Dissertation Ref. No. _____

EPIDEMIOLOGY OF TUBERCULOSIS IN BOVINE AND SWINE, AND ITS ZOONOTIC IMPLICATION IN CENTRAL ETHIOPIA

PhD DISSERTATION

BY

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PhD PROGRAMME IN VETERINARY PUBLIC HEALTH (VPH)

OCTOBER 2021

BISHOFTU, ETHIOPIA

EPIDEMIOLOGY OF TUBERCULOSIS IN BOVINE AND SWINE, AND ITS ZOONOTIC IMPLICATION IN CENTRAL ETHIOPIA

A PhD Dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Veterinary Public Health (VPH)

BY

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OCTOBER 2021

BISHOFTU, ETHIOPIA

APPROVAL FORM

Addis Ababa University
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As members of the Examining Board of the final PhD open defense, we certify that we have read and evaluated the dissertation prepared by Kassa Demissie Abdi entitled " **Epidemiology of Tuberculosis in Bovine and Swine, and its Zoonotic Implication in Central Ethiopia"** and recommend that it be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy in Veterinary Public Health.

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DEDICATION

To My Family

STATEMENT OF THE AUTHOR

First, I declare that this dissertation is my *bonafide* work and that all sources of materials used for this dissertation have been duly acknowledged. This dissertation has been submitted in partial fulfillment of the requirements for PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University's/College's Library to be made available to borrowers under rules of the Library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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BIOGRAPHICAL SKETCH

The author was born at Wollie-Deneba, Siyadebrna Wayu district of North Shoa in Amhara Region on 12th April 1972. He attended his elementary and junior educations at Deneba Elementary and Junior School and his high school education at Hailemariam Mamo Senior Comprehensive Secondary School. After successfully completed his high school education, he joined Addis Ababa University School of Veterinary Medicine in 1989 and graduated with Doctor of Veterinary Medicine (DVM) degree in 1995. After graduation, he was employed by Bureau of Agriculture in Amhara Region (1996-2004) and assigned at Metema and Chilga districts under the Department of Agriculture in North Gondar Administrative Zone. He served as a field veterinarian in various positions and different capacities such as field veterinary clinician, team leader, project coordinator and office head. Moreover, he served as a trainer and supervisor of animal health technicians (AHT), development agents (DA) and project extension agent farmers (PEAF). Then, he joined again the School of Graduate Studies of Addis Ababa University Faculty of Veterinary Medicine (FVM) to pursue specialization in Tropical Veterinary Epidemiology (2005-2006) and obtained MSc degree in 2006. He then employed at Addis Ababa Abattoirs Enterprise (AAAE) and served as main section head and acting manager of slaughter operation (2006-2007). He worked as a freelance agro-industrial consultant over the years 2007-2008. He shifted to higher education and employed as a lecturer at Debre Berhan University and served as a Director for Community Services, Dean for the College of Agriculture and Natural Resource Sciences, researcher, consultant, member in various Senate standing and other committees over the years 2010-2013. Moreover, he taught many courses of veterinary medicine for undergraduate students of animal sciences and supervised students' senior projects. He joined the School of Graduate Studies of Addis Ababa University in 2014/15 to pursue further PhD specialization in Veterinary Public Health (VPH) programme in the College of Veterinary Medicine and Agriculture.

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LIST OF ABBREVIATIONS

AAAE	Addis Ababa Abattoirs Enterprise
AAU	Addis Ababa University
AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
ALIPB	Aklilu Lemma Institute of Pathobiology
Anon	Anonymous
APHA	Animal and Plant Health Agency
APHIS	Animal and Plant Health Inspection Service
ATVET	Alage Agricultural, Technical and Vocational Education and Training
BCG	Bacille de Calmette et Guerin
BCS	Body Condition Score
BE	Base of the Ear
bP	base pair
bTB	Bovine Tuberculosis
CFIA	Canadian Food Inspection Agency
CFSPH	Center for Food Security and Public Health
CFT	Caudal Fold Test
CSA	Central Statistical Agency
CVMA	College of Veterinary Medicine and Agriculture
DBZRH	Debre Berhan Zonal Referal Hospital
DNA	Deoxyribonucleic Acid

LIST OF ABBREVIATIONS (*Cont'd*)

DR	Direct Repeat
DTH	Delayed Type Hypersensitivity
DVR	Direct Variant Repeat
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assay
EPTB	Extrapulmonary Tuberculosis
ETR	Exact Tandem Repeat
FAO	Food and Agriculture Organization
GIS	Geographic Information System
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IDF	International Dairy Federation
IU	International Unit
IFN-γ	Interferon Gamma
IRB	Institutional Review Board
IS	Insertion Sequence
LJ	Lowenstein Jensen
LSP	Large Sequence Polymorphism
MAC	*Mycobacterium avium* Complex
MCT	Mid-cervical Test
MDR/TB	Multidrug Resistant Tuberculosis

LIST OF ABBREVIATIONS (*Cont'd*)

MIRU	Mycobacterial Interspersed Repetitive Units
MLSA	Multilocus Sequence Analysis
MOH	Ministry of Health
MOTT	Mycobacteria other than Tuberculosis
mPCR	Multiplex PCR
mRNA	Messenger RNA
MSU	Mississippi State University
MTBC	*Mycobacterium tuberculosis* Complex
NAHDIC	National Animal Health Diagnostics and Investigation Center
N-PCR	Nested PCR
NTM	Non-tuberculous Mycobacteria
NVL	No Visible Lesion
OIE	Office International des Epizooties
PAT	Postaxillary Test
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
PPDA	Purified Protein Derivative_Avian
PPDB	Purified Protein Derivative_Bovine
RD	Region of Difference
rDNA	Ribosomal Deoxyribonucleic Acid
RFLP	Restricted Fragment Length Polymorphism

LIST OF ABBREVIATIONS (*Cont'd*)

RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time PCR
RT-qPCR	Real Time Quantitative PCR
SB	Spoligotype of Bovine strain
SICCTT	Single Intradermal Comparative Cervical Tuberculin Test
SIT	Spoligo-international Typing
SpolDB4	Spoligotyping Database 4
TB	Tuberculosis
TBL	Tuberculosis-like
TBLN	Tuberculous Lymphadenitis
TST	Tuberculin Skin Test
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture
VNTR	Variable Number Tandem Repeat
WAHIS	World Animal Health Information System
WGS	Whole Genome Sequencing
WHO	World Health Organization
ZN	Ziehl-Neelsen

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my spiritual thank to the Almighty GOD, who is the sustainer and Lord of everything, for His innumerable favours and benevolence to me during my studies. Special spiritual respect and thanks also need to be extended to Saint Mary.

My special gratitude goes to Professor Gobena Ameni, Professor Gezahegne Mamo and Dr. Takele Abayneh, my academic advisors, for their commitment, sensitivity, brotherhoodness, professional guidance, in-depth evaluation and edition of the manuscript in order to appear in its present form.

The author's deepest gratitude also goes to Debre Berhan University (Office of the President, Office for the Academic Vice President, College of Graduate studies, College of Agriculture and Natural Resource Sciences, Department of Animal Sciences, Human Resource Directorate, Directorate for Finance, Directorates for Procurement and Property Administration and Legal Office), Graduate Studies of Addis Ababa University, the College of Veterinary Medicine and Agriculture, Aklilu Lemma Institute of Pathobiology (ALIPB) of the Addis Ababa University, Professor Getachew Terefe, Professor Hagos Ashenafi, Professor Kebede Amenu and professor Bersisa Kumsa (Associate Deans for Graduate Programmes) for the administrative support. In addition, Addis Ababa Abattoirs Enterprise together with slaughter operators, office of meat inspection and inspectors for their cooperation to undertake swine necropsy study for seven months.

The all-rounded supports from the inception to termination of the study due to Professor Gobena Ameni should be articulated and capitalized. It was not possible to do even a piece of work without these supports. In addition, the moral, financial and material supports due to Professor Gezahegne Mamo need to be articulated. Moreover, the financial support due to Dr. Tilaye Demissie, Professor Kebede Amenu and Dr. Fanta Desissa was helpful. It is an excellent opportunity for the author to note the support made due to Zonal and Debre Berhan milkshed animal health and livestock experts in North Shoa Department of Agriculture, Alage, Bishoftu and Akaki Kality. Moreover, the livestock owners should be appreciated for their willingness to

allow their animals for the current study. My thank also goes to Aboma Zewude, Dereje Gudeta, Dereje Bekele, Misganaw Tefera and Worku Haile for their professional commitment to undertake the tuberculin skin test in cattle and swine. Moreover, the role played by Dr. Musse Girma in molecular typing of the MTBC isolates, Dr. Estifanos Lemma for mapping the study sites, Dr. Zerihun Assefa, Dr. Kassaye Aragaw, Professor Gebeyehu Goshu, Dr. Mekete Bekele, Dr. Kassahun Bekele, Dr. Yohannes Muluneh, Dr. Hailemichael Lemma, Mr. Fikru Minwalkulet, Mr. Mulugeta Jovani, Sister Tigist Fantahun, Mr. Solomon Taddese, Mr. Awulachew Solomon and Mr. Amezene Worku in support of this study is unforgettable.

The moral support, encouragement and patience of my wife, Selamawit Assefa, is above any caliber and hence it is an opportunity for me to witness once again her determination in support of my advancement. The moral support from the side of my mother (Wudnesh Abebe), Yared Assefa, Kassahun Shibeshi, all my wife's extended family, Getu Demissie with his all family members and Mulu Demissie with her all family members was an energy to indicate me the right way to success. Finally, it is very difficult to mention all people over here. However, those who contributed directly or indirectly to my study are warmly acknowledged.

EPIDEMIOLOGY OF TUBERCULOSIS IN BOVINE AND SWINE, AND ITS ZOONOTIC IMPLICATION IN CENTRAL ETHIOPIA

Kassa Demissie Abdi

PhD dissertation

Addis Ababa University (2021)

ABSTRACT

Tuberculosis is a global priority disease of humans and animals. Bovine tuberculosis has been first reported in Ethiopia in the year 1967. Since then its prevalence has been rising. The nationwide prevalence of bovine tuberculosis has been estimated (5.8%) very recently in Ethiopia and the animal level prevalence ranges from 0.8%-54.6%; the highest prevalence was being reported in intensive dairy farms in and around cities while the lowest prevalence was being recorded in grazing animals in rural areas. However, there are emerging dairy cattle farms in Debre Berhan milkshed where the current epidemiological picture of bovine tuberculosis has not yet been elucidated. Similarly, there is little scientific information on the epidemiology of tuberculosis in swine in the country although swine production has been growing in central Ethiopia since two decades following the privatization policy. Besides affecting animals and reducing their productivity, animal tuberculosis is transmitted to and causes illness in humans. However, there is little information on the magnitude of human tuberculosis of animal origin in Ethiopia that requires additional studies. This study was initiated to investigate the epidemiology of tuberculosis in bovine and swine and its zoonotic implication in central Ethiopia. A cross-sectional study was conducted on 96 herds consisting of 625 heads of dairy cattle in Debre Berhan milkshed and 11 herds comprising of 329 heads of swine raised in selected sites in central Ethiopia. Skin testing was carried out by making use of single intradermal cervical comparative tuberculin test for cattle and single intradermal comparative tuberculin test for swine. Moreover, mycobacterial culturing, gross and microscopic characterization of tuberculosis like lesions and molecular typing of mycobacteria were used. Face-to-face interview using semi-structured and open-ended questionnaire was also presented to 165 respondents (146 farm workers and 19 human health professionals) to assess their knowledge and practices on the

zoonotic transmission of tuberculosis from farm animals to humans. The finding showed that the apparent individual animal level prevalence of bovine tuberculosis was 17% (106/625; 95% CI: 14.2-20.2) at ≥4mm and 18.4% (115/625; 95% CI: 15.5-21.7) at >2mm cut-off values in 625 heads of dairy cattle tested. Whereas, the herd prevalence was 16.7% (16/96; 95% CI: 10.1-26) at ≥4mm and 22.9% (22/96; 95% CI: 15-33) at >2mm cut-off values in 96 dairy herds tested. Multivariable logistic regression analysis at ≥4mm cut-off value revealed that dairy cattle with poor body condition score (AOR = 3.7; 95% CI: 1.6-8.4; p = 0.002), in the large herd size (AOR= 29.5; 95% CI: 5.6-154.1; p = 0.000) and of exotic breed (AOR = 3.7; 95% CI: 1.3-10.7; p = 0.018) had 4, 30 and 4 times the odds of tuberculin positivity with statistical significance, respectively compared to their counterparts. The apparent individual animal level prevalence of bovine tuberculosis in swine was 3% (10/329; 95% CI: 2-6) at > 2mm cut-off value. In the total 9 clusters tested, the herd prevalence was 11% (1/9; 95% CI: 1-49) at >2mm cut-off value. In addition, the abattoir lesion prevalence of tuberculosis in swine was 4.1% (26/640; 95% CI: 2.8-6.0) on the basis of gross tuberculosis like lesions. The awareness level of farm owners/attendants towards the transmission of tuberculosis from farm animals to humans was generally low. Culturing of tuberculosis like tissue lesions from swine and human sputa from suspected active TB patients as well as molecular typing indicated *M. tuberculosis* as the principal finding. In conclusion, the current study demonstrated the endemic occurrence of animal tuberculosis in the study areas and low level of public awareness towards its zoonotic transmission. Poor body condition, large herd size and exotic breed were important predictors of bovine tuberculin positivity in dairy cattle. *M. tuberculosis* was the predominant species cycling in the study areas. The findings of the current study compliment research works done so far in Ethiopia and contribute its own share to the control options of bovine tuberculosis. The study forwarded recommendations for intervention and future research.

Keywords: *Bovine tuberculosis, Central Ethiopia, Dairy Cattle, Epidemiology, Public awareness, Swine, Tuberculin*

1. INTRODUCTION

Tuberculosis (TB) is a chronic granulomatous, debilitating and contagious disease that has existed for millennia. It remains a major important global disease which affects both humans and animals (Dibaba *et al*., 2019; WHO, 2020). TB, like other chronic inflammatory diseases, is characterized by the generation of oxygen and nitrogen free radicals. The generation of free radicals in excess of the antioxidant capacity of the host leads to cellular and eventually systemic oxidative stress. Oxidative stress resulting from the consumptive depletion of protein and non-protein antioxidants can further be complicated by inadequate dietary replenishment (Palanisamy *et al*., 2011).

