥናቱን ማቋረጥን በተመለከተ (Right to Refuse or Withdraw)

ሙረጃ ቢያስፈልገው (Who to Contact)

አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ የምርምር ገምጋሚና አጽዳቂ ቦርድ፡ ስ.ቁ.0118961396፤ e-mail:chs.irb@aau.edu.et; P.o.Box: 9086

ግዛት አልማው፣ አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፤ gizatalm@yahoo.com; Mob: 0960-080833

ክፍል 2፡ የፈቃደኝነት ቅጽ (PART 2: Certificate of Assent)

ከፍ ብሎ በክፍል 1የተዘረዘረውሙረጃ ተነቦልኛል፡ ፡ ያልተረዳኋቸውን ጉዳዮች ጠይቄ በበቂ ሁኔታ ማብራሪያ ተሰጥቶኛል፡ ፡ በዚህ ምርምር ውስጥ በራሴ ነጻ ፈቃድ ለመሳተፍ ፈቅጃለሁ፡ ፡

የተሳታፊ ስም----------

ፊርማ----------

ቀን ----------

ማን በ ብና መጻ ፍ ለ ማይ ችሉ ፤

ፈ ቃ ዳ ች ውን ለ ሰ ጡት ተ ሳ ታ ፊ የ ኮ ን ሰ ን ት ቅ ጹ ሲ ነ በ ብ ላ ች ው ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ተ ሳ ታ ፊ ው ም ያ ል ገ ባ ች ውን ጥ ያ ቄ ሲ ጠ ይ ቁ ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ሰ ለ ሆ ነ ም ተ ሳ ታ ፊ ው ፈ ቃ ዳ ች ውን ያ ለ ም ን ም ተ ጽ ዕ ኖ ሲ ሰ ጡ አ ይ ቻ ለ ሁ ፡ ፡

የ ዕ ማ ኝ ስ ም---------- የ ተ ሳ ታ ፊ ው የ አ ሻ ራ ፊ ር ማ----------

ፊ ር ማ----------

ቀ ን ----------

የ ተ መ ራ ማ ሪ ው አ ስ ተ ያ የ ት

ለ ተ ሳ ታ ፊ ው ከ ፍ ብ ሎ በ ጥ ና ቱ መ ረ ጃ ገ ጽ የ ተ ዘ ረ ዘ ሩ ት ን መ ረ ጃ ወ ች በ ማ ን በ ብ ተ ሳ ታ ፊ ው በ ት ክ ክ ል እ ን ዲ ረ ዱ እ ና እ ን ዲ ገ ነ ዘ ቡ አ ድ ር ጌ አ ለ ሁ ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ የ ተ ጠ ቀ ሱ ት ን የ ጥ ና ቱ ክ ፍ ል በ ት ክ ክ ል እ ን ዲ ገ ነ ዘ ቡ አ ስ ች ያ ለ ሁ ፡ ፡

1. ጥ ና ቱ ቃ ለ መ ጠ ይ ቅ ያ ለ ው መ ሆ ኑ ን

2. ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና ሜ ኖ ይ ሰ ጣ ሉ ፡

ስ ለ ሆ ነ ም ተ ሳ ታ ፊ ው የ ፈ ለ ጉ ት ን ጥ ያ ቄ እ ን ዲ ጠ ይ ቁ ና አ ጥ ጋ ቢ ም ላ ሽ እ ን ደ ተ ሰ ጣ ች ው አ ረ ጋ ግ ጣ ለ ሁ ፡ ፡

የ ተ መ ራ ማ ሪ ው ፤ ስ ም----------

ፊ ር ማ----------

ቀ ን ----------

[1] አ ን ድ ቅ ጂ ለ ተ ሳ ታ ፊ ው ተ ሰ ጥ ቷ ል ፡ ፡

ሐ. የ ምር ምር ተሳትፎ ፈቃድ ማጠየ ቂያ ቅጽ (ለ ወላጆች/ለ ሞግዚቶች) (Informed Consent Form forParents/Guardians)

ክፍል 1፡ የ ጥና ቱ መረ ጃ ገ ጽ (Part I: Information sheet)

መግ ቢ ያ

ሰ ላ ም!

ስ ሜ ግዛ ት አ ል ማው እ ባ ላ ለ ሁ፡ ፡ በ አ ዲ ስ አ በ ባ ዩ ኒ ቨ ር ሲ ቲ የ ጤና ሳ ይ ን ስ ኮ ሌ ጅ በ ማ ይ ክ ሮ ባ ዮ ሎ ጂ ዘ ር ፍ የ 3ኛ ዲ ግ ሪ ተ ማ ሪ ነ ኝ፡ ፡ በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች ከ ከ ብ ቶ ች ወ ደ ሰ ው ተ ላ ላ ፊ በ ሆ ነ የ ቲ ቪ በ ሽ ታ ላ ይ ምር ምሬ ን በ መ ስ ራ ት ላ ይ እ ገ ና ለ ሁ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ስ ለ ስ ራ የ ዝ ር ዝ ር መ ረ ጃ እ ነ ግ ር ሃ ለ ሁ፡ ፡ ግ ል ጽ ያ ል ነ ሆ ነ ነ ገ ር ካ ለ በ ማ ና ቸ ው ም ጊ ዜ ል ታ ስ ቆ ሜ ኝ ት ች ላ ለ ህ፡ ፡ ጉ ዳ ዩ ግ ል ጽ ከ ሆ ነ ል ህ ተ ሳ ታ ፊ እ ን ድ ት ሆ ን እ ጋ ብ ዝ ሃ ለ ሁ፡ ፡ ዛ ሬ መ ወ ሰ ን ላ ይ ኖ ር ብ ህ ይ ች ላ ል ፡ ፡ ማ ማ ከ ር የ ም ት ፈ ል ገ ው ን ሰ ው ሁ ሉ ል ታ ማ ክ ር ት ች ላ ለ ህ ፡ ፡

የ ጥ ና ቱ አ ላ ማ

ይ ህ ጥ ና ት በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች የ ሚ ካ ሄ ድ ጥ ና ት ነ ው ፡ በ ጥ ና ቱ በ ተ ጠ ቀ ሰ ው አ ካ ባ ቢ እ የ ተ ስ ፋ ፋ ያ ለ ው የ ከ ብ ቶ ች ቲ ቪ ወ ደ ሰ ዎ ች ሊ ተ ላ ለ ፍ መ ቻ ሉ ን ና በ ተ ለ ይ ም ታ ዳ ጊ ዎ ች ን ማ ጥ ቃ ቱ ን ፤ ሊ ተ ላ ለ ፍ የ ሚ ች ል ባ ቸ ው ን መ ን ገ ዶ ች/ዕ ድ ሎ ች ማ የ ት ይ ሆ ና ል ፡ ፡ የ አ ን ተ ተ ሳ ት ፎ ይ ህ ን ለ ማ ወ ቅ ይ ረ ዳ ና ል ፡ ፡

የ ተ ሳ ታ ፊ ዎ ች አ መ ራ ረ ጥ ሁ ኔ ታ

አ ን ተ የ ተ መ ረ ጥ ኸ ው ታ ዳ ጊ ዎ ች ወ ተ ት አ ዘ ው ትረ ው ስ ለ ሚ ጠ ቀ ሙ ና እ ን ስ ሳ ት ን ም ስ ለ ሚ ወ ዱ ለ በ ሽ ታ ው እ ን ደ ማ ኛ ው ም ሊ ታ ለ ጡ ይ ች ላ ሉ ከ ሚ ል እ ም ነ ት ነ ው ፡ ለ መ መ ረ ጥ የ ተ ለ የ መ መ ዘ ኛ የ ለ ም ፡ ፡ ተ ሳ ታ ፊ ሊ ሆ ኑ ከ ሚ ች ሉ ት ው ስ ጥ በ ዕ ጣ ነ ው የ ሚ መ ረ ጡ ት ፡ ፡

በ ነ ጻ ፈ ቃ ድ ስ ለ መ ሳ ተ ፍ

የ ል ጅ ህ ተ ሳ ት ፎ ሙ ሉ በ ሙ ሉ በ ነ ጻ ፈ ቀ ድ ላ ይ የ ተ መ ሰ ረ ተ ነ ው ፡ ላ ለ መ ሳ ተ ፍ በ መ ወ ሰ ን ህ የ ሚ መ ጣ ብ ህ ም ን ም ነ ገ ር የ ለ ም ፡ ፡ የ ፈ ለ ግ ኸ ው ን ሁ ሉ ማ ብ ራ ሪ ያ መ ጠ ይ ቅ ት ች ላ ለ ህ ፡ ፡ አ ን ተ ብ ቻ ሳ ት ሆ ን ል ጅ ህ ም መ ስ ማ ማ ት አ ለ ባ ት ፡ ፡