Tuberculosis is a communicable disease, a major cause of ill health, one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent (ranking above human immunodeficiency virus/acquired immunodeficiency syndrome: HIV/AIDS). In 2020, about 10 million people developed TB and 1.4 million died including 208 000 among HIV-positive people. TB can affect anyone anywhere. About 90% of the people who develop the disease are adults. There are more cases among men than women. Of those who fell sick with TB in 2020, 87% were in 30 high TB burden countries. Incidence rates at national level vary from less than 5 to more than 500 per 100 000 population per year. TB is a disease of poverty and economic distress, vulnerability, marginalization, stigma and discrimination. About a quarter of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). However, it is curable and preventable. Ethiopia is one among the 14 high burden country lists for TB, TB/HIV and multidrug-resistant tuberculosis (MDR/TB) defined by World Health Organization (WHO) for the period 2016-2020. These 14 countries accounted for 63% of the estimated global number of incident TB cases in 2020. The total TB incidence for Ethiopia based on estimated epidemiological burden of TB in 2020 for 30 high TB burden countries, WHO regions and globally is 140 per 100 000 population. HIV prevalence among incident TB cases in Ethiopia is 6.5% per 100 000 population. Similarly, the HIV-positive TB incidence for Ethiopia is 10 000 among 112 000 000 national population (WHO, 2020).

Globally, TB causes more adult death than any other single infectious diseases as 95% of cases and 98% of deaths occur in the developing world. Of notified TB cases, 95% of them are occurring in low and middle-income countries. It is responsible for one-third of all deaths of HIV-infected individuals in Africa (Kaneene and Thoen, 2004; Shitaye *et al*., 2007; Sakamoto, 2012; Ashenafi *et al*., 2013; Souza de Lima *et al*., 2016).

Bovine TB (bTB) is a chronic contagious infectious and granulomatous disease of many domestic and wild animals, swine, poultry, wild birds and people. Bovine TB has a wide host range as it occurs in almost all vertebrates and in some cold-blooded animals. It has various routes of transmission. The disease is distributed worldwide throughout the year (Thoen *et al*., 1995; Tsegaye *et al*., 2010). Bovine TB is of as great importance in Africa as it is elsewhere and it is endemic and widespread in African countries affecting the animal industries and human health posing serious public health threats (Cosivi *et al.*, 1998; Youssef and Ahmed, 2014; Dibaba *et al*., 2019). Bovine TB is characterised by progressive development of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestine and kidneys including others (Radostits *et al*., 2007; Romha *et al*., 2014).

Bovine TB has affected animals and humans health since antiquity. The epidemiology of TB in animals is complex, and is even more so in Africa, given the extreme variations in ecosystems, poor border control, and farming practices that vary from extensive to intensive and, in many instances, the movement of livestock over vast distances because of nomadism and transhumance (Dibaba *et al*., 2019). *Mycobacterium bovis* (*M. bovis*), the causative agent of bTB, has no known geographical boundaries. Despite these ominous features of *M. bovis*, to date there have been only projected global estimates of the disease burden. There has been no international effort to determine the actual disease burden owing to the non-availability of a reliable user-friendly laboratory methodology for early detection of *M. bovis* in clinical samples. Bovine TB has been controlled in farm animals in developed countries and continues to occur in developing countries. In Africa, approximately 85% of cattle and 82% of the human population live in areas where the disease is prevalent. There are limited reports from developing countries relating to the prevalence of infection with *M. bovis* in cattle. Detection of *M. bovis* in bovine

samples has become necessary as infected animals are potentially capable of infecting humans (zoonotic tuberculosis). Besides *M. bovis*, transmission of *M. tuberculosis* from infected humans to animals and back has been reported (reverse zoonosis). Hence, *M. bovis* and *M. tuberculosis* pose a potential health hazard to both animals and humans (Mishra *et al*., 2005). The infection caused by *M. bovis* is clinically indistinguishable from *M. tuberculosis* (Maas *et al*., 2002).

Cattle and swine TB was one of the commonest diseases affecting and killing millions of livestock not very long ago. The main reservoir of *M. bovis* infections in cattle is infected cattle. Infected cattle also serve as the source and reservoir of infection for bTB in humans. Humans can contract bTB by ingesting the bacteria in raw milk and milk products (Shitaye *et al*., 2007; Mandal, 2013).

Swine are susceptible to *M. tuberculosis* complex (MTBC) and *M. avium* complex (MAC) (Arega *et al*., 2013; Thoen, 2013). *M. bovis* is transmitted to swine mainly by ingestion of infected meat, milk and their products or by aerosolisation. It typically causes a persistent infection with production of granulomas of the lymph nodes, lung and may become systemic affecting most internal organs. It was originally thought that wild boars were the dead-end hosts for *M. bovis*. It has been suggested that there is a correlation between the occurrence of TB in swine and a direct or indirect contact of swine with tuberculous humans, cattle or birds. The presence of TB in swine in virtually all countries where swine are farmed has long been reported. *M. avium* infection in swine causes lesions indistinguishable from those caused by *M. tuberculosis* in humans or *M. bovis* in cattle (Weeks, 1985; Maas *et al*., 2002; Tibbo *et al*., 2008; Meng *et al*., 2009; Arega *et al*., 2013; Romha *et al*., 2014). *M. avium* can affect all species of birds, swine and cattle. *M. tuberculosis* primarily affects humans but can also be transmitted to swine, cattle and dogs (Admassu *et al*., 2015).

It is important to note that tuberculous lesions, in swine due to *M. bovis*, have been reported in wild swine in several countries in Europe in recent years. In industrialized countries most tuberculous lesions in swine are caused by bacteria of the *M. avium* complex (MAC: *M. avium* subspecies hominisuis, *M. avium* subspecies avium and *M. intracellulare*). Lesions are most

often observed in lymph nodes associated with the gastrointestinal tract. However, it is important to emphasize that some swine may develop progressive disease involving the liver, spleen and other organs of the abdominal and thoracic cavities (Arega *et al*., 2013; Thoen, 2013).

The incidence of TB in swine depends mainly on its exposure to tuberculous products from cattle, birds or man. It was suggested that transmission of TB from birds to swine could occur on farms where the species are kept in close proximity. Nevertheless, the epidemiology of avian TB in swine was still thought to be in need of clarification (Windsor *et al*., 1984).

Tuberculosis in cattle is a chronic disease that usually takes many months to a year before the development of clinically evident infections. There is a high prevalence of latent and hidden cases as well. Most infected cattle only manifest the clinical signs at adult age. Cases of bTB are occurring in swine either as a direct contact with infected cattle on smaller mixed species farms; some cases, however, have occurred in independent outdoor swine herds possibly as a result of domestic and wildlife contamination of the environment. There is no compulsory slaughter of individual cattle/swine or cattle/swine herds infected with bTB. The presence of bTB and its growing threat to swine is a complex issue with key animal health strategy implications. Smaller farmers must be aware of the risks and consider the environmental risks to their swine caused by proximity to cattle (Meng *et al*., 2009; Mandal, 2013; White, 2016).

Research indicated bTB is endemic in Ethiopia. Prevalence is varying from 0.8-10% in extensive rural farming systems while higher prevalence has been reported in intensively managed dairy farms (Firdessa *et al*., 2012). The overall contribution of *M. bovis* to human TB in Ethiopia is minor but greater in specific areas. Monitoring of zoonotic transmission is needed in urban areas in Ethiopia with high rates of bTB associated with intensive farming of imported dairy cattle and among pastoralist populations from which human *M. bovis* cases were identified. Zoonotic transmission of *M. bovis* can be excluded as the predominant cause of the high national incidence of TBLN in Ethiopia. Mapping of disease by spoligotyping and mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) analysis showed an integrated distribution of the tuberculous lymphadenitis (TBLN) and pulmonary TB forms, which suggested

that cases of TBLN arise from within the pulmonary TB transmission network rather than from an external zoonotic source (Firdessa *et al*., 2013).

1. 1. Rationale

The occurrence of bTB in domestic animals together with companionship between livestock owners and their animals is a potential danger of transmission to humans given that *M. bovis* is inherently resistant to pyrazinamide which is one of the safest first line anti-TB drugs. It clearly shows the difficulty of treating human patients once infected with *M. bovis*. This problem is exacerbated by the absence of government policy to study and control bTB in Ethiopia.

Cattle and swine TB are transmissible to humans. Humans acquire bTB from cattle through ingestion of raw milk and beef as well as aerosolization by close contact and sharing night shelter. On the other hand, humans share TB from swine via aerosolization during close contact. The experience of mixed farming system of livestock in Ethiopia facilitates interspecies transmission of animal TB through sharing feed, water and shelter.

Researchers reported the endemic occurrence of bTB in dairy cattle in Debre Berhan milkshed (Kiros, 1998; Shimeles, 2008; Woldemaryiam *et al*., 2021) and swine in central Ethiopia (Shitaye *et al*., 2006; Arega *et al*., 2013). These reports are the only available information on TB in cattle and swine in the current study areas. Some of these studies involved small sample size and had limited geographical coverage to describe the status of bTB. Specifically, the studies so far carried out on bTB in dairy cattle did not cover most of the dairy farms included within Debre Berhan milkshed. On the other hand, the studies so far conducted on bTB in swine are abattoir based and did not use single intradermal comparative tuberculin (SICT) test as a screeing test to diagnose bTB. Hence, this study is the first of its kind in involving farmed swine and using SICT test as a screeing test. Cattle are the principal hosts and the best biological indicators of bTB which served as a control to study same in swine.

There are currently emerging dairy and swine farms in central Ethiopia because of the encouraging privatization policy of the government to satisfy the growing demand for milk and pork. The demand for milk is increasing from time to time due to establishment of new towns and increase in population size. In addition, the demand for pork by the Chinese people is also increasing as a result of the strong bond created between the government of Ethiopia and China. The number of foreign pork consumer people in general is increasing from time to time. In majority of the cases, however, the awareness level of farm owners towards the zoonotic transmission of bTB from animals to humans is generally low. Expansion of farms and low level of public awareness about the zoonotic transmission of animal TB to humans create favourable conditions for TB to occur in animals and be transmitted to humans. Therefore, the current status of bTB in cattle and swine, risk factors (at animal, herd and environment levels), levels of awareness of farm owners and farm biosecurity issues were not studied in detail in the current study areas. This study was thus initiated to elucidate the epidemiology and public health significance of bTB in dairy cattle and swine which would contribute its share towards the launching of practical and cost-effective control methods of same in Ethiopia.

1. 2. Research Questions

1. What is the prevalence of bTB in dairy cattle of Debre Berhan milkshed?
2. What is the prevalence of TB in swine in selected sites of central Ethiopia?
3. Which risk factors play important role to precipitate bTB in cattle and swine in central Ethiopia?
4. What is the species of TB cycling in swine and dairy farmers (in Debre Berhan milkshed) in central Ethiopia?
5. What is the awareness level of farm owners/workers on zoonotic importance of bTB in humans in central Ethiopia?

1. 3. Hypotheses

Ho1: The prevalence of bTB is high in dairy farms of Debre Berhan milkshed, central Ethiopia.
Ho2: The prevalence of TB in swine is low in central Ethiopia.
Ho3: There are important risk factors precipitating bTB in cattle and swine in central Ethiopia.
Ho4: MTBC are the major cause of swine and human TB in central Ethiopia.
Ho5: The awareness of farm owners about the transmission of TB from animals to humans is low.

1. 4. Objectives

1. 4. 1. General objective

●To investigate the epidemiology of TB in bovine and swine, and its zoonotic implication in farm owners/animal attendants in central Ethiopia.

1. 4. 2. Specific objectives

► To estimate the prevalence of bTB in dairy cattle of Debre Berhan milkshed,
►To estimate the prevalence of TB in swine at ATVET, Bishoftu and Akaki Kality, as well as investigate the gross and microscopic lesions of TB in swine,
►To assess important risk factors associated with the occurrence and transmission of TB in bovine and swine,
►To isolate and identify mycobacteria from swine tissues and human sputa using mycobacterial culturing and molecular typing, and
►To assess the knowledge and practices of farm owners and/or animal attendants on the zoonotic transmission potential of animal TB to humans.

2. LITERATURE REVIEW

2. 1. Tuberculosis

The name TB comes from the nodules called **'tubercles'** (or nodular granulomas) which are formed in the lymph nodes of affected animals (Verma *et al*., 2014). TB is a term that encompasses various diseases caused by bacteria of the MTBC and other mycobacterial species such as MAC (Kaneene and Thoen, 2004; Sakamoto, 2012).

Tuberculosis is a complex and multi-species disease which can be of three types: human, bovine and avian TB. Gross lesions of TB were most often in the lungs which have a predilection for the lung tissues rich in oxygen supply (Maas *et al*., 2002; Merck, 2006; Malama *et al*., 2013; Ejeh *et al*., 2014).

The World Organisation for Animal Health (OIE) classified bTB as a List B disease, meaning those transmissible diseases with socioeconomic and/or public health implications that are significant to be controlled (Cosivi *et al*. 1998). *M. bovis* is a zoonotic organism and should be treated as a risk/hazard group III organism with appropriate precautions to prevent the occurrence of human infection (OIE, 2018). Infection with *M. bovis* has been reported in 69% of the tropical countries around the world and in 80% of African countries. The potential for causing widespread dissemination of infection and development of clinical disease is probably greater in intensively managed animals and multi-species systems with high population densities than in natural free-ranging ecosystems ((Dibaba *et al*., 2019).

Theoretically swine could form a huge reservoir for *M. bovis* but fortunately they are slaughtered before they reach the infectious stage. The marked decrease in *M. bovis* infections in cattle is being accompanied by a similar decrease in infections in swine. Mycobacteria were isolated from 566 swine in Germany in 1971-75 and *M. bovis* infection was found in only 1% and not a single

case of *M. tuberculosis* infection. In 1961-70, the situation had been quite different in that 4.3% of *M. bovis* has been found. The Veterinary Service Laboratories in Ames, Iowa, USA, examined tissues from tuberculous swine collected between 1971 and 1974. Of the 1,591 isolations, 15 strains (1%) were *M. bovis* and none was *M. tuberculosis*. In general, mammalian tubercle bacilli are tending to disappear from swine, but lesions caused by other mycobacteria still occur, and cannot be differentiated at meat inspection or by histology. If either *M. bovis* or *M. tuberculosis* is found, an attempt must be made to trace its origin (Kleeberg, 1984). Increasing isolates of *M. tuberculosis* in livestock raises the question if they may become a reservoir of human TB (Cosivi *et al*., 1998).

Swine TB due to *M. avium* is minimally contagious in swine herds but it has great economic impact because meat from a large number of slaughtered swine of American farmers and meat packing industry cannot be sold as roasts, chops or hams. Moreover, the spread of *M. avium* by contact and the danger of transmitting the disease to the offspring are minimal too. Instead many carcasses must be cooked prior to processing or they may even be condemned. The swine are healthy and show no clinical signs of disease. The meat inspector finds the lesions when the animals are slaughtered (Weeks, 1985).

2. 2. Aetiology

The taxonomic classification of mycobacteria is described as follows (Radostits *et al*., 2007; Anon, 2009; Ejeh *et al*., 2014):

Kingdom: Bacteria

Phylum: Actinobacteria

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

Species: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, etc.

Mycobacteria share common characteristics with bacteria such as *Corynebacterium*, *Nocardia*, and *Rhodococcus*. These bacteria also express unique mycolic acids in their cell envelope that play a critical role in the structure and function of the cell wall. Their cell wall is rich in lipids called mycolic acid that provides them the thick waxy coat. The waxy cell wall confers many of the unique characteristics of *Mycobacterium*: acid-fastness, extreme hydrophobicity, greatly contributing for the bacteria resistance to many disinfectants, common laboratory stains, antibiotics and physical injuries, drying, acidity/alkalinity as well as distinctive immunostimulatory properties. The mycolic acid probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients. Mycobacteria of the MTBC are aerobic, non-motile, non-capsular, non-sporulating, weakly Gram positive, slow-growing and non-photochromogenic acid-fast bacilli that appear microscopically as straight or slightly curved rods, 1 to 4µm in length and 0.3 to 0.6µm wide. Mycobacteria are facultative intracellular bacteria that multiply within phagocytic cells particularly macrophages and monocytes. They have a slow generation time of about 15-20hours. Isolation of the bacteria can require 3 to 8 weeks of incubation because they are slow growing. Results of experimental studies indicated that the strain, dose, prevailing conditions for growth of the organism and route of inoculation may influence the time required to produce disease (Kaneene and Thoen, 2004; Strain *et al*., 2011; Sakamoto, 2012; Birhanu *et al*., 2015).

M. tuberculosis, M. bovis, M. bovis BCG, *M. canettii, M. africanum, M. pinnipedii, M. microti, M. caprae*, the *dassie* and the *oryx* bacillus and the recently discovered *M. mungi* are closely related species that form the MTBC pathogenic subgroup. These are generally regarded as host adapted but they have the ability to spillover into other species. *M. bovis* is the main aetiological agent /primary cause/ of bTB and appears to be strongly cattle adapted but is able to infect humans. Cattle are the primary hosts for *M. bovis* (Anon, 2009). However, it does not appear to easily be transmitted between people. *M. tuberculosis* and *M. bovis* are the most important species in MTBC which commonly cause human and animal TB, respectively with concomitant negative consequences for human and animal health and economic costs (Strain *et al*., 2011; Katale *et al*., 2012; Vordermeier *et al*., 2012; Rekha *et al*., 2015). A new species, *M. bovis* subspecies caprae previously classified as *M. tuberculosis* subspecies caprae has been identified

as a cause of infection in goats and humans in Spain and goats, cattle, deer and swine in Europe (Radostits *et al*., 2007).

Detailed phylogeographic and statistical analysis was performed on African diversity of *M. bovis*. For that, 1504 genotypes using 48 markers (43 spoligotype spacers and 5 VNTRs; 43 biallelic and 5 multiallelic) to which 45 new genotypes were added from Mozambique. Ancient lineages are mostly from Eastern Africa (Ethiopia, Tanzania) and Northern Africa (Algeria, Tunisia) although some haplotypes with minor frequencies from Central Africa are also present. On an overview analysis of the network, Eastern and Northern Africa seem to display a deeper ancestry of *M. bovis* compared with other African regions. Most of the *M. bovis* genotypes radiate into several clades from a single major coalescence point (Figure 1) (Inlamea *et al*., 2020).

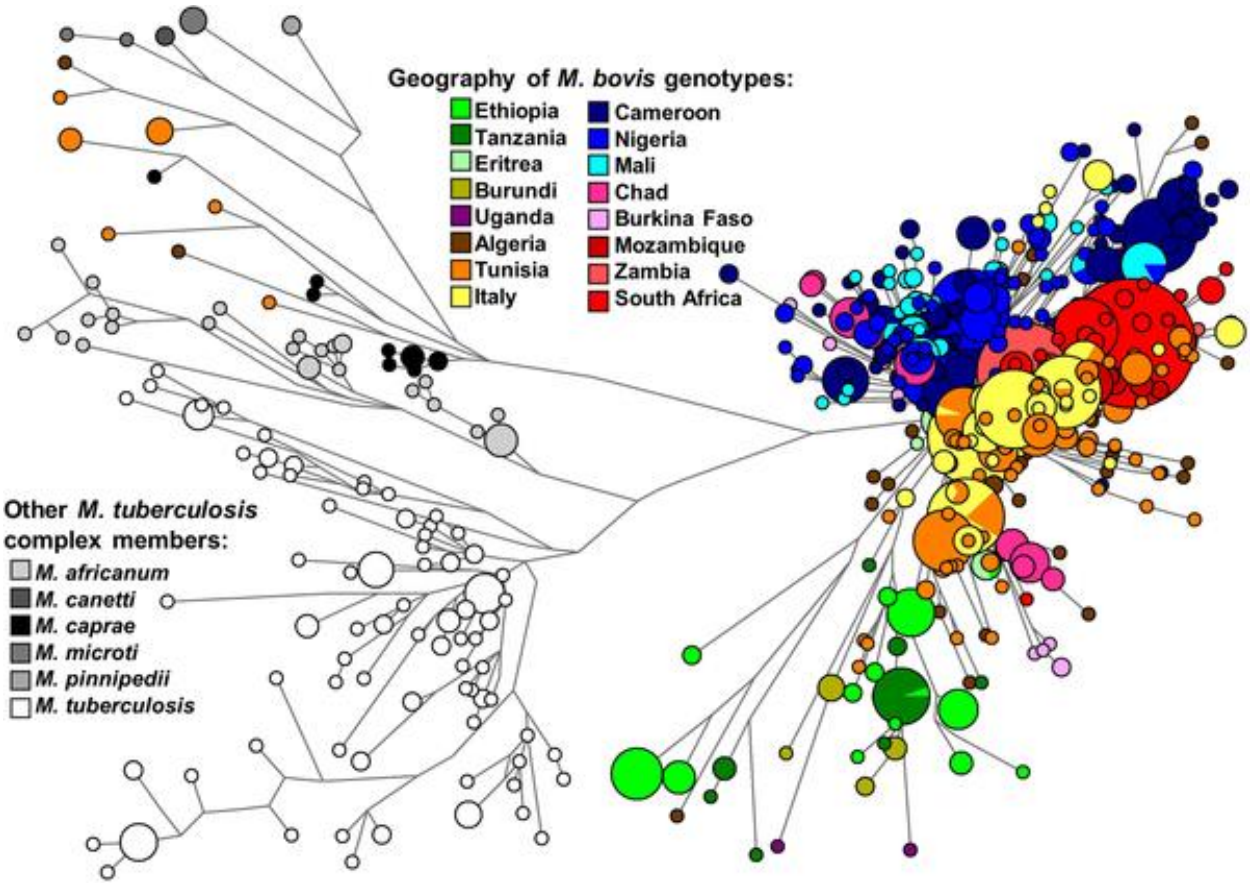

Figure 1. Phylogenetic tree of MTBC in Africa (Inlamea *et al*., 2020)

M. bovis is the most universal pathogen among mycobacteria and affects many vertebrate animals (Anon, 2009). All species and age groups including humans are susceptible to *M. bovis*. Cattle, goats and swine are the most susceptible whereas sheep and horses are showing a high natural resistance (Radostits *et al*., 2007). Moreover, it is one of the causative agents of human extrapulmonary tuberculosis (Katale *et al*., 2012).

Infection of animals by *M. tuberculosis* has been reported but again it is generally accepted that this represents spillover of infection from humans to animals and that animal populations cannot sustain *M. tuberculosis*. This apparent attenuation of *M. tuberculosis* in animal hosts is all the more intriguing given that genome studies have shown us that the *M. bovis* genome is merely a reduced version of the *M. tuberculosis* genome; hence, *M. bovis* does not have any **"virulence"** loci for animals *per se* that have been lost in *M. tuberculosis*. Instead it appears likely that differential expression of a range of genes between *M. tuberculosis* and *M. bovis* explains their specific host predilections (Whelan *et al*., 2010).

Swine are natural hosts for mycobacterial infections including those due to *M. bovis* (Weeks, 1985; Bolin *et al*., 1997). Swine TB is caused by *M. tuberculosis*, *M. bovis* and *M. avium*. It causes tubercles in various organs, pneumonia, enteritis and hepatopathy. Moreover, it causes clinical disease in both European and African suids (Fowler, 1996). The most common cause of swine TB is *M. avium* serotype 4 but infection with mammalian tubercle bacilli including *M. tuberculosis*, *M. bovis* and *M. africanum* occur coincident with infections of cattle, wildlife and human beings with these agents. Mycobacterial agents are present in the environment and there are many serotypes of *M. avium*. The organisms are very resistant to temperature changes and can survive a long time in soil, water and bedding (Weeks, 1985; Bolin *et al*., 1997).

Mycobacteriosis (NTM: non-tuberculous mycobacteria; environmental mycobacteria; atypical mycobacterial; MOTT: mycobacteria other than tuberculosis) is associated with members of the *M. avium*-intracellulare complex which includes *M. avium* subsp. avium, *M. avium* subsp. paratuberculosis, *M. avium* subsp. sylvaticus and *M. intracellulare* and occasionally to other less well-defined mycobacterial species such as *M. kansasii*, *M. chelonae*, *M. fortuitum*, *M. aquae*, *M.*

cookii and *M. scrofulaceum*. Accuracy in the identification of these organisms has been enhanced by the use of modem molecular techniques (Radostits *et al*., 2007; Mamo, 2014). Some of the NTM isolated and characterized in pastured cattle in Ethiopia were *M. avium* subspecies tropicalis, *M. intracellulare, M. gordonae, M. arupense, M. holsaticum, M. acapulcensis, M. colombiense, M. engbaekii, M. monacense, M. mucogenicum, M. nonchromogenicum, M. peregrinum, M. vaccae* and *M. fortuitum*. These subspecies have been isolated from typical granulomatous TBLs in different tissues including lung, thoracic lymph nodes (broncheal and mediastinal lymph nodes), retropharyngeal lymph nodes, mesenteric lymph nodes and hepatic lymph nodes. However, the pathogenesis of NTM subspecies and their significance in the epidemiology of animal TB infection is poorly understood (Mamo, 2014).

M. avium serotypes 1, 2, 4, 5 and 8 were isolated from tissues of affected swine. These serotypes are responsible for approximately 85% of the mycobacterial infections found in swine of the United States. The *M. avium*-intracellulare complex consists of 28 serovars and two species. Currently, serotypes 1-6, 8-11 and 21 are *M. avium* and serotypes 7, 12-20, and 25 are *M. intracellulare*. The remaining serotypes do not fit with either species. The classical *M. avium* serovars 1-3 are the cause of TB in domestic and wild birds. Serovars 1, 2, 4-6, 8-10 and 21 appear to be the most common in infections of domestic livestock. Individual swine may be infected with more than one serovars. Recently, it is proposed to restrict the designation of *M. avium* subsp. avium to the "**bird-type**" isolate (serotypes 1-3) and to use the designation *M. avium* subsp. hominissuis for serotypes 4--6, 8-11 and 21 (Songer *et al*., 1980; Radostits *et al*., 2007).

The *M. avium*-intracellulare complex comprises ubiquitous opportunistic pathogens of a large range of species in livestock. They have got most importance in swine. TB associated with these organisms in livestock is not usually manifested clinically and is not a major disease problem but infected animals react to the mammalian tuberculin test are creating difficulty in *M. bovis* TB eradication programmes. Outbreaks in swine herds can cause significant losses because of carcass condemnation. In swine, a significant proportion of reactors to tuberculin are due to

infection with organisms of this complex and infected cattle and swine are potential sources of infection for the increasing number of MAC infections in humans (Radostits *et al*., 2007).

2. 3. Epidemiology

Bovine TB is found throughout the world, widespread in cattle around the world, present in many wildlife species and domestic animals. This has serious implications for international trade and export of animals and animal products including beef (Le Roex *et al*., 2013).

Improved surveillance and accurate reporting by a country's Veterinary Services contribute to the prevention and control of bTB at the animal source. Forty-four percent of countries reported bTB via the OIE World Animal Health Information System (WAHIS) between January 2017 and June 2018. Only a quarter of the affected countries were applying all the relevant control measures. Of the 188 countries and territories reporting their bTB situation to the OIE, 82 countries (44%) were affected with a widespread distribution of the disease (Figure 2). Among the 82 affected countries, 29 (35.4%) countries reported the presence of bTB in both livestock and wildlife. In addition, two (2.4%) countries reported bTB presence in only wildlife compared to 52 (62.2%) which indicated the infection in only livestock. Moreover, among these 82 affected countries, 66 (80.5%) provided quantitative data for outbreaks via WAHIS, demonstrating relatively good reporting of the global situation of this disease. The persistence of infection in wildlife poses challenges for disease control in some countries due to the potentially significant impact of wildlife as reservoirs and spillover hosts to domestic animals (OIE, 2019).

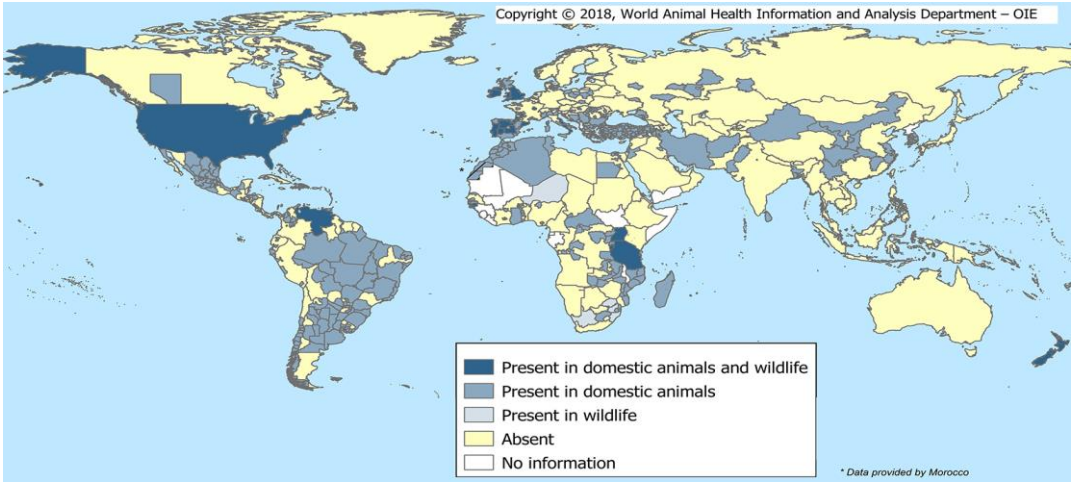

Figure 2. Global epidemiology of bTB and its control (OIE, 2019)

Note: Ethiopia is referred by absent because there is no government launched programme to study the epidemiology of bTB and to institute the appropriate control measures at a national level. The efforts so far exerted are either from the sides of few professionals or institutions or both in synergy (Personal view).