የ ጥ ና ቱ ሂ ደ ቶ ች

በ ዚ ህ ጥ ና ት ው ስ ጥ ተ ሳ ታ ፊ ዎ ች የ ሚ ሰ ተ ፋ ታ ዳ ጊ ዎ ች ፡

1ኛ፡ ቃ ለ መ ጠ ይ ቅ ይ ሰ ጣ ሉ ፡ ፡

2ኛ፡ ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና አ ስ ፈ ላ ጊ ሆ ኖ ሲ ገ ኝ ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና ሙ ና ይ ሰ ጣ ሉ ፡

ተ ሳ ታ ፊ ዎ ች የ ሚ ያ ደ ር ጉ ት ቆ ይ ታ (ጊ ዜ)

ቃ ለ - መ ጠ ይ ቁ ግ ማ ሽ ሰ ዓ ት ያ ህ ል ጊ ዜ ይ ወ ስ ዳ ል ፡ ፡ ና ሙ ና በ ግ ም ት አ ን ድ ሰ ዓ ት ሊ ወ ስ ድ ይ ች ላ ል ፡ ፡

ስ ጋ ቶ ች ና ም ቾ ት ማ ጣ ት ሁ ኔ ታ ዎ ች

ልጅህ ከአንገት ከሚገኝ ዕጢና ሙኖ ሲወሰድ መጠነኛ ህመምሊኖረውይችላል፡፡ ነገር ግን በጤና ላይ የሚያስከትለውችግር የለም፡ ፡

ጥቅሞች

በጥናቱ መሳተፍ የሚያስገኝውቀጥተኛ ጥቅምየለም፡ ፡ ነገር ግን የልጅህ ተሳትፎ ስለበሽታውየተሻለ እንድናውቅና ለቁጥጥር የሚረዳሃሳብእንድናመነጨይረዳናል፡፡ የላቦራቶሪ ምርመራ ውጤት በሽታው መኖሩን የሚያረጋግጥከሆነ በአቅራቢያ ወደሚገኙጤና ጣቢያ ሪፈር ተደርገህ በአገር አቀፍ በተዘጋጀውየቲቪ ህክምና መመሪያ በሚያዘውመሰረት ህክምናውን የምታገኝ ይሆናል፡ ፡

ወጪሞተካትን በተመለከተ

ልጅህ በጥናቱ በመሳተፉ ምንምአይነት ማበረታቻአይከፈለወትም፡ ፡ ነገር ግን ካሉበት ቦታ/ጤና ጣቢያ ሆስፒታል ለመምጣት ትራንስፖርት መጠቀምካለበወት ለዚህ የሚሆን ገንዘብይከፈላል፡ ፡ ሆስፒታሎቹ ጥቁር አንበሳ ወይምቅዱስ ጳውሎስ ናቸው፡ ፡ የምትመጣውአንድጊዜ ብቻ ይሆናል፡ ፡

ምስጢር አጠባብቅን በተመለከተ

ማንኛውምአይነት መረጃ ከጥናት ቡድኑ አባለት ውጪሌላ ወገን እንዲያውቀውአይሆንም፡ ፡ የልጅህ ሰምምቃለ-መጠይቁ ላይ አይጻፍም፡ ፡ በምትኩ ሌላ የምስጢር ቁጥር ይሰጠዋል፡ ፡ ስለሆነ ምየሰጡት መረጃ ምስጢራዊነቱ የተጠበቀነው፡ ፡

የውጤት አገላለጽ ሁኔታ

ይህ የምርምር ስራ በምርምር መጽሄቶች ላይ የሚታተምይሆናል፡ ፡ ነገር ግን የተሳታፊወችን መረጃ በሚልጽ መልኩሳይሆን መረጃውተተንትኖ የሚታተምይሆናል፡ ፡

በጥናቱ ያለመሳተፍን ወይምጥናቱን ማቋረጥን በተመለከተ

ልጅወት በጥናቱ ያለመሳተፍ ወይምበማናቸውምጊዜ ማቋረጥ ሙብቷነው፡ ፡

መረጃ ቢያስፈልገወ

አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ የምርምር ገምጋሚና አጽዳቂ ቦርድ፡ ስ.ቁ.0118961396፤ e-mail:chs.irb@aau.edu.et; P.o.Box: 9086

ግዛት አልማው፣ አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፤ gizatalm@yahoo.com; Mob: 0960-080833

ክፍል 2፡ የፈቃደኝነት ቅጽ

ከፍ ብሎበክፍል 1የተዘረዘረውመረጃ ተነቦልኛል፡ ፡ ያልተረዳኋቸውን ጉዳዮች ጠይቄ በበቂ ሁኔታ ማብራሪያ ተሰጥቶኛል፡ ፡ ልጄበዚህ ምርምር ውስጥ በራሴ ነጻ ፈቃድ ለመሳተፍ ፈቅጃለሁ፡ ፡

የወላጅ/የሞግዚት ስም----------

ፊርማ----------

ቀን ----------

ማን በ ብና መጻ ፍ ለ ማይ ችሉ ፤

ፈ ቃ ዳ ች ውን ለ ሰ ጡት ተ ሳ ታ ፊ የ ኮ ን ሰ ን ት ቅ ጹ ሲ ነ በ ብ ላ ች ው ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ተ ሳ ታ ፊ ው ም ያ ል ገ ባ ች ውን ጥ ያ ቄ ሲ ጠ ይ ቁ ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ሰ ለ ሆ ነ ም ተ ሳ ታ ፊ ው ፈ ቃ ዳ ች ውን ያ ለ ም ን ም ተ ጽ ዕ ኖ ሲ ሰ ጡ አ ይ ቻ ለ ሁ ፡ ፡

የ ዕ ማ ኝ ስ ም---------- የ ተ ሳ ታ ፊ ው የ አ ሻ ራ ፊ ር ማ----------

ፊ ር ማ----------

ቀ ን ----------

የ ተ መ ራ ማ ሪ ው አ ስ ተ ያ የ ት

ለ ተ ሳ ታ ፊ ው ከ ፍ ብ ሎ በ ጥ ና ቱ መ ረ ጃ ገ ጽ የ ተ ዘ ረ ዘ ሩ ት ን መ ረ ጃ ወ ች በ ማ ን በ ብ ተ ሳ ታ ፊ ው በ ት ክ ክ ል እ ን ዲ ረ ዱ እ ና እ ን ዲ ገ ነ ዘ ቡ አ ድ ር ጌ አ ለ ሁ ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ የ ተ ጠ ቀ ሱ ት ን የ ጥ ና ቱ ክ ፍ ል በ ት ክ ክ ል እ ን ዲ ገ ነ ዘ ቡ አ ስ ች ያ ለ ሁ ፡ ፡

1. ጥ ና ቱ ቃ ለ መ ጠ ይ ቅ ያ ለ ው መ ሆ ኑ ን

2. ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - አ ክ ታ ና ከ አ ን ገ ት ላ ይ ከ ሚ ገ ኝ እ ጢ የ ሚ ወ ሰ ድ ና ሜ ኖ ይ ሰ ጣ ሉ ፡

ስ ለ ሆ ነ ም ተ ሳ ታ ፊ ው የ ፈ ለ ጉ ት ን ጥ ያ ቄ እ ን ዲ ጠ ይ ቁ ና አ ጥ ጋ ቢ ም ላ ሽ እ ን ደ ተ ሰ ጣ ቸ ው አ ረ ጋ ግ ጣ ለ ሁ ፡ ፡

የ ተ መ ራ ማ ሪ ው ፤ ስ ም----------

ፊ ር ማ----------

ቀ ን ----------

[1] አ ን ድ ቅ ጂ ለ ተ ሳ ታ ፊ ው ተ ሰ ጥ ቷ ል ፡ ፡

<u>የ ምር ምር ተሳትፎ ፈቃድ ማጠየ ቂያ ቅጽ (ለ እ ን ስ ሳ ት ባ ለ ቤ ቶ ች)</u>

ክ ፍ ል 1፡ የ ጥ ና ቱ መ ረ ጃ ገ ጽ

መ ግ ቢ ያ

ሰ ላ ም!

ስ ሜ ግ ዛ ት አ ል ማ ው እ ባ ላ ለ ሁ፡ ፡ በ አ ዲ ስ አ በ ባ ዩ ኒ ቨ ር ሲ ቲ የ ጤ ና ሳ ይ ን ስ ኮ ሌ ጅ በ ማ ይ ክ ሮ ባ ዮ ሎ ጂ ዘ ር ፍ የ 3ኛ ዲ ግ ሪ ተ ማ ሪ ነ ኝ፡ ፡ በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች የ ከ ብ ቶ ች የ ቲ ቪ በ ሽ ታ ያ ለ በ ት ን ሁ ኔ ታ ና አ ጋ ላ ጭ ሁ ኔ ታ ዎ ች ላ ይ ም ር ም ሬ ን በ መ ስ ራ ት ላ ይ እ ገ ኛ ለ ሁ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ስ ለ ስ ራ የ ዝ ር ዝ ር መ ረ ጃ እ ነ ግ ር ሃ ለ ሁ፡ ፡ ግ ል ጽ ያ ል ነ ሆ ነ ነ ገ ር ካ ለ በ ማ ና ቸ ው ም ጊ ዜ ል ታ ስ ቆ መ ኝ ት ች ላ ለ ህ፡ ፡ ጉ ዳ ዩ ግ ል ጽ ከ ሆ ነ ል ህ /ሽ ተ ሳ ታ ፊ እ ን ድ ት ሆ ን እ ጋ ብ ዝ ሃ ለ ሁ፡ ፡ ዛ ሬ መ ወ ሰ ን ላ ይ ኖ ር ብ ህ ይ ች ላ ል፡ ፡ ማ ማ ከ ር የ ም ት ፈ ል ገ ው ን ሰ ው ሁ ሉ ል ታ ማ ክ ር ት ች ላ ለ ህ፡ ፡

የ ጥ ና ቱ አ ላ ማ

ይ ህ ጥ ና ት በ አ ዲ ስ አ በ ባ ና ዙ ሪ ያ ከ ተ ሞ ች የ ሚ ካ ሄ ድ ጥ ና ት ነ ው ፡ በ ጥ ና ቱ በ ተ ጠ ቀ ሰ ው አ ካ ባ ቢ እ የ ተ ስ ፋ ፋ ያ ለ ው የ ከ ብ ቶ ች ቲ ቪ አ ሁ ን ያ ለ በ ት ን ደ ረ ጃ ለ ማ ወ ቅ ይ ረ ዳ ል፡ ፡ የ አ ን ተ እ ን ስ ሳ ቶ ች /የ እ ር ባ ታ ጣ ቢ ያ በ ዚ ህ ጥ ና ት ው ስ ጥ መ ካ ተ ት /መ ሳ ተ ፍ ይ ህ ን ለ ማ ወ ቅ ይ ረ ዳ ና ል፡ ፡

የ አ መ ራ ረ ጥ ሁ ኔ ታ

የ አ ን ተ እ ን ስ ሳ ቶ ች /የ እ ር ባ ታ ጣ ቢ ያ የ ተ መ ረ ጥ ኸ ው አ ን ድ ሃ ላ ፊ ነ ት እ ን ደ ሚ ሰ ማ ው ዜ ጋ ል ት ሳ ት ፍ ት ች ላ ለ ህ በ ሚ ል ዕ ም ነ ት ነ ው ፡ ለ መ መ ረ ጥ የ ተ ለ የ መ መ ዘ ኛ የ ለ ም፡ ፡ ተ ሳ ታ ፊ ሊ ሆ ኑ ከ ሚ ች ሉ ት ው ስ ጥ በ ዕ ጣ ነ ው የ ሚ መ ረ ጡ ት፡ ፡

በ ነ ጻ ፈ ቃ ድ ስ ለ መ ሳ ተ ፍ

የ እ ን ስ ሳ ቶ ች ህ /የ እ ር ባ ታ ጣ ቢ ያ ህ ተ ሳ ት ፎ ሙ ሉ በ ሙ ሉ በ ነ ጻ ፈ ቀ ድ ላ ይ የ ተ መ ሰ ረ ተ ነ ው ፡ ላ ለ መ ሳ ተ ፍ በ መ ወ ሰ ን ህ የ ሚ መ ጣ ብ ህ ም ን ም ነ ገ ር የ ለ ም፡ ፡

የ ጥ ና ቱ ሂ ደ ቶ ች

በ ዚ ህ ጥ ና ት ው ስ ጥ የ ሚ ሳ ተ ፉ እ ን ስ ሳ ቶ ች /እ ር ባ ታ ጣ ቢ ያ ዎ ች፡

1ኛ፡ የ እ ን ስ ሳ ት ቲ ቪ ም ር መ ራ ይ ደ ረ ግ ላ ቸ ዋ ል፡ ፡ ይ ህ ም የ ሚ ሰ ራ ው አ ን ገ ት ቆ ዳ ላ ይ የ ቲ ቪ መ ድ ሃ ኒ ት መ መ ር መ ሪ ያ ቅ መ ም በ መ ው ጋ ት ና የ ቆ ዳ ው ን ዕ ብ ጠ ት ከ ሶ ስ ት ቀ ና ት በ ኋ ላ በ መ ለ ካ ት የ ሚ ሰ ራ ነ ው ፡

2ኛ፡ የ አ ን ገ ት ላ ይ የ ቆ ዳ ም ር መ ራ ው ጤ ት ቲ ቪ እ ን ዳ ለ ባ ት የ ሚ ያ ሳ ይ ከ ሆ ነ ለ ላ ቦ ራ ቶ ሪ ም ር መ ራ - ወ ተ ት (50ሲ ሲ) ይ ወ ሰ ዳ ል፡ ፡

የ ሚ ያ ስ ፈ ል ግ የ ቆ ይ ታ (ጊ ዜ)

ጥ ና ቱ አ ን ድ ጊ ዜ ብ ቻ የ ሚ ሰ ራ ሲ ሆ ን ሶ ስ ት ቀ ን ይ ወ ስ ዳ ል፡ ፡

ስጋቶች

የመመርመሪያ መድሃኒቱየታወቀየሚያስከትለውችግር የለም፡ ፡ ሆኖምበዚህ ስራወቅት እንስሳቶችን መያዝ ስለሚጠይቅ አልፎ አልፎ አንድ አንድ ልዩ ባህሪ ያላቸውእንስሳት ሊደናገጡና ራሳቸውምላይ ሆነ ሰራተኞች ላይ አደጋ ሊያደርሱይችላሉ፡ ፡ እንዲህ አይነት እንስሳት ጠባያቸውአስቀድሞየሚጣወቅ ከሆነ እንዳይካተቱ ይደረጋል፡ ፡ ከዚህ ባለፈምአስፈላጊ መሳሪወችን የምንጠቀምይሆናል፡ ፡

ጥቅሞች

በጥናቱመሳተፍየሚያስገኝውቀጥተኛ ጥቅምየለም፡ ፡ ነገር ግን የእርሶ ተሳትፎ ስለበሽታውየተሻለ እንድናውቅና ለቁጥጥር የሚረዳሃሳብእንድናመነ ጭይረዳናል፡ ፡ የላቦራቶሪ ምርመራ ውጤት በሽታው መኖሩን የሚያረጋግጥከሆነ ቀጥሎበእንስሳቱ ላይ ስለሚወስዱት እርምጃና ተያያዥየማግለያ አካሄዶችን በተመለከተ ምክረሃሳብበነጻ የሚያገኙይሆናል፡ ፡

ገንዘብነክ ጉዳዮችን በተመለከተ

በጥናቱበመሳተፈወየሚያስከትልበወት ምንመምወጪአይኖርም፡ ፡ ለላቦራቶሪምሆነ ማንኛውም ከምርምሩ ጋር የተያያዘ ነገር የሚከፍሉት ነገር የለም፡ ፡

ምስጢር አጠባብቅን በተመለከተ

ማንኛውምአይነት መረጃ ከጥናት ቡድኑ አባለት ውጪሌላ ወገን እንዲያውቀውአይሆንም፡ ፡ የእርባታው ስምምቃለ-መጠይቁ ላይ አይጻፍም፡ ፡ በምትኩ ሌላ የምስጢር ቁጥር ይሰጠዋል፡ ፡ ስለሆነ ምየሰጡት መረጃ ምስጢራዊነቱየተጠበቀነው፡ ፡ ስለሆነ ምቢዝነስወላይ ከጥናቱጋር በተያያዘ የሚደርስ ችግር የለም፡ ፡

የውጤት አገላለጽ ሁኔታ

ይህ የምርምር ስራ በምርምር መጽሄቶች ላይ የሚታተምይሆናል፡ ፡ ነገር ግን የተሳታፊወችን መረጃ በሚገልጽ መልኩ ሳይሆን መረጃውተተንትኖ የሚታተምይሆናል፡ ፡

መረጃ ቢያስፈልገወ

አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ የምርምር ገምጋሚና አጽዳቂ ቦርድ፡ ስ.ቁ.0118961396፤ e-mail:chs.irb@aau.edu.et; P.o.Box: 9086

ግዛት አልማው፣ አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፤ gizatalm@yahoo.com; Mob: 0960-080833