Bovine TB is more prevalent in Africa, parts of Asia and Latin America. Sporadic (particularly 11% in Africa) and endemic occurrences of bTB have been reported in developing countries such as in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries. In developed countries that have had rigorous bTB control programmes in place for many years, TB in animals is now a rarity with occasional severe outbreaks occurring in a small group of herds. The prevalence of the disease is high in the tropical and sub-tropical countries and is usually signaled by detection in carcasses at abattoirs (Radostits *et al*., 2007; Admasu *et al*., 2014; Verma *et al*., 2014). *M. bovis* infection occurs more frequently in older cattle than yearlings and calves. Larger herds of cattle had a higher rate of bTB (Katale *et al*., 2012).

The origin of bTB in Africa was believed, until recently, to be associated with the importation of infected cattle mainly from Europe and other continents essentially during the past three centuries (Malama *et al*., 2013; Wichatitsky *et al*., 2013; Caron *et al*., 2014). New studies have revealed the existence of at least three clonal complexes of *M. bovis* which each appears to occur predominantly or exclusively in a geographically localized region of the world. The presence of *M. bovis* strains belonging to the European 1 clonal complex in South Africa, Tanzania and

Zambia may therefore indeed be explained by historical livestock trade links between the UK and these African countries. Two additional clonal complexes, African 1 and African 2, have been detected in several countries in Westcentral and East Africa, respectively but very rarely outside Africa. The origin of these two *M. bovis* complexes is unknown and there is a possibility that their progenitors evolved in cattle in Africa any time between the appearance of classic *M. bovis* over 2000 years ago and colonial times. Given the exceptional diversity of African wild mammal species, especially ruminants, presumed to be immunologically naive to the infection. The disease might spillover to wild African species especially those that are taxonomically related to the domestic bovid reservoir host (Wichatitsky *et al*., 2013).

Detection of contact-neighbour herds where bTB infection could have been expanded from the farm of origin is possible by employing epidemiologic investigation systems (De Kantor *et al*., 2008). Many factors could contribute to *M. bovis* hotspot foci including the presence of mycobacteria in the environment, intensive management practices and overcrowding at watering points, in communal grazing areas and auction markets. The prevalence of *M. bovis* in cattle has been reported to be higher in intensive system than in pastoral production system. The similarity of *M. bovis* isolates from different geographical locations was attributed to free movement of cattle, sale and a decline in the provision of public veterinary services. These all impeded bTB control programmes that might contribute to its spread. Flooding has also been suggested as a propagating factor of *M. bovis* in the environment (Katale *et al*., 2012).

There is a risk of bTB spillover from wildlife to livestock and vice versa although the environmental pathways and frequency need to be determined. Infection of wild animals by bTB caused by *M. bovis* is raising concern worldwide. The first reported diagnosis of *M. bovis* infection in African free-ranging wild mammals was during the 1920s followed by the confirmation of the African buffalo (*Syncerus caffer*) as a maintenance host during the late 1990s. But it is unclear when African wildlife first became exposed to the pathogen. So far, more than 60 wild mammal species worldwide have been shown to be infected with *M. bovis* although only a few have been demonstrated to play the role of maintenance hosts (Anon, 2009; Malama *et al*., 2013; Wichatitsky *et al*., 2013; Caron *et al*., 2014).

The epidemiology of bTB is influenced by many risk factors such as genetic, behavioural, biological or environmental which have effects on transmission, establishment of infection and expression of disease. The incidence of bTB in cattle progeny is also affected by hereditary and maternal influences. Factors associated with the occurrence of animal TB are sex, breed and management (Verma *et al*., 2014).

M. bovis and *M. avium* readily affect wild swine. Warthogs were involved in the epidemiology of TB in Africa, Cape buffalo and domestic cattle in Uganda. Diseases caused by these organisms may be transmitted to domestic livestock and this may complicate government disease eradication programmes (Fowler, 1996).

The evidence in the East African region generally suggests low prevalence of bTB in both domestic and wild animals with notable exceptions in intensive dairy husbandry systems in some countries such as Ethiopia, Uganda and Tanzania as well as in pastoral system in Uganda. The spillover of bTB into humans in the region is poorly documented except Ugandan studies reported an average of ~3% of human TB infection. In Ethiopia, despite the very high prevalence of extrapulmonary TB (EPTB) in humans which is suggestive of bTB infection, *M. bovis* was only isolated in 4 out of 964 EPTB patients (Firdessa *et al*., 2013). Three out of 173 pulmonary TB patients were *M. bovis* positive and none of the suspected lymphadenitis TB cases were positive in another study among pastoralists in close physical contact with livestock in Southeastern Ethiopia. Bovine TB infection of buffalo and baboons has also been confirmed in Kenya and several wildlife species have been found positive to serological rapid tests in Ethiopia (Tschopp *et al*., 2010; Wichatitsky *et al*., 2013; Caron *et al*., 2014).

Bovine TB infections may be maintained, independently or not, within livestock and wildlife populations whereas human infections result from pathogen spillover from animals and very rarely from human-to-human transmission (Wichatitsky *et al*., 2013; Caron *et al*., 2014). Most of the studies of bTB in Ethiopia were conducted in Addis Ababa, Amhara, Oromia and Southern regions while no valid published study was obtained from Benishangul-Gumuz, Harari and Dire Dawa regions. On the other hand, few studies were undertaken in Afar, Gambella, Somali and

Tigray regions. Variable animal level prevalence of bTB was recorded in the districts of the regions ranging from 0.8-54.6%. The highest prevalence was being reported in intensive farms in and around cities while the lowest prevalence was being recorded in grazing animals in rural areas (Figure 3) (Sibhat *et al*., 2017).

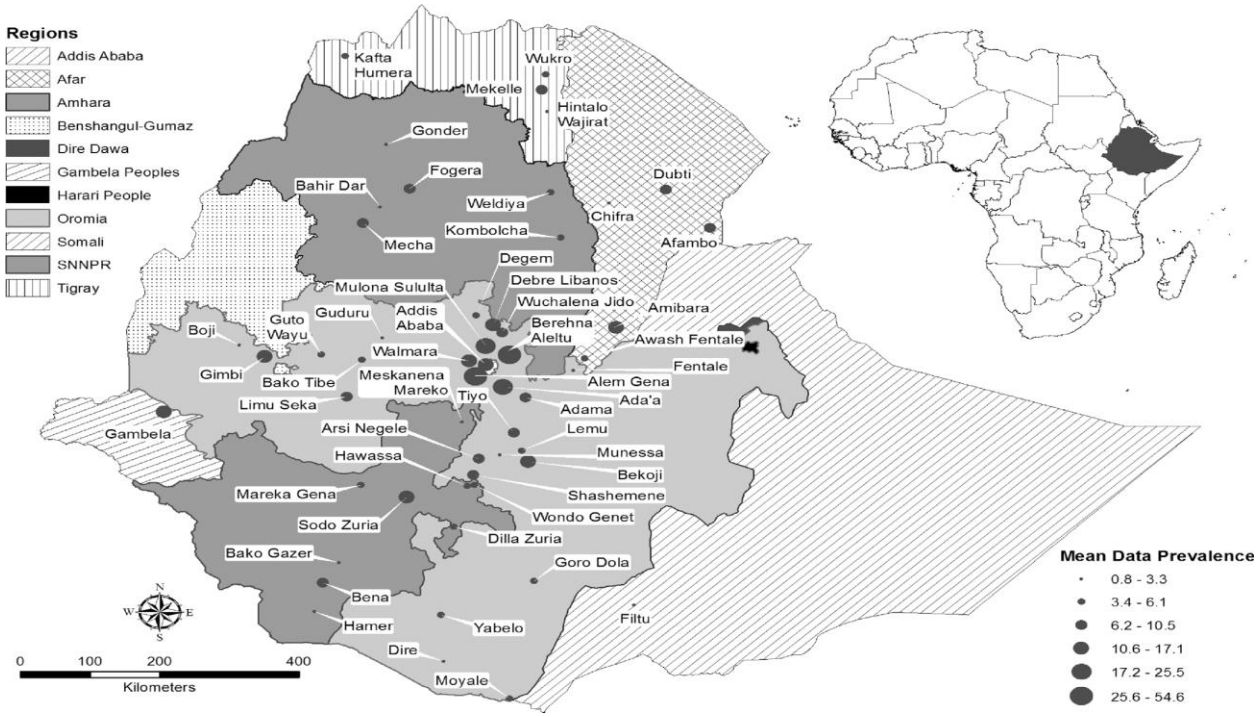

Figure 3. Epidemiology of bTB in Ethiopia (Sibhat *et al*., 2017)

2. 3. 1. Occurrences

Bovine TB has a global distribution that occurs in every country of the world (Dibaba *et al*., 2019) and is of major importance in dairy cattle. It is under strict control in most developed countries but is still a major cause of loss in many developing countries (Tschopp *et al*., 2011). Lymphadenitis in swine associated with tubercle bacilli organisms is reported from all continents (Radostits *et al*., 2007).

The species of animals reported to be spillover hosts include sheep, goats, horses, swine, dogs, cats, ferrets, camels, llamas, many species of wild ruminants including deer and elk; elephants,

rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warthogs, large cats (including lions, tigers, leopards, cheetahs and lynx) and several species of rodents. Most mammals may be susceptible. Little is known about the susceptibility of birds to *M. bovis* although they are generally thought to be resistant (Anon, 2009).

Bovine TB is still widespread in Africa, parts of Asia and some Middle Eastern countries (Anon, 2009). Africa is assumed to bear the highest consequences of zoonotic TB worldwide because of the frequent and concurrent presence of multiple risk factors (Muller *et al*., 2013). Bovine TB in animals has been reported from 33 of 43 African countries. Human bTB cases have been described in some Sahelian countries like Ghana, Niger, Uganda, and Tanzania and in immigrants from Chad. The representative proportion of bTB in human TB is estimated at less than 5% worldwide (Gumi *et al*., 2012; Caron *et al*., 2014).

Several studies conducted since 2006 have confirmed that bTB is endemic in cattle of Ethiopia and prevalence varies from 3.5-50% depending on the geographical locations, breeds and husbandry practices (Gumi *et al*., 2012; Caron *et al*., 2014). Moreover, the prevalence varies from 0.8% to around 10% in extensive rural farming systems while higher prevalences have been reported from regions in Ethiopia where intensive husbandry systems are more common (Firdessa *et al*., 2012). Furthermore, bTB is endemic in the cattle population of the Ethiopian highlands but the prevalence varies by areas depending on the prevailing breeds (exotic *taurin* breeds versus local *Zebu* breeds) and farming practices. High prevalence (7.9- 78.7%) was found in periurban and/or urban areas which are characterized by high numbers of dairy farms, exotic breeds and their crosses kept under intensive or semi-intensive husbandry systems. In contrast, low bTB prevalences (0–2.4%) were found in cattle among mixed farming smallholders in rural areas in the highlands where they keep Zebu cattle in smaller numbers under traditional management system (Tschopp *et al*., 2010; Caron *et al*., 2014). The geographical distributions of *Mycobacterium* isolates are depicted by (Figure 4) (Berg *et al*., 2009) and *Mycobacterium* strains are depicted by (Figure 5) (Conceicao *et al*., 2020).

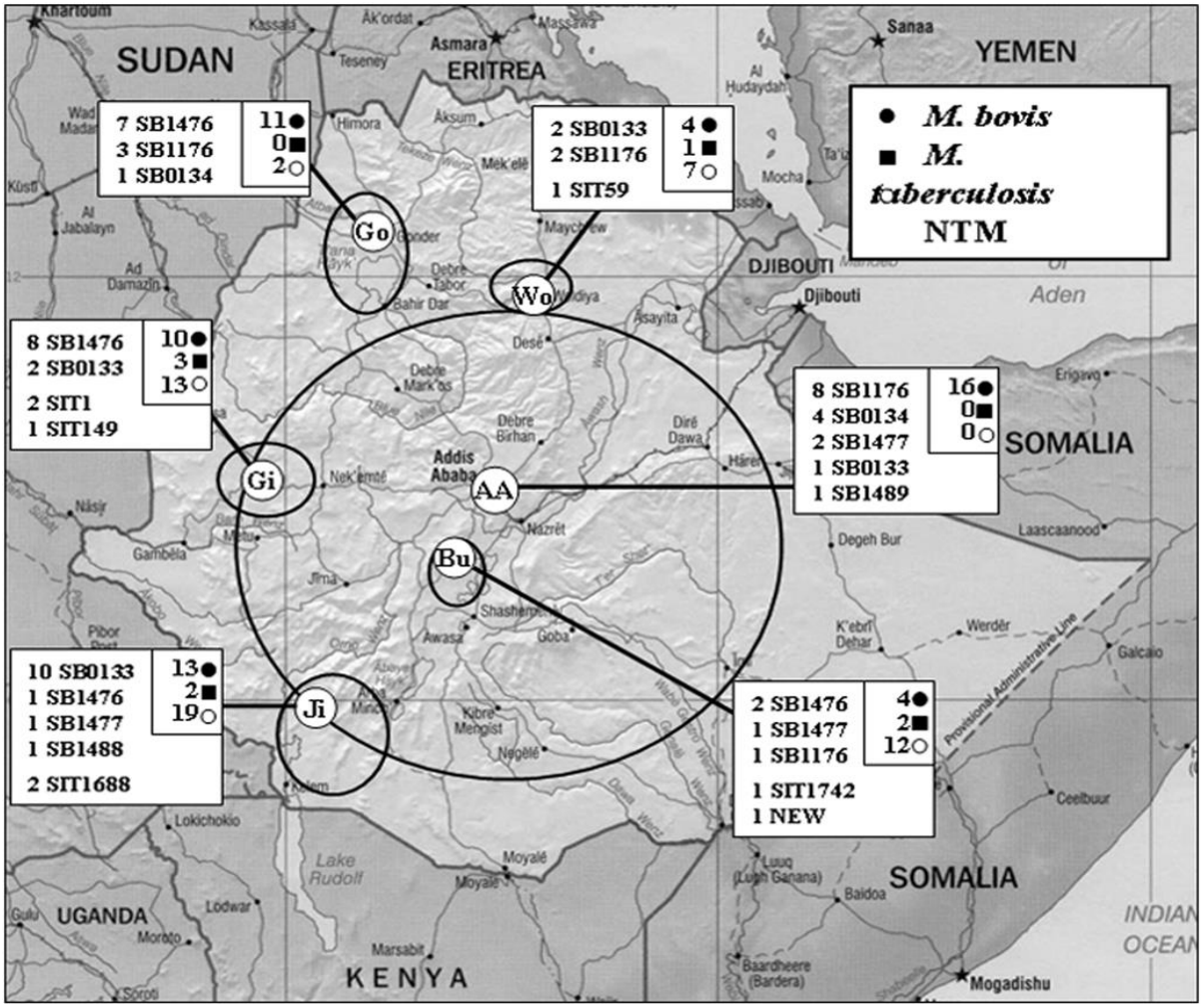

Figure 4. Geographical distribution of *Mycobacterium* isolates from cattle slaughtered in Ethiopian local abattoirs. The isolates from respective abattoirs are indicated in respective box and the characterized spoligotype patterns are also indicated by number. Approximate area coverage for each abattoir is shown by a solid circle. doi:10.1371/journal.pone.0005068.g001 (Berg *et al*., 2009)