ክፍል 2፡ የፈቃደኝነት የምስክር ወረቀት

ከፍ ብሎበክፍል 1የተዘረዘረውመረጃ ተነቦልኛል፡ ፡ ያልተረዳኋቸውን ጉዳዮች ጠይቄበበቂ ሁኔታ ማብራሪያ ተሰጥቶኛል፡ ፡ በዚህ ምርምር ውስጥበራሴነጻ ፈቃድ እንስሳቶቼ/የእርባታጣቢያ ፈቅጃለሁ፡ ፡

የእርባታውባለቤት/ተወካይ ስም----------

ፊርማ----------

ቀን ----------

ማን በ ብና መጻ ፍ ለ ማይች ሉ ፤

ፈ ቃ ዳ ች ውን ለ ሰ ጡት ተ ሳ ታ ፊ የ ኮ ን ሰ ን ት ቅ ጹ ሲ ነ በ ብ ላ ች ው ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ተ ሳ ታ ፊ ው ም ያ ል ገ ባ ች ውን ጥ ያ ቄ ሲ ጠ ይ ቁ ተ መ ል ክ ቻ ለ ሁ ፡ ፡ ሰ ለ ሆ ነ ም ተ ሳ ታ ፊ ው ፈ ቃ ዳ ች ውን ያ ለ ም ን ም ተ ጽ ዕ ኖ ሲ ሰ ጡ አ ይ ቻ ለ ሁ ፡ ፡

የ ዕ ማ ኝ ስ ም---------- የ እ ር ባ ታ ው ባ ለ ቤ ት /ተ ወ ካ ይ ፊ ር ማ----------

ፊ ር ማ----------

ቀ ን ----------

የ ተ መ ራ ማ ሪ ው አ ስ ተ ያ የ ት

ለ ተ ሳ ታ ፊ ው ከ ፍ ብ ሎ በ ጥ ና ቱ መ ረ ጃ ገ ጽ የ ተ ዘ ረ ዘ ሩ ት ን መ ረ ጃ ው ች በ ማ ን በ ብ ተ ሳ ታ ፊ ው በ ት ክ ክ ል እ ን ዲ ረ ዱ እ ና እ ን ዲ ገ ነ ዘ ቡ አ ድ ር ጌ አ ለ ሁ ፡ ፡ ከ ዚ ህ ቀ ጥ ሎ ያ ሉ ት ን ም በ ት ክ ክ ል እ ን ዲ ገ ነ ዘ ቡ አ ስ ች ያ ለ ሁ ፡ ፡

1ኛ ፡ እ ን ስ ሳ ቱ የ ቲ ቪ ምር መራ ይ ደ ረ ግ ላ ች ዋ ል ፡ ፡ ይ ህ ም የ ሚ ሰ ራ ው አ ን ገ ት ቆ ዳ ላ ይ የ ቲ ቪ መ ድ ሃ ኒ ት መ መ ር መ ሪ ያ ቅ መ ም በ መ ው ጋ ት ና የ ቆ ዳ ው ን ዕ ብ ጠ ት ከ ሶ ስ ት ቀ ና ት በ ኋ ላ በ መ ለ ካ ት የ ሚ ሰ ራ ነ ው ፡ ፡

2ኛ ፡ የ አ ን ገ ት ላ ይ የ ቆ ዳ ምር መራ ው ጤት ቲ ቪ እ ን ዳ ለ ባ ት የ ሚ ያ ሳ ይ ከ ሆ ነ ለ ላ በ ራ ቶ ሪ ምር መራ - ወ ተ ት (50ሲ ሲ) ይ ወ ሰ ዳ ል ፡ ፡

ስ ለ ሆ ነ ም ተ ሳ ታ ፊ ው የ ፈ ለ ጉ ት ን ጥ ያ ቄ እ ን ዲ ጠ ይ ቁ ና አ ጥ ጋ ቢ ም ላ ሽ እ ን ደ ተ ሰ ጣ ች ው አ ረ ጋ ግ ጣ ለ ሁ ፡ ፡

የ ተ መ ራ ማ ሪ ው ፤ ስ ም----------

ፊ ር ማ----------

ቀ ን ----------

[1] አ ን ድ ቅ ጂ ለ ተ ሳ ታ ፊ ው ተ ሰ ጥ ቷ ል ፡ ፡

Gucaa Hirmanaa Heyyamm a Qoronnoo (Ga'eesootaf)

Kutaa 1: Fuula Ragaa Qorannoo

Seensa

Nagaa wajjin

Ani Gizaat Almaawu Jedhama. Addis Ababa Univarsiiti colleejji saayinsii fayyaa irraa gosa barnoota mikroobiyilooji digrii sadaffaan baradha (PhD) yeroo amma kana magaalaa Finfinne fi nanaawaa finfinneetti argamaan irratti qorannoo dhukuba sombaa beeyladaa irraa gara namaatti dadarbaa jiru irraattin hojachaa jira.kanatti ansuun wa'ee hojii kiyyaa ragaan sif ibsa.wanti ifaa hin tannee yoo jiraatee yeroo kamiyyuu dhaabdee na gafachuu ni dandeesa.yadni isaa ifaa yoo ta'e immoo akka irratti hirmatuun si afeera.hardha yaada kee kennuf yoo sif hin mijanne mari'atee naf kennu dandeesa.

kayyoo qoranichaa

Qorannoon kun Finfinnee fi magaaloot nannawaa finfinnee jiran irratti kan gageefamudha.Qoranichaa irratti nannowaan jiraan irratti dhukuba sombaa beeyladaa irraa gara namaatti dadarbuu danda'uu fi karaa ittin dadarbuu ilaaluu ta'a.Hirmaanaan kee kana baruuf na gargaara.

Akkataa filatamuu keessani

ulaagaan isin itti filatamtan ulaagaa addaaf osoo hint taane caarraandhaan waan ta'eef namoota biraa irraa adda taatanii akka hin taane hubataanii ragaa barbaachisu bu'aa qoraanoo kanaaf akka nuuf keenitan isin gaafanna.

Fedhiidhaan kan hirmaatan

Hirmaataan guutuumaan guutuutti fedhii irraatti kan hunda'edha.Hirmaachuu dhabuu keef wantti isaa ga'u tokkolee hin jiru.

Adeemsa Qoranichaa

Qoranno kan keessatti hirmaatootni kan keennan;

1ffaa Gaafannoo afaanii ni kennu

2ffaa Qorannoo labiraatoorii ancuufaa fi Barbaachisummaa ta'ee yoo Argame dhiitoo mormaa irraatti argamuu saamuudaa ni keennu.

Turtii (yeroo) itti fudhatu

Gaafannoo Afaanii fi qorannoo labiraatooriif waliigala sa'a 1;30 ni gaha.

Yaadoowaan

Qorannoon kun gaafaannoowaan jiraan keessaa galii ji'an argatuu gaafachuu ta'a.Gaafannon kun galii dhuunfaa wan ta'eef yoo sitti hin tolee,deebiisuu dhiisuu ni dandeesa.Dhukubni xiqoon Samuudaa dhiitoo mormaraa fudhatamuun jirachuu danda'a.Hata'u malee fayyumaarratti rakkon geesisuu geesisuu hin jiru.

Fayyidaa argamu

Hirmaatootni qorannoo kanaa faayidaan kallattin argatan hin jiru.Hata'u malee hirmaanan keessan wa'ee dhukubaa kanaa sirritti akka beeknuu fi to'annoo isaaf yaadni keessan nu gargara.qorannoon labiraatoorii dhukubichii jirachuu kan mirkanneesuu yoo ta'ee,. Buufeta Fayyaa dhihoottii Argamuttii Reeferii gochuun Akkes Biyyoolessaattii Qajeelfama qorannoo TB Bu'ura godhateen yaaliin skke kenna muuf ta'a.

Baasii bakka buusuu

Qorannoo kana irratti hirmaachuu keef onnachiiftuun kanfalamu tokkolee hin jiru.Hata'u malee Hospitalaa dhufuuf geejibaa si barbachiisa yoo ta'e baasiin geejibaa si kanfalama. Bakkeec Hospiitaala dhuftanii Xiggur Anbassaa yku Quidduus Phaul woos yoota'an kan dhuftani. Al tokkoo gopdhufta

Iccitti eguu ilaalchiisee

Odeefannoo kamilee miseensota qorannoon ala namni biraa akka baruu ta'uu hin qabu.Gaafannoo afaaniin kenitan irratti maqaan keessan hin bara'u.Bakka bu'iinsa maqaaf lakkofsi iccitti ni keenamaaf waan ta'eef odeefannoo keenitaaniif iccitiin isaa kan egaamedha.

Haala ibsa bu'aa

Qorannoon kun bifa baruuleettin ni maxanfama.Hata'u malee ragaa hirmaatoota akkataa ibsuun osoo hin ta'in ragaawan ibsuun kan maxannu ta'a.