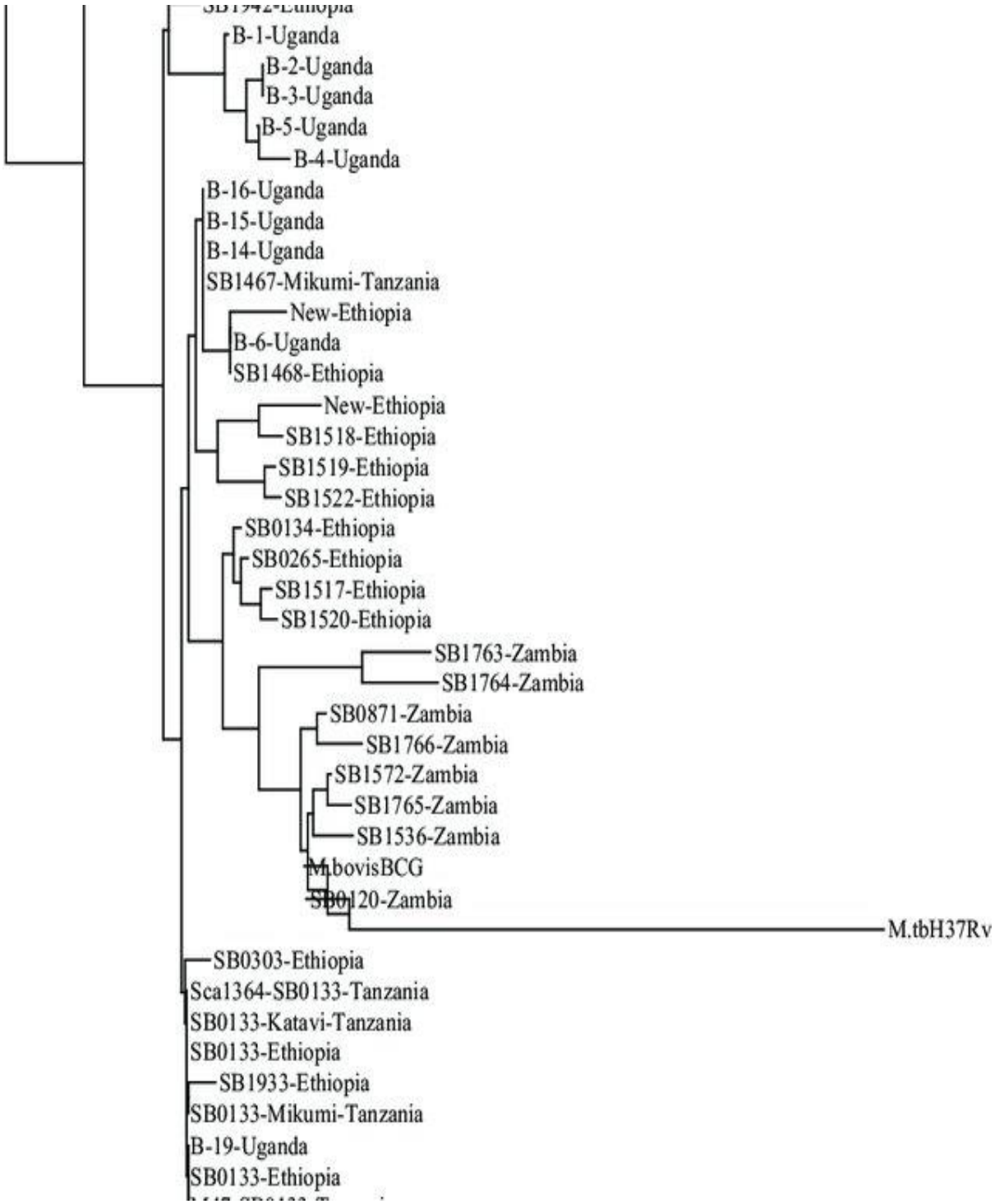

Figure 5. Spacer oligotyping and phylogenomic perspectives of *M. bovis* strains (Conceicao *et al*., 2020)

2. 3. 2. Sources of infection

***Cattle*:** Infected cattle are the main sources of infection for other cattle, other animals and human beings. *M. bovis* was isolated from tuberculin skin tested positive cows' milk (4/30: 13.3%) based on conventional biochemical characterization (Ameni *et al*., 2001). The isolation of *M. bovis* from milk suggests a risk of infection of humans through consumption of raw milk (Tschopp *et al*., 2011). Calves may be born with TB from infected dams. Animals with gross lesions that communicate with airways, on skin or intestinal lumen are obvious disseminators of infection. Cattle in the early stages of the disease, before any lesions are visible, may also excrete viable mycobacteria in nasal and tracheal mucus. Moreover, TB may remain **''silent''** for years. Unfortunately, infected animals that appear healthy may be capable of transmitting infection (Maas *et al*., 2002; Radostits *et al*., 2007). *M. bovis* (spoligotypes SB0133 and SB0303) was isolated from human sputa collected from pastoralists in Southeastern Ethiopia (Gumi *et al*., 2012). Cattle serve as sources of infection to swine (Arega *et al*., 2013).

The *M. bovis* isolates from human pulmonary TB patients matched with both the dominant spoligotype of the animal isolates in Southeastern Ethiopia (SB0133) and with SB0303 which has been isolated from cattle in central Ethiopia as well as in other countries of East Africa indicated cattle-to-human transmission. The most common spoligotype pattern among the animal isolates was SB0133. Previously this spoligotype was reported as the second most dominant strain in Ethiopian cattle and it is a common type in East Africa (Biffa *et al*., 2010; Berg *et al*. 2011). All *M. bovis* isolates (4 in number) from humans showed typical bovine spoligotype profiles (Firdessa *et al*., 2013).

Reports of person-to-person spread of *M. bovis* infection are rare strengthening the belief that *M.bovis* is not as infective for humans as *M. tuberculosis* (Admassu *et al*., 2015).

***Human*:** The isolation of *M. tuberculosis* by biochemical characterization from cows' milk with tuberculous mastitis, which is of rare occurrence, indicated a possible reciprocal transmission between cattle and man. Several other workers have similarly isolated *M. tuberculosis* from milk (Elias *et al*., 2008). Berg and others (2009) isolated *M. tuberculosis* by molecular techniques

from tuberulous tissue (2/18: 11%) collected from cattle slaughtered at Butajira abattoir. *M. tuberculosis* (SIT149) was isolated for the first time in Afar by molecular techniques from tuberculous tissue collected from tuberculin reactor goat suggesting the possibility of its transmission from human to goat (Mamo *et al*., 2012). Similar single strain of *M. tuberculosis* (SIT 149 of the E-A lineage) which is dominant in Ethiopia has been isolated in a camel with disseminated TB lesions from pastoral region in Southeastern Ethiopia. This is the first known report of *M. tuberculosis* from a camel in Ethiopia indicating the likelihood of human-to-camel transmission. The authors considered it as a rare event (Gumi *et al*., 2012). Isolation of *M. tuberculosis* from gross TBLs was frequently reported in Ethiopian cattle (Berg *et al*., 2009; Ameni *et al*., 2010).

Different studies have reported the isolation of *M. tuberculosis* from domestic and wildlife animals. Recent studies in Ethiopia have reported transmission of *M. tuberculosis* from humans to cattle. Molecular characterization of isolates from five cattle in Sellale, central Ethiopia revealed the presence of *M. tuberculosis* strains (SIT149 and SIT53). These cattle were tuberculin reactors and with visible lesions of milder severity. These two strains were the most commonly isolated ones from farmers who possess cattle. Cattle are likely to be exposed primarily through inhalation of droplets of cough from active pulmonary TB cases of farmers and less often by ingestion of pasture contaminated with expelled bacilli in sputum, urine and faeces from infected farmers but the exposure may not lead to disease. Similarly, the traditional animal husbandry practice of chewing tobacco and spitting its juice into the oral cavity of cattle could also be considered as a means of transmission of *M. tuberculosis* from farmers to their cattle. Chewing tobacco is widely practiced in Sellale and spitting its juice into the oral cavity of cattle is usually done with the assumption of deworming against many gastrointestinal parasites. It was learnt that the local custom of spitting chewed tobacco or tobacco juice into the mouths of cattle has animal husbandry significance. According to the respondents, animals fed on tobacco had good body condition and in a better health as compared to animals that did not feed on tobacco. As a result, this custom has widely been accepted and practiced by the community in Sellale. Both men and women chew tobacco for this purpose (Ameni *et al*., 2013).

An increasing number of *M. tuberculosis* was isolated from cattle in recent years (Radostits *et al*., 2007) and *M. tuberculosis* seems to be more frequently transmitted in Ethiopia from humans to livestock than *M. bovis* from cattle to humans (Gumi *et al*, 2012). Unfortunately, infected people that appear healthy may be capable of transmitting infection. *M. tuberculosis* is occasionally isolated from cattle or swine with tuberculous lesions but in rare occasions. Swine may develop minor lesions in their lymph nodes. *M. tuberculosis* infections in swine are usually the result of feeding offal from a tubercular household or contact with a tuberculous attendant (Maas *et al*., 2002; Radostits *et al*., 2007). In humans, only 10% of people infected with *M. tuberculosis* will develop TB disease in their lifetimes (Admassu *et al*., 2015).

***Wildlife Reservoirs*:** A large number of wildlife/feral species are naturally infected with *M. bovis*. Certain wildlife species appear to be significant maintenance hosts and reservoirs for infection in cattle in some areas of the world while most wildlife animals are unimportant as sources for infection to cattle. These reservoirs escape traditional test and slaughter control programmes and result in regions where the disease remains endemic in cattle herds. Different reservoirs that serve as maintenance and spillover hosts in different countries of the world are indicated hereunder (Radostits *et al*., 2007):

►The epidemiology of bTB in cattle and infection of cattle is believed to be from infected badgers' (*Meles meles*) urine and faecal contamination of pastures in Southwest England and Ireland.

►Spread infection to cattle through comingling or sharing of winter feed resulting in foci of herd infections by infected red deer in Great Britain and Ireland.

►Infection to cattle is believed to occur when there is cattle-possum contact on the pasture-bush margin and curious cattle sniff moribund brush-tail possums (*Trichosurus vulpecula*) in New Zealand.

►Mule deer (*Odocoileus hemionus*), white tailed deer (*0. virginianus*), elk (*Cervus elaphus canadensis*) and bison (*Bison bison*) can act as maintenance hosts in North America.

►Buffaloes (*Syncerus caffer*) can act as maintenance hosts in South Africa.

►Water buffaloes (*Bulbalis bulbalis*) can act as maintenance hosts in Australia.

***Birds and Environment*:** Tuberculous birds may continue to be sources of infection for swine although other environmental sources may be more significant. Improper handling of bird wastes

fed to swine also may allow transmission of the disease. Soil and water are other possible reservoirs of infection for swine. Pathogenic mycobacteria may survive for more than 4 years in soil and litter contaminated by birds with TB. Studies have shown that MAC often found in samples of sawdust and wood shavings used for swine bedding are sources of *M. avium* subspecies hominisuis in swine. The mycobacteria may multiply under proper conditions of moisture and temperature which could explain the seasonal occurrence of TB in some herds. The presence of lesions in the intestinal wall with subsequent swine-to-swine transmission probably is due to shedding of mycobacteria in the faeces. Granulomatous lesions of lungs, mammary glands and uterus may also occur with the potential for transmission of organisms from these sites. Thus, the addition of infected breeding stock could introduce the disease within a herd and transmission from infected sows to their litters may maintain the disease within a herd (Thoen, 2013).

Organisms of the *M. avium*-intracellulare complex are ubiquitous in nature and cosmopolitan in free-ranging, captive and domestic birds. It can be isolated from soil, plants, water, animal feed and animal bedding. Avian TB likely exists in free-ranging wild birds wherever there are major bird concentrations. Infected birds nesting in animal or feed buildings are the most common sources of *M. avium* subspecies avium of serotypes 1-3 which can contaminate feed and water supplies. In contrast, isolates of *M. avium* subspecies horninissuis (serotypes 4-6, 8-11 and 21) are commonly isolated from the environment and can be isolated from various species of **flies** and **beetles** that inhabit the ground, bedding and feed in farm environments. The organisms are resistant to survive in acidic and humid environments of peat bogs as well as decomposed faeces. The lipopolysaccharide bacterial wall promotes survival in environments inside and outside barns for extended periods of time. Seasonal trends of TB in wild birds have not been documented. The chronic nature of this disease guarantees its presence year-round for both wild and captive birds. The location of primary lesions is an indication of the route of exposure. Intestinal lesions suggest ingestion of *M. avium* in contaminated feed or water. Lesions in the lungs and other areas of the respiratory tract suggest inhalation as the route of exposure (Radostits *et al*., 2007).

2. 4. Methods of Transmission

Transmission involved a general principle that has been proposed to be approximately 20% of the host population contributes 80% of the net transmission potential of a very wide varieties of diseases (the **''20/80''** rule), i.e. a small proportion of a population having a disproportionate effect on the overall transmission of disease (Strain *et al*., 2011). In addition to a broad host range, *Mycobacterium* also occurs in numerous biotopes including water, soil, protozoa, aerosols and fresh tropical vegetation (Verma *et al*., 2014).