Odeefannoo Dabalataaf

CHS IRB [Telephone 011 896 1396; email: chs.irb@aau.edu.et; P.o.Box 9086]

Gizaat Almawu: gizatalm@yahoo.com; mob: 0960-08-08-33

Kutaa 2; Guea Fedhii Rbsu

kutaa 1ffaa keessatti ragaawwan jiraan naf dubbifamaani,kan naf hin galee gaaffadheeibsii gahaa ta'e naf keenamee jira.Qorannoo kana irratti fedhii kiyyaan hirmaachuuf eyyamera.

Maqaa hirmaataa_____

Mallatoo_____

Guyyaa_____

Barreesuu fi Dubbiisuu kan hin dandeenyeef

Hirmaatoota yaadaa isaanii keennaniif unkii konsantii (consent) yeroo dubbifamuuf argeera.Hirmaataaniis wan hin galeef yeroo gaafatuu argeera waan ta'eef,hirmaataan yaadaa isaa dhibaa tokko malee yeroo kennu argeera.

Maqaa Ragaa_____ Mallatoo hirmaataa _____

Mallatoo_____

Guyyaa_____

yaadaa Qoratichaa

Hirmaatootaaf olitti ragaa qoranichaa dubiisuun hirmaatootni sirritti akka akka hubataan taasiseen jira.Itti ansuun kan jiruus akka sirritti hubataan taasiseera.

1.Qoranichii gaafannoo afaanii qabaachuu isaa

2. Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa akka kenamuu waan ta'eef hirmaatootni qorannoo kanaa gaaffii barbaadaan akka gaafatanii fi deebiin qubsaa kan keenameef ta'uu nan mirkaneesa.

Maqaa Qorataa_____

Mallatoo_____

Guyyaa_____

Gucaa Hirmanaa Heyyamm a Qoronnoo (umrii 12-17 Jirsu)

Kutaa 1: Fuula Ragaa Qorannoo

Seensa

Nagaa wajjin

Ani Gizaat Almaawu Jedhama. Addis Ababa Univarsiiti colleejji saayinsii fayyaa irraa gosa barnoota mikroobiyilooji digrii sadaffaan baradha (PhD) yeroo amma kana magaalaa Finfinne fi nanaawaa finfinneetti argamaan irratti qorannoo dhukuba sombaa beeyladaa irraa gara namaatti dadarbaa jiru irraattin hojachaa jira.kanatti ansuun wa'ee hojii kiyyaa ragaan sif ibsa.wanti ifaa hin tannee yoo jiraatee yeroo kamiyyuu dhaabdee na gafachuu ni dandeesa.yadni isaa ifaa yoo ta'e immoo akka irratti hirmatuun si afeera.hardha yaada kee kennuf yoo sif hin mijanne mari'atee naf kennu dandeesa.

kayyoo qoranichaa

Qorannoon kun Finfinnee fi magaaloot nannawaa finfinnee jiran irratti kan gageefamudha.Qoranichaa irratti nannowaan jiraan irratti dhukuba sombaa beeyladaa irraa gara namaatti dadarbuu danda'uu fi karaa ittin dadarbuu ilaaluu ta'a.Hirmaanaan kee kana baruuf na gargaara.

Akkataa filatamuu keessani

Akkataan ati itti filatamtee ulaagaa adda hin qabu.Hirmaatoota warrii ta'an carraadhani kan filatamaan.

Fedhiidhaan kan hirmaatan

Hirmaataan guutuumaan guutuutti fedhii irraatti kan hunda'edha.Hirmaachuu dhabuu keef wantti isaa ga'u tokkolee hin jiru.

Adeemsa Qoranichaa

Qoranno kan keessatti hirmaatootni kan keennan;

1ffaa Gaafannoo afaanii ni kennu

2ffaa Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa ni keennu.

Turtii (yeroo) itti fudhatu

Gaafannoo Afaanii fi qorannoo labiraatooriif waliigala sa'a 1;30 ni gaha.

Yaadoowaan

Dhukubni xiqoon Samuudaa dhiitoo mormaraa fudhatamuun jirachuu danda'a.Hata'u malee fayyumaarratti rakkon geesisuu geesisuu hin jiru.

Fayyidaa argamu

Hirmaatootni qorannoo kanaa faayidaan kallattin argatan hin jiru.Hata'u malee hirmaanan keessan wa'ee dhukubaa kanaa sirritti akka beeknuu fi to'annoo isaaf yaadni keessan nu gargara.qorannoon labiraatoorii dhukubichii jirachuu kan mirkanneesuu yoo ta'ee,ragaa isaa ogeesaa fayyaa dhimmi isaa ilaalatuuf keenuun bilisaan yaalaa akka argatan ni ta'a.

Baasii bakka buusuu

Qorannoo kana irratti hirmaachuu keef onnachiiftuun kanfalamu tokkolee hin jiru.Hata'u malee Hospitalaa dhufuuf geejibaa si barbachiisa yoo ta'e baasiin geejibaa si kanfalama.

Iccitti eguu ilaalchiisee

Odeefannoo kamilee miseensota qorannoon ala namni biraa akka baruu ta'uu hin qabu.Gaafannoo afaaniin kenitan irratti maqaan keessan hin bara'u.Bakka bu'iinsa maqaaf lakkofsi iccitti ni keenamaaf waan ta'eef odeefannoo keenitaaniif iccitiin isaa kan egaamedha.

Haala ibsa bu'aa

Qorannoon kun bifa baruuleettin ni maxanfama.Hata'u malee ragaa hirmaatoota akkataa ibsuun osoo hin ta'in ragaawan ibsuun kan maxannu ta'a.

Odeefannoo Dabalataaf

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Gizaat Almawu: gizatalm@yahoo.com; mob: 0960-08-08-33

Kutaa 2;Waraqaa ragaa eyyamuumaa

kutaa 1ffaa keessatti ragaawwan jiraan naf dubbifamaani,kan naf hin galee gaaffadheeibsii gahaa ta'e naf keenamee jira.Qorannoo kana irratti fedhii kiyyaan hirmaachuuf eyyamera.

Maqaa hirmaataa_____

Mallatoo_____

Guyyaa_____

Barreesuu fi Dubbiisuu kan hin dandeenyeef

Hirmaatoota yaadaa isaanii keennaniif unkii konsantii (consent) yeroo dubbifamuuf argeera.Hirmaataaniis wan hin galeef yeroo gaafatuu argeera waan ta'eef,hirmaataan yaadaa isaa dhibaa tokko malee yeroo kennu argeera.

Maqaa Ragaa_____ Mallatoo hirmaataa _____

Mallatoo_____

Guyyaa_____

yaadaa Qoratichaa

Hirmaatootaaf olitti ragaa qoranichaa dubiisuun hirmaatootni sirritti akka akka hubataan taasiseen jira.Itti ansuun kan jiruus akka sirritti hubataan taasiseera.

1.Qoranichii gaafannoo afaanii qabaachuu isaa

2. Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa akka kenamuu waan ta'eef hirmaatootni qorannoo kanaa gaaffii barbaadaan akka gaafatanii fi deebiin qubsaa kan keenameef ta'uu nan mirkaneesa.

Maqaa Qorataa_____

Mallatoo_____

Guyyaa_____

Gucaa Hirmanaa Heyyamm a Qoronnoo (Warrotaaf/Guddiftootaaf)

Kutaa 1: Fuula Ragaa Qorannoo

Seensa

Nagaa wajjin

Ani Gizaat Almaawu Jedhama. Addis Ababa Univarsiiti colleejji saayinsii fayyaa irraa gosa barnoota mikroobiyilooji digrii sadaffaan baradha (PhD) yeroo amma kana magaalaa Finfinne fi nanaawaa finfinneetti argamaan irratti qorannoo dhukuba sombaa beeyladaa irraa gara namaatti dadarbaa jiru irraattin hojachaa jira.kanatti ansuun wa'ee hojii kiyyaa ragaan sif ibsa.wanti ifaa hin tannee yoo jiraatee yeroo kamiyyuu dhaabdee na gafachuu ni dandeesa.yadni isaa ifaa yoo ta'e immoo akka irratti hirmatuun si afeera.hardha yaada kee kennuf yoo sif hin mijanne mari'atee naf kennu dandeesa.

kayyoo qoranichaa

Qorannoon kun Finfinnee fi magaaloot nannawaa finfinnee jiran irratti kan gageefamudha.Qoranichaa irratti nannowaan jiraan irratti dhukuba sombaa beeyladaa irraa gara namaatti dadarbuu danda'uu fi karaa ittin dadarbuu ilaaluu ta'a.Hirmaanaan kee kana baruuf na gargaara.

Akkataa filatamuu keessani

Akkataan ati itti filatamtee,akkataa lamii itti gaafatamuumaan itti dhaga'amuutti hirmaaachuu ni dandeesa.Filamuu keef ulaagaa adda hin qabu.Hirmaatoota warrii ta'an carraadhani kan filatamaan.

Fedhiidhaan kan hirmaatan

Hirmaataan mucaa kee guutuumaan guutuutti fedhii irraatti kan hunda'edha.Hirmaachuu dhabuu keef wantti isaa ga'u tokkolee hin jiru.

Adeemsa Qoranichaa

Qoranno kan keessatti hirmaatootni kan keennan;

1ffaa Gaafannoo afaanii ni kennu

2ffaa Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa ni keennu.

Turtii (yeroo) itti fudhatu

Gaafannoo Afaanii fi qorannoo labiraatooriif waliigala sa'a 1;30 ni gaha.

Yaadoowaan

Qorannoon kun gaafaannoowaan jiraan keessaa galii ji'an argatuu gaafachuu ta'a.Gaafannon kun galii dhuunfaa wan ta'eef yoo sitti hin tolee,deebiisuu dhiisuu ni dandeesa.Dhukubni xiqoon Samuudaa dhiitoo mormaraa fudhatamuun jirachuu danda'a.Hata'u malee fayyumaarratti rakkon geesisuu geesisuu hin jiru.

Fayyidaa argamu

Hirmaatootni qorannoo kanaa faayidaan kallattin argatan hin jiru.Hata'u malee hirmaanan keessan wa'ee dhukubaa kanaa sirritti akka beeknuu fi to'annoo isaaf yaadni keessan nu gargara.qorannoon labiraatoorii dhukubichii jirachuu kan mirkanneesuu yoo ta'ee,ragaa isaa ogeesaa fayyaa dhimmi isaa ilaalatuuf keenuun bilisaan yaalaa akka argatan ni ta'a.

Baasii bakka buusuu

Qorannoo kana irratti mucaan kee hirmaachuu isaaf onnachiiftuun kanfalamu tokkolee hin jiru.Hata'u malee Hospitalaa dhufuuf geejibaa si barbachiisa yoo ta'e baasiin geejibaa si kanfalama.

Iccitti eguu ilaalchiisee

Odeefannoo kamilee miseensota qorannoon ala namni biraa akka baruu ta'uu hin qabu.Gaafannoo afaaniin kenitan irratti maqaan mucaa keessanii hin bara'u.Bakka bu'iinsa maqaaf lakkofsi iccitti ni keenamaaf waan ta'eef odeefannoo keenitaaniif iccitiin isaa kan egaamedha.

Haala ibsa bu'aa

Qorannoon kun bifa baruuleettin ni maxanfama.Hata'u malee ragaa hirmaatoota akkataa ibsuun osoo hin ta'in ragaawan ibsuun kan maxannu ta'a.

Qoranichaa irratti hirmaachuu dhabuu yookiin addan kutuu.

Mucaan keessan qoranichaa irratti hirmaachuu yookiin addan kutuuf mirga ni qaba.

Odeefannoo Dabalataaf

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Gizaat Almawu: gizatalm@yahoo.com; mob: 0960-08-08-33

Kutaa 2;Waraqaa ragaa eyyamuumaa

kutaa 1ffaa keessatti ragaawwan jiraan naf dubbifamaani,kan naf hin galee gaaffadheeibsii gahaa ta'e naf keenamee jira.Qorannoo kana irratti mucaan kiyyaa hirmaachuuf eyyamera.

Maqaa warraa/ guddiftuu_____

Mallatoo_____

Guyyaa_____

Barreesuu fi Dubbiisuu kan hin dandeenyeef

Hirmaatoota yaadaa isaanii keennaniif unkii konsantii (consent) yeroo dubbifamuuf argeera.Hirmaataaniis wan hin galeef yeroo gaafatuu argeera waan ta'eef,hirmaataan yaadaa isaa dhibaa tokko malee yeroo kennu argeera.

Maqaa Ragaa_____ Mallatoo hirmaataa _____

Mallatoo_____

Guyyaa_____

yaadaa Qoratichaa

Hirmaatootaaf olitti ragaa qoranichaa dubiisuun hirmaatootni sirritti akka akka hubataan taasiseen jira.Itti ansuun kan jiruus akka sirritti hubataan taasiseera.

1.Qoranichii gaafannoo afaanii qabaachuu isaa

2. Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa akka kenamuu waan ta'eef hirmaatootni qorannoo kanaa gaaffii barbaadaan akka gaafatanii fi deebiin qubsaa kan keenameef ta'uu nan mirkaneesa.

Maqaa Qorataa_____

Mallatoo_____

Guyyaa_____

Gucaa Hirmanaa Heyyamm a Qoronnoo (abbotti beladdotaf)

Kutaa 1: Fuula Ragaa Qorannoo

Seensa

Nagaa wajjin

Ani Gizaat Almaawu Jedhama. Addis Ababa Univarsiiti colleejji saayinsii fayyaa irraa gosa barnoota mikroobiyilooji digrii sadaffaan baradha (PhD) yeroo amma kana magaalaa Finfinne fi nanaawaa finfinneetti argamaan irratti qorannoo dhukuba sombaa beeyladaa irraa gara namaatti. mallattro dhukuti chae qaban irrati qoranno gochaan jire. Odeefanno waa'ee qorannoo ku akka itti aamuttan ibsuuf yaale. Waan ifa hintaane tokkoo yoo jiraatte yeroo kamittiyyuu nodhabsisu dandeessu. Dhimmidi ifa kan hin taane you ta'ee qoodata hojichae akk taatan sin afera. Iyurtoo hardha gochuu dhisu dandeesu akkesumas namoota marisisisur barbeadaa hundae marisisuu dandcassu.

kaayyoo qorannichae

qorannon kuni magaloota Finfine kanene sululta Holotto

kane kana fakkaran kellaa qorannoon kanne addemsifamu qorano dha

qoranno iddoo ittii caqafmmee nannoo dhukkubinii somb (TB) beladdotta irraattii akkotti babalcha jiru hamo sadarkaa idaa bekudhof ni gargaraa

bufanni Horjisaa kciee qorannoo kanna kcisaa mokannii Hirmahisu kanna gochu dha nigagroo

simafilannoo qooddatoota

bufanni bedaddotaa keciee kan filtamman akk namma ittigaffata mumma dhagahammutti

akkaa sabba toico hirmachudhaf hin dandessaa ichame amana dhii

Filatammu ulgaa kammiyyuu hin jiru

Hirmatottaa Hin to'ku kanea jdhaman kessa corrad an filtan uii

Feddan kan himaatan

buffanii horisa kunii gutumma qutu fedlie irrati kan hunda'ee.

Hirmachu dhiisun keiee bablaa wanee yolello sirae kane gahu hin jiru

Adeamra qoranno

Horssisa beildda qorannooo kuma keissa Hirmatan

1^{ffaa} beladddoratff dhukkubba somba wolamsaa hin godhmafii

Kunise kaneee Hojarmu Horrii irrattii morma issannii irartti gogaa issanni irratti somba(TB) qoricha qraniodhanf warannudhan

Hamma dhita goggaa guyyaa boddaa kane Hojtamu ta'aa.

2ffaa Mormma irrattii gaggaa walansun bu'aan Somba(TB) jirachu issaa yoggaa aggansisee irraa deebii walansudhaff anani horii sanii irraa laboratoridhof sc 50 hinfudhatanna

Yeroo turti

qorannon yeroo tokko kanee hojatamu yoo tahu

Guyyaa 3

Yaaddoo

qorichii qorannoo dhaf kane fayyaddamenu Horma rakko Hin Fiddu

ta'uu illee Hojiin kun yaggaa Hojatamu beladdoto qabun dirqama wan to'eef beladdan wareanni fi amala addu addu qaban offi irratti to'ee Hojatotofis bala fidu dhanda'uu

Horiwon amala akassi bufta kana kessattii galu hin qaban ykn Meshawan addo adda Fayyada Hojachudhaf dirqamea

Fiyyidae

bu'aan walnisii Laboratorii dhukbinnii sunil jirachudhaf Sunii irrachudhaf kane Mirkanessu yoo jirate

beladdorra san irrattii ajaiaa tarkanfii irratti fludhatamu wal qabatee Fayyaddamma irrattii ilachissee gorsaa yadda. lolan kane kenamu ta'aa.