Transmission of an infection in air droplets is direct transmission. Direct contact with infected animals (aerosol inhalation) or contact with infected animal excreta can spread TB. When milk is the main/ideal vehicle then transmission is said to be indirect (Kleeberg, 1984). The importance of these routes varies between species (Anon, 2009; OIE, 2009; Malama *et al*., 2013; Verma *et al*., 2014; Youssef and Ahmed, 2014). Inhalation /aerosolization/ is the almost invariable portal of entry of *M. bovis* in housed cattle with close contact and it is considered to be the principal mode of transmission even in those at pasture (Radostits *et al*., 2007). Transmission by air droplets is however more frequent than was previously thought. When *M. tuberculosis* infection decreases while cattle TB is still present, most pulmonary TB cases may be due to *M. bovis* (Kleeberg, 1984).

Tubercle bacilli of *M. bovis* are excreted in the exhaled air (respiratory discharges), sputum, faeces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk from infected mammary glands, urine, semen, vaginal and uterine discharges (lochia in cows with tuberculous endometritis) and discharges from open peripheral lymph nodes. Natural transmission of *M. bovis* can occur between domestic and wild animals of the same or different species, from animals to humans and, more rarely, from humans to animals or between humans. *M. bovis* infection in cattle can be transmitted by a number of routes and transmission of infection is possible both in the field and indoors. The possible routes of infection include the respiratory, alimentary, percutaneous and the teat canal. However, the percutaneous and

transplacental routes of infection are unusual and intrauterine infection of calves occur only when bTB is common (Shimeles, 2008).

It is the route/mode of infection that determines the clinical signs of the disease. Adult cattle are usually infected by inhaling invisible infected droplet nuclei (containing the bacteria) into their lungs after expelled from the lungs of infected animals (Mandal, 2013). Calves are often infected by ingesting contaminated raw milk from infected mammary glands of cows. The ingestion of infected milk by young animals is a common method of transmission where the disease is endemic but mammary infection occurs late in the course of the disease and is less common in countries with advanced control programmes. Calves can also become infected through breaks in the skin. Other uncommon routes of infection include intrauterine infection at coitus, by the use of infected semen or of infected insemination or uterine pipettes and intramammary infection by the use of contaminated teat siphons or by way of infected cups of milking machines (Radostits *et al*., 2007). Cutaneous, genital /venereal/ and congenital infections have been seen but are rare. An animal can spread the disease to many other herd mates before it begins to manifest clinical signs. Therefore, movement of undetected infected domestic animals and contact with infected wild animals are the major ways of spreading the disease (Anon, 2009; OIE, 2009; Malama *et al*., 2013; Verma *et al*., 2014; Youssef and Ahmed, 2014).

The survival of the organism in the environment is influenced by temperature, moisture, exposure to the desiccating effect of sunlight and ultraviolet light. The organism can survive for long periods in faeces and soil but most studies showed that survival on pasture is measured in weeks rather than months and that environmental contamination of pasture is not of major importance in the epidemiology of the disease in cattle (Radostits *et al*., 2007).

Infection by ingestion is possible at pasture when faeces contaminate the feed and communal drinking water and feed troughs but a **large infective dose** is required. Under natural conditions, stagnant drinking water may cause infection up to 18 days after its last use by a tuberculous animal whereas a running stream does not represent an important source of infection to cattle in downstream fields (Radostits *et al*., 2007; Australian Government, 2012).

The feeding of tuberculous cattle carcasses to swine has also caused a severe outbreak of the disease. Unusual sources of infection are infected cats, goats or even humans. Stockmen with genito-urinary infections have transmitted infection to cattle through urinating in the cattle environment (Radostits *et al*., 2007). Ingestion appears to be the primary route of transmission in swine, ferrets, cats and probably deer. In addition, cats can be infected by the respiratory route or via percutaneous transmission in bites and scratches. Non-human primates are usually infected by inhalation. Aerosol transmission also seems to be the main route of spread in badgers but transmission in bite wounds can be significant. Badgers with advanced disease can shed *M. bovis* in the urine and organisms have been found in the faeces. Due to behavioural changes, badgers and possums are most likely to transmit *M. bovis* to cattle during the late stages of disease (De Kantor *et al*., 2008; Anon, 2009; Australian Government, 2012; Verma *et al*., 2014).

The frequent occurrence of EPTB is a result of transmission by ingestion and not of a particular affinity of *M. bovis* for certain abdominal organs. Enormous numbers of tubercle bacilli can be excreted by a cow with tuberculous mastitis. The milk of one cow excreting *M. bovis* with enough viable bacilli could contaminate the milk of 100 cows when their milk is mixed in factories. Any remaining infected milk is left to farmers to feed their calves. One bulk transportation may also result in large quantities of milk becoming infected. TB only becomes a serious problem in cattle when a dairy industry is established particularly when European breeds are introduced. Thus, the dairy industry contributed heavily to the spread of *M. bovis* in cattle. Pockets of high-density dairy farming concentrated around the towns are heavily infected (Kleeberg, 1984; Wichatitsky *et al*., 2013; Caron *et al*., 2014).

M. tuberculosis infection is largely spread from human-to-human whereas *M. bovis* infection has been identified as a zoonotic disease with most cases of human infection attributable to animal sources. Although many mycobacterial species are environmental, *M. tuberculosis* and *M. bovis* are strictly parasitic. The mycobacteria other than tuberculosis complex (MOTT**)** which includes *M. avium* subsp avium and *M. avium* subsp intracellulare isolated from animals have been also isolated from immunocompromised (i. e, those with human immunodeficiency virus /HIV/ infection) humans but seldom from immunocompetent humans. Recently, there has been

increased interest among public health officials in drug-resistant strains of *M. tuberculosis*, *M. bovis*, and *M. avium* because several have been isolated from HIV infected and non-immunocompromised humans (Kaneene and Thoen, 2004; Sakamoto, 2012).

Swine infected with some serovars excrete the organism in their faeces. The use of dirt floors or deep litter in swine, rather than bare concrete or slats, increases the risk of infection and the development of macroscopic lymphadenitis in large numbers of swine. The length of time that swine are kept on the litter is also important and severe outbreaks can occur for the entire period from weaning to slaughter. Sawdust, straw, peat and wood shavings have all been found to be highly infected. Serovars 4 and 8, in particular, have been associated with infection from bedding (Radostits *et al*., 2007).

The classic *M. avium* serovars 1-3 are the cause of TB in domestic and wild birds which are infected by ingestion of contaminated feed or soil and excrete large numbers of organisms in faeces. Although infection in domestic livestock is commonly contracted from domestic poultry, from soilborne infection or from pen floors or feeds contaminated by wild birds, swine-to-swine transmission can also occur (Radostits *et al*., 2007). Infection could persist within a herd and considered that the disease might be maintained in some herds by swine-to-swine transmission. The normal picture on a farm of a single animal is to **become infected but to show no sign of disease until it has been slaughtered**. Even when sows are diseased, no **''open''** lesion has been seen. It was surprising that none of the sows were positive to the tuberculin test considering the number of swine with lesions. It was indicated that 80% of swine are infected via the tonsil although lesions are usually first observed in submaxillary lymph node. Transmission to the pulmonary lymph nodes occurred after haematogenous spread to the lungs. Lesions in liver and spleen are thought to develop later than in the lungs unless there is a heavy bacteraemia (Windsor *et al*., 1984).

The migration of bacteria through mucus and lymphoid tissue in the gut is facilitated by simultaneous digestion of nutrients. Milk products such as yoghurt and cream cheese made from unpasteurized milk have been found to contain TB bacilli 14 days after manufacture and butter

as long as 100 days after manufacture (Kleeberg, 1984). Large numbers of organisms may be shed in the late stages of infection. Asymptomatic and anergic carriers occur. All infected cattle may not transmit the disease (De Kantor *et al*., 2008; Anon, 2009; Australian Government, 2012; Verma *et al*., 2014).

2. 5. Risk Factors

The main risk factors of bTB in African cattle populations include the type of production system (intensive dairy farms and use of upgraded *B. taurus* breeds), animal movements (importing animal herds and transhumance) and absence or inefficiency of bTB surveillance and control measures (Wichatitsky *et al*., 2013).

2. 5. 1. Environmental risk factors

M. bovis can survive for several months in the environment particularly in cold, dark and moist conditions. At 12-24°C (54-75°F), the survival time varies from 18 to 332 days depending on the exposure to sunlight. This organism is infrequently isolated from soil or pastures grazed by infected cattle. Although *M. bovis* can be cultured from artificially stored samples for nearly two years under some conditions, it appears to survive in natural pastures for, at most, a few weeks. In a recent study, *M. bovis* remained viable for 4 to 8 weeks in dry or moist soil samples in 80% shade at 34°C (93°F). In another study, it was destroyed within four days on New Zealand pastures in either summer or winter (Anon, 2009).

Housing predisposes to the disease as does high stocking density. The disease is more common and serious where intensive husbandry is practiced. The closer the animals are in contact the greater is the chance that the disease will be transmitted. In spite of the low overall incidence in countries where cattle are at pasture all the year round, individual herds with 60-70% morbidity may be encountered (Radostits *et al*., 2007).

2. 5. 2. Host risk factors

Zebu (*Bos indicus*) type cattle are thought to be much more resistant to bTB than European cattle (*Bos taurus*) and the effects on *Bos indicus* are much less severe. But under intensive feedlot conditions, a morbidity of 60% and a depression of weight gain can be experienced in tuberculous zebu cattle. TB may also be encountered in elk, wild deer of various species, water buffalo, camels, bison, wild carnivores, monkeys and other wild fauna as well as birds. Most are dead end-hosts but some may act as important reservoirs of infection for cattle (Radostits *et al*., 2007).

The disease levels in swine reflect those in the local cattle population from which the infection is driven either by the ingestion of dairy products or by grazing over the same pasture with infected cattle. The lower relative prevalence in swine is due to a number of factors particularly the tendency of the disease to remain localized in this species and the early age of slaughter. Prevalence is higher in older swine. When the disease is common among dairy cattle in an area, 10-20% of the local swine are likely to be infected. A high prevalence has been observed in swine bedded on shavings infected with *M. bovis*. Feral swine can be infected but the prevalence also reflects the prevalence in local cattle populations and they appear to be dead end-hosts (Radostits *et al*., 2007).

The main risk factors of bTB associated to the environment, host and agent are classified into animal, herd and region/country levels and are depicted by (Figure 6) (Humblet *et al*., 2009).

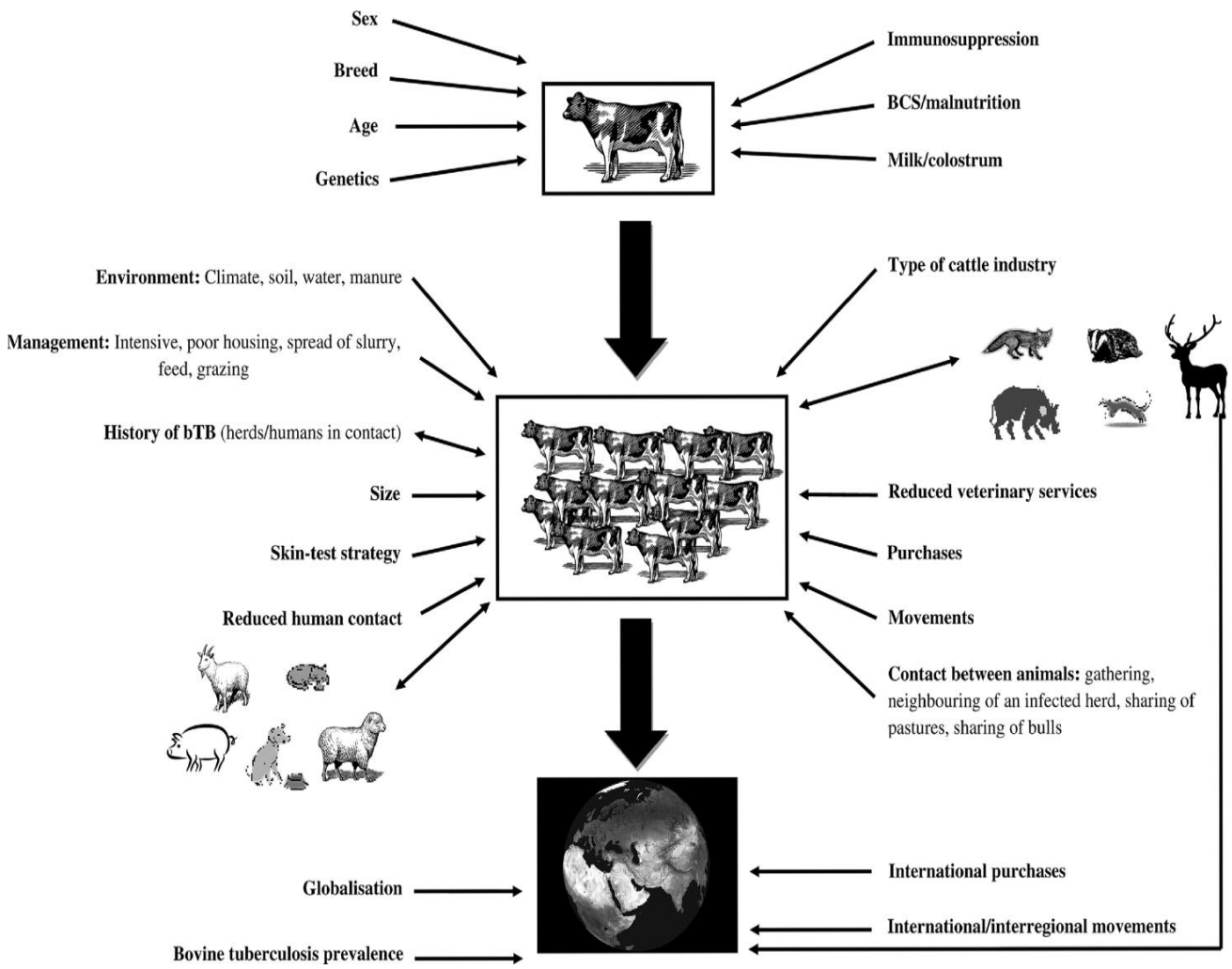

Figure 6. Main risk factors of bTB classified into animal, herd and region/country levels (Humblet *et al*., 2009)

2. 5. 3. Pathogen risk factors

The causative organism is moderately resistant to heat, desiccation and many disinfectants. It is readily destroyed by direct sunlight unless it is in a moist environment. The bacilli may remain viable for weeks in warm, moist and protected positions (Radostits *et al*., 2007).

2. 6. Economic Importance

Controlling a chronic disease like bTB is expensive (Tschopp and Assefa, 2016). Bovine TB is still common in less developed countries and severe economic losses can occur from livestock deaths, chronic disease and trade restrictions /market impairments/. Apart from these, advanced TB may lead to death of the animals (Radostits *et al*., 2007; Verma *et al*., 2014). In some situations, this disease may also be a serious threat to endangered species (Anon, 2009). Bovine TB is of major importance in dairy cattle causing a high morbidity and loss of production as infected animals lose 10-25% of their productive efficiency. Direct losses due to the infection become evident by decrease in 10-18% milk and 15% reduction in meat production. It can also be a financial burden to farmers owning infected cattle. It has been suggested that cattle with bTB have a reduced productivity affecting milk yield, animal welfare and causes poor carcass quality /value/ as well as reduced pulling power in traditional farming system (Radostits *et al*., 2007; Firdessa *et al*., 2012).

The economic loss caused by bTB and its impact is enormous due to high cost of eradication programmes and has serious consequences for movements of animals and their products, biodiversity, public health and significant economic effect. Bovine TB has serious economic impacts not only for the animal owners but also for the regional and national economies through decreased animal productivity besides being a public health threat (Tschopp, *et al*., 2013). Worldwide agricultural losses due to bTB are estimated at around $3 billion annually (Ali *et al*., 2013). The economic losses due to the presence of bTB in Mexico could be calculated in the order of US$ 450 million as a consequence of limited exportation of livestock to the United States of America (Enriquez-Cruz *et al*., 2010). The eradication of bTB in the US did cost 538 million USD between the years 1917-1992. Its current cost in the UK is £100 million per year (Tschopp and Assefa, 2016). Additional economic losses are suffered through the slaughter of livestock and regular bTB testing incurs a vast expense (Le Roex *et al*., 2013). Moreover, bTB is an economic burden through cost of control, loss in trade markets and cost of eradication programmes (Tschopp *et al*., 2011). The economic loss incurred by cattle dealers due to bTB

infection in cattle was estimated at US$1 million in Togo (West Africa) in 1985 based on the meat condemned at abattoirs because of the presence of bTB lesions (Wichatitsky *et al*., 2013).

The lesions of swine TB cannot be differentiated from those caused by, for example, *M. tuberculosis* on gross examination. Therefore, caution is necessary. This, unfortunately, reduces the value of the carcass by 50%. If lesions are found in three body cavities, the meat is condemned thereby causing a total loss. Swine TB has a devastating economic effect on individual producers. Miliary TB of both lungs with evidence of TB elsewhere is the reason enough for total condemnation of the carcass (Songer *et al*., 1980; Windsor *et al*., 1984; Weeks, 1985).

Major financial loss from clinical disease does not occur but at slaughter organs with tuberculous lesions are discarded and the entire carcass may be condemned or require heat treatment before being released for human consumption. The meat is cooked if lesions are found in two body cavities before being released for human consumption (Radostits *et al*., 2007).

Carcasses found to have granulomas compatible with TB is to be cooked at 77°C (170°F) for 30 minutes. Carcasses processed in this manner lose most of their commercial value to the extent of 75% and the additional labour in cooking is an added expense. Also, many processing plants fail to have facilities for cooking; therefore, the carcasses are subsequently condemned. It is important to emphasis that tissues from swine naturally infected with *M. avium* have not been utilized because of anticipated bad publicity for government agencies and the pork industry (Thoen, 2013).

2. 7. Zoonotic Importance

Host genetic factors are strongly associated with the development of TB as 5-10% of *M. tuberculosis* infected immune competent individuals develop the disease in their life time. Several studies have associated alleles of the Human Leukocyte Antigen (HLA) class II to TB

which is the most common in pulmonary TB patients showing strong association with susceptibility to the disease (Souza de Lima *et al*., 2016). Humans are considered as the principal reservoir hosts for *M. tuberculosis*. The human-to-human infection cycle rotates; however, tubercle bacilli have a wide host range and *M. tuberculosis* has been detected in fish, reptiles, birds and mammals including marine animals. Naturally, the first infection of these animals with *M. tuberculosis* is caused by humans and then infection may occur between animals which in turn become the source of infection in humans (Rekha *et al*., 2015).

The period of communicability of both bovine and human TB is extremely long compared to that of other airborne and foodborne infections. It is agreed today that man is as susceptible to the bovine as to the human tubercle bacillus. Although there have been reports of the transmission of *M. bovis* from man-to-man, the disease does not seem to spread in the same way as TB caused by *M. tuberculosis*. Most cases end blind without further transmission. EPTB frequently remains undiagnosed during life time. Only a few cases of infection acquired from human urine have been described. Infection is usually family-based and primarily affects children though some cattle were infected by urine from persons infected with *M. bovis* in the Netherlands. Of 50 patients held to be responsible for infecting herds of cattle, 24 had urinary TB. These patients infected a total of 259 cattle or 41% of those in the affected herds. The British group believed that some bovine strains had spread from person-to-person but in most cases it was very difficult to determine the source of infection (Kleeberg, 1984; Wichatitsky *et al*., 2013; Caron *et al*., 2014).

Zoonotic TB is a form of TB in people caused by *M. bovis* which belongs to the MTBC. It often affects sites other than the lungs (extrapulmonary) but in many cases is clinically indistinguishable from TB caused by *M. tuberculosis* (Anon, 2017). The sources of zoonotic TB (inhalation, contact and ingestion) are depicted by (Figure 7).

Figure 7. Zoonotic tuberculosis (Anon, 2017): close contact between a cow and a woman facilitates *M.bovis* transmission via inhalation, a woman cutting meat may facilitate *M.bovis* contact transmission through broken skin with a knife, undercooked meat and underboiled milk may facilitae *M.bovis* transmission through ingestion.

M. bovis and *M. tuberculosis* are extremely closely related **genetically**, **antigenically** and **phylogenetically**. MTBC share more than 99.95% chromosomal identity at the nucleotide level and have a virtually identical 16S rRNA/16S rDNA/ gene sequence but differ widely in terms of **their host tropisms**, **phenotypes** and **pathogenicity** (Bolin *et al*., 1997; OIE, 2009; Vordermeier *et al*., 2012; Mamo, 2014; Birhanu *et al*., 2015). Bovine TB is more prevalent in dairy workers exposed to poor control areas of *M. bovis*. The pulmonary form of bTB is more developed in occupational groups working with animals infected with *M. bovis* on farm or slaughterhouse than the alimentary form of the disease (Verma *et al*., 2014). Lesions produced in humans infected with *M. bovis* and *M. tuberculosis* are similar **clinically**, **radiologically** and **histologically** but differ somewhat in their **tissue distributions**. The primary route of infection of *M. bovis* is more commonly oral as opposed to a respiratory route of infection for *M. tuberculosis*. This results in lesions more often in cervical and abdominal lymph nodes in *M. bovis* infections than in *M. tuberculosis* infections. However, the distinction is far from absolute; primary pulmonary *M. bovis* infections do occur and respiratory tract disease was found in up to 89% of *M. bovis* infections described in the last three decades (Bolin *et al*., 1997; OIE, 2009; Vordermeier *et al*., 2012; Mamo, 2014; Birhanu *et al*., 2015).

The public health significance of MAC infections in man is now recognized as the most common secondary bacterial infection in patients with acquired immunodeficiency syndrome (AIDS). The

bacteria are most often of environmental origin. *M. avium* and other mycobacteria abound in the environment and occur in food and drinking water; therefore, it is not surprising that they are present in the human alimentary tract. Thus, the alimentary tract could be the source of frequently disseminated *M. avium* infections in patients with AIDS (Thoen, 2013).

There are many authenticated cases of *M. avium* infection in people although humans are considered highly resistant to this organism. Avian TB is generally considered noncontagious from an infected person to an uninfected person. Infections with atypical mycobacteria are not uncommon in humans and it is more likely to occur in persons with preexistent diseases especially those involving the lungs and have higher prevalence in persons whose immune systems are impaired by an illness such as AIDS or steroid therapy. Members of the *M. avium*-intracellulare complex cause both pulmonary infections in immunocompetent individuals and disseminated diseases in acquired immunodeficiency syndrome (Radostits *et al*., 2007).

Animals or animal products may be a source for human infection but direct associations are difficult to prove (Radostits *et al*., 2007) and TB is one of the diseases of cattle and swine transmitted to humans (Fowler, 1996). Although not clinically ill, human workers have been found to be infected on farms when the disease occurred in swine. It is likely that infections in humans and animals on the same farm come from one source but it is also possible that spread from animals to humans occurs (Radostits *et al*., 2007). Therefore, the typing of strains isolated from patients is the only effective means of identifying the causative organisms. Hence, in many regions of the world, *M. tuberculosis* infection is so widespread and occurs so early in life that most persons coming into contact with *M. bovis* are protected because of immunity conferred by *M. tuberculosis* (Kleeberg, 1984). Resurgence of the disease in developed countries in association with wildlife reservoirs has resulted in a spillover into human populations. The widespread occurrence of TB in exotic animals maintained in captivity adds to the public health importance of these infections (Radostits *et al*., 2007).

2. 8. Pathogenesis

Developing technologies support the fact of identical pathogenesis because of unusually high conserved sequence similarity in genome of TB causing bacteria in more than 99.95% animals. Basic pathogenic mechanisms are more or less the same in case of bTB and human TB (Verma *et al*., 2014). TB spreads in the body by two stages (Radostits *et al*., 2007):

►the primary complex, and ►post primary dissemination

The primary complex consists of the lesion at the point of entry and in the local lymph node. A lesion at the point of entry is common when infection is by inhalation. When infection occurs via the alimentary tract, a lesion at the site of entry is unusual although tonsillar and intestinal ulcers may occur. More commonly, the only observable lesion is in the pharyngeal or mesenteric lymph nodes. A visible primary focus develops within 8 days after entrance of the bacilli. Calcification of the lesions commences about 2 weeks later. The developing necrotic focus is soon surrounded by granulation tissue, monocytes and plasma cells and the pathognomonic **''tubercle''** is established. Bacilli pass from this primary focus and 90-95% of cases in cattle is in the regional lymph nodes of the respiratory tract and cause the development of a similar lesion there. The lesions in the lungs in cattle occur in the caudal lobes in 90% of the cases. In calves fed tuberculous milk, the primary focus is likely to be in the pharyngeal or mesenteric lymph nodes with hepatic lesions as the major manifestation of postprimary spread (Radostits *et al*., 2007).

Postprimary dissemination from the primary complex may take the form of acute miliary TB, discrete nodular lesions in various organs or chronic organ TB caused by endogenous or exogenous reinfection of tissues rendered allergic to tuberculo-protein. In the latter case, there may be no involvement of the local lymph node. Depending upon the sites of localization of infection, clinical signs vary because the disease is always progressive and there is constant underlying toxaemia which causes weakness, debility and eventual death of the animal (Radostits *et al*., 2007).

The disease in cattle, horses, sheep and goats is progressive and generalized TB is common. Localization as non-progressive abscesses in the lymph nodes of the head and neck in swine is

the most common finding (Radostits *et al*., 2007). On the other hand, tuberculous lesions occurring in swine were characterized by **advanced calcification** and by a **lack**, in most cases, of **viable mycobacteria**. The lesions were limited in all cases to the lymph nodes of the digestive tract. These findings indicated that the exposure was by way of the gastrointestinal tract and the infection was initiated early in the life of swine (Songer *et al*., 1980).

M. avium after ingestion penetrates the wall of the pharynx near the tonsils or the wall of the small intestine and becomes localized in the submandibular and/or mesenteric lymph nodes. Small lesions develop in these lymph nodes. The health and condition of the infected swine usually are not affected which is often impossible to establish a clinical diagnosis in these animals. It should also be noted that in herds in which TB has been diagnosed, *M. avium* has been isolated from the lymph nodes of swine that were negative to skin tests, presented no lesions in the tissues and had no signs of illness (Thoen, 2013).

The study on *M. bovis* is preferred over *M. tuberculosis* infection in swine for the following reasons (Bolin *et al*., 1997):

►first, swine are naturally infected with each of these organisms and the lesions produced are indistinguishable. Therefore, it is likely that swine are equally susceptible to *M. bovis* and *M. tuberculosis*.

►second, because these organisms are so closely related, many studies in TB are done with *M. bovis.* In fact, much of the work on the pathogenesis and prevention of TB has been done in animal models infected with *M. bovis*.

The hosts' response to mycobacteria and ability to control the infection involve local as well as systemic factors. Local factors such as production of **cytokines** (e.g., interferon-γ) may result in activation of macrophages at the site of infection with subsequent control of mycobacterial replication. Lack of activated macrophages at the local site of infection may lead to extensive replication of the mycobacteria within nonactivated macrophages and extension of the lesions (Bolin *et al*., 1997). IL-4 and other Th2 cytokines are elevated in patients with active TB and high pulmonary IL-4 expression has correlated with cavitation. IL-4 has a pathogenic role during

late TB disease by downregulating protective Th1 responses. Th2-skewed responses are also more commonly observed in TB patients living closer to the equator possibly due to helminth coinfection, high exposure to saprophytic mycobacteria or higher *M. tuberculosis* inoculum. This effect may be responsible for the uneven efficacy of BCG vaccination in different parts of the world (Sakamoto, 2012).

Human *M. tuberculosis* infections usually begin by inhalation of aerosol droplets containing tubercle bacilli directly expectorated from an individual with **"open"** pulmonary disease. The infectious dose for a person is reported to be between 1 and 200 bacilli. However, a single aerosol droplet can contain from 1 to 400 bacilli. It is unclear what is considered a biologically relevant dose. The bacilli travel to the alveoli where they are rapidly phagocytosed by alveolar macrophages. These macrophages are stimulated to produce proinflammatory cytokines and chemokines driving the recruitment of more leukocytes to the site of infection. Neutrophils and monocytes arrive first, phagocytose additional bacteria, secrete more cytokines and chemokines and begin to organize the early granuloma. Dendritic cells also phagocytose *M. tuberculosis* and then migrate to regional lymph nodes to present mycobacterial antigens to lymphocytes. Interestingly, the upper lung lobes of human favour bacillary growth due to higher oxygen pressure and delayed immune responses (Sakamoto, 2012).