Dhimma qarshi wal ilaaattu

qorannoo Hirmachuu keessannif rakkoo Hommayyuu basii hin qabuii

Laboratorisse ta'ee waa hundaffuu walansaa saniff qbattee wall qabattee kaffittii homayyu hin qabu

Iccitti eapuu ilaallatte

waa Hundaafu biffaa oddeffanno qorannoo garee ykn misen saa alaa dhimma issaa ilaltu alaa akka bekuu win godhamu

Mafaan Horsisaa sannii gaffi ittii gafatammu irratti hin bareffamu

Kan birraa Lakkofisaa Iccittidhan hin kenamo

wan ta'eeff odddeffannon Iccittin kenamissun kan eggamedha

wan ta'ccf bu'aa issin qabdanni irraa qorannon sunii rackkoo homayyuu hin qabu

Akkatae ibse bu'aa qoranno

qorannon kun hojii qorannoo matseetii kan bahu to'aa

Haa' ta'uu malee oddeffannon Hirmatoa kan waan iffaa to'aan Osso wn taiin addan babach

Odeefannoo Dabalataaf

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Gizaat Almawu: gizatalm@yahoo.com; mob: 0960-08-08-33

Kutaa 2;Waraqaa ragaa eyyamuumaa

kutaa 1ffaa keessatti ragaawwan jiraan naf dubbifamaani,kan naf hin galee gaaffadheeibsii gahaa ta'e naf keenamee jira.qorannoo kan kessa feedhan kottin bilisaan eyyammu keenen jira horsina bcladotaa kiyya .

Maqaa Horsisaa abba qabenyaa/ ittiafatommmjiaa_____

Mallatoo_____

Guyyaa_____

Barreesuu fi Dubbiisuu kan hin dandeenyeef

Hirmaatoota yaadaa isaanii keennaniif unkii konsantii (consent) yeroo dubbifamuuf argeera.Hirmaataaniis wan hin galeef yeroo gaafatuu argeera waan ta'eef,hirmaataan yaadaa isaa dhibaa tokko malee yeroo kennu argeera.

Maqaa Ragaa_____ Mallatoo hirmaataa _____

Mallatoo_____

Guyyaa_____

yaadaa Qoratichaa

Hirmaatootaaf olitti ragaa qoranichaa dubiisuun hirmaatootni sirritti akka akka hubataan taasiseen jira.Itti ansuun kan jiruus akka sirritti hubataan taasiseera.

1.Qoranichii gaafannoo afaanii qabaachuu isaa

2. Qorannoo labiraatoorii ancuufaa fi dhiitoo mormaa irraatti argamuu saamuudaa akka kenamuu waan ta'eef hirmaatootni qorannoo kanaa gaaffii barbaadaan akka gaafatanii fi deebiin qubsaa kan keenameef ta'uu nan mirkaneesa.

Maqaa Qorataa_____

Mallatoo_____Guyyaa_____

Annex 10: Identification of TB suspects

This will be following the MoH, (2008) algorism

1. Pulmonary TB:

A person is a suspect of Pulmonary Tuberculosis when presenting with persistent cough for two weeks or more. Cough is usually with expectoration, with or without blood stained sputum and can be accompanied by one or more of the following symptoms:

• Weight loss;

• Chest pain;

• Shortness of breath;

• Intermittent fever;

• Night sweats;

• Loss of appetite;

• Fatigue and malaise

2. Extra-pulmonary TB

Tuberculous lymphadenitis:

• Slowly developing and painless enlargement of lymph nodes, followed by matting and eventual drainage of pus.

Suggested clinical characteristics to assist the diagnosis of extrapulmonary tuberculosis (ETB)

Suspect ETB in patients with

Cough for two weeks or more *or*

- Unintentional weight loss with
  - Night sweats *and*
  - Temperature >37.5 °C or feels feverish
- Breathlessness (effusion/ pericarditis) *or*
- Enlarged glands in neck/ armpit *or*
- Chest X-ray
  - Miliary or diffuse shadowing
  - Large heart (especially if symmetrical and rounded)
  - Pleural effusion
  - Enlarged lymph nodes inside the chest
- Chronic headache or altered mental state

Suspect disseminated tuberculosis in all people living with HIV who experience rapid or marked weight loss, fever and night sweats

Establish HIV status if ETB is suspected

- Advise and arrange for rapid HIV testing if status is unknown or last test was negative
  - Explain that this will affect the way that this illness is investigated and treated
  - Discuss the need for antiretroviral treatment if HIV-related tuberculosis is diagnosed
  - If consent is given, try to arrange testing on the same day

Look and listen for

- Lymph nodes swelling in the neck or armpits (if present with other types of ETB it may provide the only way to confirm the diagnosis) **Possible tuberculosis lymphadenitis**
- Signs of fluid in the chest
  - Absent breath sounds
  - Reduced chest wall movement
  - Dull to percussion

  Possible tuberculosis pleural effusion
- Signs of fluid around the heart
  - Heart sounds distant
  - Swollen legs and/or abdomen
  - Neck and hand veins distended with arm held above the shoulder

  Possible tuberculosis pericarditis
- Signs of meningitis
  - neck stiffness
  - confusion
  - abnormal eye movements

  Possible tuberculosis meningitis

Tuberculous pleurisy

• Pain while breathing in, dull lower chest pain, intermittent cough, breathlessness on exertion.

TB of bones and/or joints

• Localized pain and/or swelling, discharge of pus, muscle weakness, paralysis, stiffness of joints.

Intestinal TB

• Loss of appetite and weight, abdominal pain, diarrhoea or constipation, mass in the abdomen, fluid in the abdominal cavity (ascites).

Tuberculous meningitis

• Headache, fever, vomiting, neck stiffness and mental confusion of insidious onset

Tuberculous pleurisy

• Pain while breathing in, dull lower chest pain, intermittent cough, breathlessness on exertion.

TB of bones and/or joints

• Localized pain and/or swelling, discharge of pus, muscle weakness, paralysis, stiffness of joints.

Intestinal TB

• Loss of appetite and weight, abdominal pain, diarrhoea or constipation, mass in the abdomen, fluid in the abdominal cavity (ascites).

Tuberculous meningitis

• Headache, fever, vomiting, neck stiffness and mental confusion of insidious onset

Annex 11: Description of the herd demography and characteristics

Table. Description of the herd demography and characteristics

Characteristics	Levels	Herd size			
		Small (n=212)	Medium (n=49)	Large (n=38)	Total (n=299)
Calf (0-1yr)	Crossbreed	381	257	360	998
	Zebu	34	5	1	40
	Exotic (pure)	0	0	1	1
Heifer	Crossbreed	360	191	413	964
	Zebu	15	4	1	20
	Exotic (pure)	0	0	0	0
Cow	Crossbreed	1116	703	1486	3305
	Zebu	37	15	52	104
	Exotic (pure)	8	2	0	10
Bullock/Steers (1-2 yrs)	Crossbreed	17	27	24	68
	Zebu	6	2	3	11
	Exotic (pure)	0	2	1	3
Bull/Oxen	Crossbreed	32	14	31	77
	Zebu	52	11	6	69
	Exotic (pure)	0	0	5	5
Total cattle	Total	2058	1233	2384	5675
Other animals	Sheep	549	310	500	1359
	Goats	99	56	77	232
	Equine	142	24	34	200
	Dogs	260	87	58	405
	Cats	167	69	18	254
	Swine	6	45	1511	1562
	Poultry	5963	6952	7541	20456

Annex 13: Description of risk factors

Table. Description of risk factors

Risk factors	Level	Description
Herd size	(4, 20]	Exclusive of 4 but inclusive of 20
	(20, 37]	
	(37,168]	
Age (yrs)	(0.1, 2]	Exclusive of 0.1 but inclusive of 2
	(2, 4]	
	(4, 6]	
	(6, 8]	
	(8, 10]	
Source	Purchased	Bought from market or farm
	On farm bred	Reared at the farm
Breed	Cross and exotic	Cross : Pure Holstein Friesian X Zebu, Jersey X Zebu , Exotic:pure HF& Jersey
	Zebu	Indigenous local Zebu breed
Sex	Female	
	Male	
Farm age (yrs)	(4, 20]	Farm age since establishment
	(20, 35]	
	(35, 68]	
bTB history at farm	Yes	Yes: bovine TB detected during tuberculin testing the last three years
	No	No: Not detected
Contact with other domestic animals	Yes	Presence of possible contact between herd and other domestic animals (e.g. goat/swine/cat/sheep/swine/equine/poultry)
	No	No possible contact between herd and other domestic animals (e.g. goat/swine/cat/sheep/swine/equine/poultry)

Stocking density (no. cattle/m^2)	High	High: > $0.5m^2$
	Satisfactory	Satisfactory: equals $0.5m^2$: one cattle need (1m *2m) $2m^2$ area ($1/2=0.5m^2$)
	Less	Less: < $0.5m^2$
Ventilation	Very good	Above half of the four sides of the wall opened/meshed
	Satisfactory	Above half of the two sides of the wall opened/meshed
	Poor	Closed wall with few windows
Viral disease outbreak	Yes	Experience of the incidence of viral disease (FMD, LSD, BVD, etc) in cattle during the last one calendar year
	No	No experience of the incidence of viral disease (FMD, LSD, BVD, etc) in cattle during the last one calendar year
Biosecurity measures	Absent	No measures (e.g.no visitors allowed) taken to minimize possibility of disease transmission
	Present	Measures (e.g.no visitors allowed) taken to minimize possibility of disease transmission
Neighbor herd	Yes	Adjacent herd present
	No	No adjacent herd
House type	Free movement	Cows are not restrained and are able to move freely, to enter and leave
	Loose	Cows are confined together on a platform and secured at neck by rope or neck chains
	Cubicle	Each cow has a separate stall with or without neck tie
Regular de-worming	Yes	Practice of de-worming of the herd once/twice in a year
	No	No practice of de-worming of the herd once/twice in a year
Stages of lactation (months)	(0, 2]	
	(2,4]	
	(4,8]	

CURRICULUM VITAE

PERSONAL DATA

Name: Gizat Almaw Enyew

Sex: Male

Date of Birth: September 15, 1974

Marital status: Married

Nationality: Ethiopian

Address: National Animal Health Diagnostic and Investigation Center

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E-mail **: gizatalm@yahoo.com** or **tiwawyedegera@yahoo.com**

EDUCATION

- 1994-1999: Faculty of Veterinary Medicine, Addis Ababa University (DVM degree)
- 2002-2004: Faculty of Veterinary Medicine, Addis Ababa University (MSc in tropical veterinary medicine)
- 2015 to Present: PhD student of Medical Microbiology, Addis Abeba University, Ethiopia

SHORT-TERM TRAININGS

- 26th August - 9th October 2008: Tamil Nadu Veterinary and Animal Sciences University, India. Laboratory Animal Husbandry and Medicine (Certificate)
- May 21 - July 27, 2012: Molecular Epidemiology of Foodborne Pathogens at The Ohio State University-Eastern Africa One Health Summer Institute Held in Ethiopia (Certificate)

 Dangerous Goods Regulations Initial I from March 18-22, 2013 (Certificate)

 Mycobacterium isolation techniques, pcr and spoligotyping techniques for 5 days (September, 2015) at Armaeur Hansen Research Institute, Ethiopia

RESEARCH AND LABORATORY EXPERIENCE

 2011-present: Employee of National Animal Health Diagnostic and Investigation Laboratory (NAHDIC), Ethiopia as a researcher in bacteriology laboratory. And I have been involved in salmonellosis and bovine tuberculosis projects.

TEACHING EXPERIENCE

 Instructor of veterinary public health, immunology and other courses at Faculty of Veterinary Medicine, University of Gondar (2006 to April, 2011)

PROFESSIONAL EXPERIENCE

 District Veterinary Practitioner of Ministry of Agriculture, Ethiopia (20002002)

PREVIOUS APPOINTMENTS

 Head department of veterinary microbiology, immunology and public health, faculty of veterinary medicine, university of Gondar Chairman of faculty research and publication committee, faculty of veterinary medicine, university of Gondar Member of senate standing committee of research and publication, university of Gondar

PUBLICATIONS

Book (e-book)

Gizat Enyew. 2013. Bovine mastitis and antibiotic resistance patterns of major pathogens. LAMBERT Academic Publishing, Germany. **ISBN**: 978-3-659-36566-9

Chapters in Yearbook

Wubete A., Amare E., **Alimaw G**., Sahile M., Abie G., Koran T., Tamiru M., Yimesigen 2013. Prevalence of Bovine Tuberculosis in Debire Birhan and Fiche smallholder dairy farms. In: Gari G., Wubete A., Chaka H., Almaw G. (eds). NAHDIC yearbook. p. 90-96. Ministry of Agriculture, Ethiopia.

Journals

1. Almaw G, Conlan AJK, Ameni G, Gumi B,Alemu A, Guta S, et al. 2021. The variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia provides opportunities for targeted intervention. PLoS ONE 16(7): e0254091. https://doi.org/10.1371/journal.pone.0254091

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DECLARATION

I the undersigned declare that this PhD Thesis is my own original work and has not been presented for a degree in any other university and all sources of materials used for the Thesis have been duly acknowledged.

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PLOS ONE

RESEARCH ARTICLE

The variable prevalence of bovine tuberculosis among dairy herds in Central Ethiopia provides opportunities for targeted intervention

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Abstract

Bovine tuberculosis (bTB) is an important disease for dairy productivity, as well as having the potential for zoonotic transmission. Previous prevalence studies of bTB in the dairy sector in central Ethiopia have suggested high prevalence, however, they have been limited to relatively small scale surveys, raising concerns about their representativeness. Here we carried out a cross sectional one-stage cluster sampling survey taking the dairy herd as a cluster to estimate the prevalence of bTB in dairy farms in six areas of central Ethiopia. The survey, which to date is by far the largest in the area in terms of the number of dairy farms, study areas and risk factors explored, took place from March 2016 to May 2017. This study combined tuberculin skin testing and the collection of additional herd and animal level data by questionnaire to identify potential risk factors contributing to bTB transmission. We applied the single intradermal cervical comparative tuberculin (SICCT) test using >4mm cut-off for considering an individual animal as positive for bTB; at least one reactor animal was required for a herd to be considered bTB positive. Two hundred ninety-nine dairy herds in the six study areas were randomly selected, from which 5,675 cattle were tested. The overall prevalence of bTB after standardisation for herd-size in the population was 54.4% (95% CI 48.7–60%) at the herd level, and it was 24.5% (95% CI 23.3–25.8) at the individual animal level. A Generalized Linear Mixed Model (GLMM) with herd and area as random effect was used to explore risk factors association with bTB status. We found that herd size, age, bTB history at farm, and breed were significant risk factors for animals to be SICCT positive. Animals from large herds had 8.3 times the odds of being tuberculin reactor (OR: 8.3, p-

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value:0.008) as compared to animals from small herds. The effect of age was strongest for animals 8–10 years of age (the oldest category) having 8.9 times the odds of being tuberculin reactors (OR: 8.9, p-value: <0.001) compared to the youngest category. The other identified significant risk factors were bTB history at farm (OR: 5.2, p-value:0.003) and cattle breed (OR: 2.5, p-value: 0.032). Our study demonstrates a high prevalence of bTB in central Ethiopia but with a large variation in within-herd prevalence between herds, findings that lays an important foundation for the future development of control strategies.

Introduction

Bovine tuberculosis (bTB) is a chronic disease of cattle primarily caused by *Mycobacterium bovis* (*M. bovis*), which has zoonotic potential and can also infect other domestic and wild animals. The disease is prevalent in most of Africa, parts of Asia and the Americas, and in several European countries. Many industrialised countries have managed to reduce or eliminate bTB in their livestock sectors through test-and-slaughter, however significant pockets of infection remain in wildlife [1]. In Africa the disease is endemic due to a lack of control measures. This has economic implications for the growth of the livestock sector, especially the dairy sector, and poses the risk of zoonotic TB transmission which is exacerbated by the existence of concomitant infections such as HIV/AIDS [2]. In Ethiopia, the demand for milk is expanding rapidly due to increased urbanization and population pressure; Ethiopia is the second most populous country in Africa with an estimated population of 110 million people [3]. Since the introduction of intensive dairy farming in central Ethiopia in the 1950s to provide the Emperor and his establishment with milk, the dairy sector has steadily increased. This increase has accelerated during the last 30 years—trying to meet the demand from increased urbanization and the need to supply milk and milk products to the city dwellers [4]. Although the dairy sector is most developed in central Ethiopia, urban centers across the country have more recently seen an increase in dairy farming. This most developed dairy belt in Ethiopia is expected to be challenged with diseases of intensification such as bTB [5, 6]. This is believed to

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MICROBIOLOGY SOCIETY

OPEN DATA OPEN ACCESS

Population structure and transmission of *Mycobacterium bovis* in Ethiopia

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Abstract

Bovine tuberculosis (bTB) is endemic in cattle in Ethiopia, a country that hosts the largest national cattle herd in Africa. The intensive dairy sector, most of which is peri-urban, has the highest prevalence of disease. Previous studies in Ethiopia have demonstrated that the main cause is *Mycobacterium bovis*, which has been investigated using conventional molecular tools including deletion typing, spoligotyping and Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR). Here we use whole-genome sequencing to examine the population structure of *M. bovis* in Ethiopia. A total of 134 *M. bovis* isolates were sequenced including 128 genomes from 85 mainly dairy cattle and six genomes isolated from humans, originating from 12 study sites across Ethiopia. These genomes provided a good representation of the previously described population structure of *M. bovis*, based on spoligotyping and demonstrated that the population is dominated by the clonal complexes African 2 (Af2) and European 3 (Eu3). A range of within-host diversity was observed amongst the isolates and evidence was found for both short- and long-distance transmission. Detailed analysis of available genomes from the Eu3 clonal complex combined with previously published genomes revealed two distinct introductions of this clonal complex into Ethiopia between 1950 and 1987, likely from Europe. This work is important to help better understand bTB transmission in cattle in Ethiopia and can potentially inform national strategies for bTB control in Ethiopia and beyond.