2. 9. Clinical Findings

Tuberculosis is usually a chronic debilitating disease and usually has a prolonged course in cattle but it can occasionally be acute and rapidly progressive. Early infections are often asymptomatic because no symptoms occur in early stage of the disease. The symptoms of bTB usually take months or years to develop in cattle; however, in late stage, the usual clinical signs include loss of appetite, weight-loss, weakness, a mild (a lowgrade) fluctuating fever, intermittent hacking cough, progressive emaciation, diarrhoea, large prominent lymph nodes, anorexia and induration of udder. On the other hand, infections can also remain dormant for years and reactivate during periods of stress or in old age (Anon, 2009; Verma *et al*., 2014). In cattle, clinical evidence of TB is usually lacking until very extensive lesions have developed. For this reason, its diagnosis

in individual animals and an eradication programme were not possible prior to the development of tuberculin by Koch in 1890. In some animals, the only symptom may be abscesses of unknown origin in isolated lymph nodes and symptoms may not develop for several years. In other cases, the disease may be disseminated with a rapid fulminating course. However, the bacteria can also lie dormant in the host without causing disease (OIE, 2009).

Some general signs are also evident although signs referable to localization in a particular organ usually attract attention to the possible occurrence of TB. Some cows with extensive miliary tubercular lesions are clinically normal but in most cases progressive emaciation unassociated with other clinical signs occurs. In spite of good appetite, TB can be suspected due to the fact that a capricious appetite and fluctuating temperature are commonly associated with the disease. The hair coat may be rough or sleek. Affected animals tend to become more docile and sluggish but the eyes remain bright and alert. These general signs often become more pronounced after calving (Radostits *et al*., 2007; Verma *et al*., 2014).

Lungs: Pulmonary involvement is characterized by a chronic cough due to bronchopneumonia. The cough is never loud or paroxysmal, occurring only once or twice at a time and is low, suppressed and an intermittent hacking moist. It is easily stimulated by squeezing the pharynx or by exercise and is most common in the morning or in cold weather. In the advanced stages when much lung has been destroyed, dyspnoea, moist cough or tachypnoea with increased rate and depth of respiration becomes apparent. At this stage, abnormalities may be detected by auscultation and percussion of the chest. Areas with no breath sounds and dullness on percussion are accompanied by audible squeaky crackles. It is most often audible over the caudal lobes. TB pleurisy may occur but is usually symptomless because there is no effusion. Involvement of the broncheal lymph nodes may cause dyspnoea because of constriction of air passages and enlargement of the mediastinal lymph node is commonly associated with recurrent and then persistent ruminal tympany. In the terminal stage, infected animals become extremely emaciated and develop **acute respiratory distress**. In some animals, the retropharyngeal or other lymph nodes enlarge and may rupture and drain. Greatly enlarged/large prominent lymph nodes can also obstruct blood vessels, airways or the digestive tract (Radostits *et al*., 2007; OIE, 2009; Verma *et al*., 2014).

Intestine: Intermittent diarrhoea and constipation may be seen if the digestive tract is involved (OIE, 2009) and rarely tuberculous ulcers of the small intestine cause diarrhoea. Enlargement of retropharyngeal lymph node causes dysphagia and noisy breathing due to pharyngeal obstruction. Pharyngeal palpation or endoscopy reveals a large, firm and rounded swelling in the dorsum of the pharynx. Chronic and painless swelling of the submaxillary, prescapular, precrural and supramammary lymph nodes is relatively rare (Radostits *et al*., 2007).

Uterus and testes: Reproductive disorders include uterine TB which is uncommon with bovine strains except in advanced cases. Spread by contiguity from the uterus causes peritonitis, bursitis and salpingitis. The lesions in the salpinx are taking the form of small enlargements containing a few drops of yellow fluid. In tuberculous metritis, there may be infertility or conception may be followed by recurrent abortion late in pregnancy or a live calf is borne which in most cases dies quickly of generalized TB. Lesions similar to those of brucellosis occur on the placenta. In cows that fail to conceive, there may be a chronic purulent discharge heavily infected with the organism and the condition is very resistant to treatment. A number of cows will have an associated tuberculous vaginitis affecting chiefly the ducts of Gartner. Rare cases of tuberculous orchitis are characterized by the development of large, indurated and painless testicles (Radostits *et al*., 2007).

Mastitis: Tuberculous mastitis is of major importance because of the danger to public health, spread of the disease to calves and the difficulty of differentiating it from other forms of mastitis. Its characteristic feature is a marked induration and hypertrophy which usually develops first in the upper part of the udder particularly in the rear quarters. Palpation of the supramammary lymph nodes is essential in all cases of suspected tuberculous mastitis. Enlargement of the nodes with fibrosis of the quarter does not necessarily indicate TB but enlargement without udder induration suggests either TB or **lymphomatosis**. In the early stages, the milk is not macroscopically abnormal but very fine floccules appear later and settle after the milk stands leaving a clear amber fluid. Later, the secretion may still be an amber fluid only (Radostits *et al*., 2007).

Tuberculosis infection is usually sporadic in swine herds but in some herds it can be enzootic. The naturally occurring disease is non-progressive and usually restricted to the lymph nodes of the head, neck and mesentery. Occasional generalized cases occur and an outbreak of pulmonary TB associated with *M. avium* has been recorded in swine. The lesions may be free of suppuration and resemble neoplastic tissue but granulomatous and occasionally caseous lesions in lymph nodes also occur. Similar lesions are associated with *Rhodococcus equi*. Granulomatous lesions which develop in the tonsils and intestinal wall result in the passage of organisms in the faeces for at least 55 days and transmission to incontact swine occurs readily. Tuberculous lesions in cervical lymph nodes of swine usually cause no clinical abnormality unless they rupture to the exterior. Generalized cases present a syndrome similar to that seen in cattle although tuberculous involvement of the meninges and joints is more common. Generalized cases of TB in cattle are denoted by the presence of miliary TB with small, transparent and shot-like lesions in many organs or by pulmonary lesions which are not well-encapsulated and caseated. The presence of bronchopneumonia or hyperaemia around pulmonary lesions is highly suggestive of active disease. Cases with tuberculous mastitis or discharging tuberculous metritis must also be considered as likely to be potent spreaders of infection (Radostits *et al*., 2007).

2. 10. Diagnosis and Clinical Pathology

2. 10. 1. Tuberculin skin test

Antemortem evaluations are critical components of TB control programmes throughout the world. One of the most reliable and practical methods of diagnosis in infected domestic animals at this time is assessment via the tuberculin skin test which is the standard and official OIE prescribed antemortem test for the diagnosis of bTB infection in live animals in the field as well as international trade (OIE, 2009). The tuberculin skin test has been used to identify animals exposed to pathogenic mycobacteria. Tuberculin skin testing of livestock has been the cornerstone of on-farm active surveillance of bTB for many years. Testing is also applied for the purpose of import, export, entry into semen production centres and to support disease investigations (CFIA, 2019). Occasionally, the sputum and other body fluids may be collected from live animals for mycobacteriological examination (Anon, 2009).

Tuberculin skin test is primarily a screening test with low sensitivity (Verma *et al*., 2014). This test has been practiced since more than a century in bTB control campaigns all over the world and it is still largely used for field testing (De Kantor *et al*., 2008). This test involves the intradermal administration of purified protein derivatives /PPDs/_*in vivo* diagnostica. Injection of tuberculins into animals infected with mycobacteria is allergic to the mycobacterial tuberculo-protein contained in tuberculins (Kaneene and Thoen, 2004; OIE, 2009). Tuberculin is a concentrated sterile culture filtrate of tubercle bacilli which is prepared from cultures of *M. tuberculosis* or *M. bovis* after being grown on glycerinated beef broth and more recently on synthetic media which provides a means of detecting the disease. Bovine tuberculin is more potent and specific (Anon, 2009; OIE, 2009). The basis of tuberculin testing is the induction of delayed type hypersensitivity (DTH) reaction to the intradermal injection of antigenic substances derived from a laboratory strain of *M. Bovis* (CFIA, 2019). A positive reaction constitutes a diffuse swelling at the injection site and local inflammation of the skin as a result of DTH reaction (Kaneene and Thoen, 2004; OIE, 2009).

The hypersensitivity response results in the accumulation of cells and fluid in the dermis which creates swelling at the injection site. In test positive animals, the intradermal skin reaction develops within 24 hours post-injection reaching a maximum by 48-72 hours which is characterized by erythema and swelling but rarely to the point of induration or ulceration (CFIA, 2019). On the other hand, reactions at the injection site fail to develop in uninfected animals. The sensitivity and specificity of the intradermal test often depend on the field conditions, prevalence of infection and other factors (Kaneene and Thoen, 2004; OIE, 2009).

The tuberculin test involves measuring the skin thickness before any injection, injecting PPDs into the measured area of a skin fold and remeasuring as well as recording any subsequent swelling at the site of injection two to three days later depending on the species of animals being tested. The reaction is read and compared after 48-72hours post-injection. In cattle, it can be read between 48-72hours for maximum sensitivity and at 96 hours for maximum specificity. The same skin calipers calibrated into mm will be used before and after PPD injection. Moreover, the same person should measure the skin thickness before and after injection. Most farms during a

bTB breakdown are required to test cattle/swine every 60 days (Butler *et al*., 2010; Thoen, 2013).

Mixed avian and mammalian tuberculins may be used when no attempt is being made to determine the type of infection. The single intradermal comparative cervical tuberculin test (SICCTT) in cattle and the single intradermal comparative tuberculin test (SICTT) in swine can be performed using both biologically balanced PPDs of *M. avium* and *M. bovis* (Thoen, 2013) to differentiate between animals infected with *M. bovis* and those responding/sensitizing/ to bovine tuberculin as a result of exposure to other environmental mycobacteria. This sensitization can be attributed to the antigenic cross reactivity among mycobacterial species and related genera. The comparative test depends on the greater sensitivity to homologous tuberculin. Both avian and bovine tuberculins are injected simultaneously in bovine into two separate sites on the same side of the mid-neck horizontally, 12cm apart, one above the other and the test is read 72hours later (Radostits *et al*., 2007; Anon, 2009). *M. avium* and *M. bovis* may be injected in swine at the left and right sides of the base of the ears, respectively to get useful information about exposure to either MTBC or to MAC organisms (Thoen, 2013).

The maximum skin thickening may not occur until 48hours after injection if the swine is infected with either *M. bovis* or *M. avium* (Radostits *et al*., 2007). Positive reactions usually include swelling and redness and they may vary in size and intensity. Haemorrhage and ulceration may occur at the site of injection. The reliability of the tuberculin test when used on individual swine has been questioned. The tuberculin test can be used successfully as a herd test although false positive reactors occur. Enzyme linked immunosorbent assays (ELISA) have been described for obtaining information on the presence of mycobacterial antibodies in the sera of the swine naturally exposed to clinically significant mycobacteria. However, these tests have not come into widespread use since some animals fail to develop detectable antibodies in their sera for several weeks or months following natural exposure (Thoen, 2013).

Avian tuberculin PPD is used as a control for nonspecific reactions. The greater of the two reactions indicates the organism responsible for the sensitization. The test is not generally

intended for primary use in detecting reactors but only to follow up known reactors to determine the infecting organism. Its use as a primary test is recommended when a high incidence of avian TB or Johne's disease is anticipated or when vaccination against Johne's disease has been carried out. The comparative test is adequate to differentiate between vaccination against Johne's disease and TB. The distinction is easier when the time between vaccination and testing is longer (Radostits *et al*., 2007; Anon, 2009).

All animals over 3 months of age should be tested and positive reactors disposed of according to local legislation. Infected herds are usually quarantined and animals that have been in contact with reactors are traced. Affected herds are re-tested periodically to eliminate cattle that may shed the organism. Suspicious reactors are retested at intervals appropriate to the test used. At the initial test, a careful clinical examination should be conducted on all animals to insure that there are no advanced clinical cases which will give negative reactions to the test. Doubtful cases and animals likely to have reduced sensitivity particularly old cows and those that have calved within the previous 6 weeks may be tested by one of the special sensitivity or serological tests or re-tested subsequently. The comparative test should be used where infection with *M. paratuberculosis* or *M. avium* is anticipated or where a high incidence of reactors occurs in a herd not showing clinical evidence of the disease (Radostits *et al*., 2007; Anon, 2009).

False-positive reactors (no gross lesion reactors) may occur due to animals sensitized to other mycobacterial allergens including those of human or avian TB or Johne's disease, relatively non-pathogenic mycobacteria, e.g. skin TB and ingestion of non-pathogenic mycobacteria in permanent waters inhabited by birds or poultry litter fed to cattle/swine when the birds are infected with *M. avium*, animals sensitized to other allergens, e.g. *Nocardia farcinicus*, animals injected with irritants at the injection site prior to reading of the tuberculin test and when compensation rates for reactors exceed true cattle/swine prices (Radostits *et al*., 2007). **False-negative** responses are sometimes seen in animals soon after infection (early cases i.e. 6 weeks after infection), in the late/advanced stage of TB, in those that have recently calved (cows which have calved within the preceding 6 weeks), in those with poor immune responses, those that were retested within 8-60 days after single intradermal testing, old cattle/swine, low-potency

tuberculin or bacterial contamination of the tuberculin and variable doses with multi-dose syringes (Radostits *et al*., 2007; Anon, 2009; Verma *et al*., 2014).

Care must be taken in placing tuberculin injections as sensitivity varies from place to place in the skin of animals. The cervical fold /neck skin/ is much more sensitive than that of the caudal /anal/ fold which the latter provides greater specificity. The subjective method of palpation is more accurate. The neck area has the advantages that reactions are more pronounced, animals can be retested immediately and the area is more sanitary. Its disadvantages are that restraint of each animal is necessary and the proportion of no-visible-lesion (NVL) reactors increases. The exact dose for the particular tuberculin should be strictly adhered to when the cervical skin test is used. A dose of one-tenth (0.1mL) PPD is recommended for herds of unknown status and 0.2mL in known infected herds when cases with low sensitivity are to be carefully sought. The method of injection of tuberculin also has some importance when the cervical site is used. A careful intradermal injection produces the largest swelling. Variations in technique appear to have little effect on the size of reaction when the caudal fold is used (Songer *et al*., 1980; Radostits *et al*., 2007; Verma *et al*., 2014). The amount of tuberculin used and the site of injection in swine have varied depending on the investigator (Thoen, 2013). Mid-cervical test (MCT) is the standard official screening test for TB in cervids. The test involves an intradermal injection of 0.1mL of both avian and bovine tuberculins (PPDs) in the mid-cervical area (approximately 8cm square) with observation and palpation at 72 hours ± 6 hours (CFIA, 2019). Tuberculin skin tests of domestic animals with various interpretation criteria are depicted in (Table 1).

Infection in swine exposed to *M. avium* is usually associated with the lymph nodes of the head and the digestive tract and rarely spreads to other locations. Diagnosis of TB by physical examination of live swine is usually impossible. Visual examination of infected sites at slaughter cannot differentiate lesions of TB from those caused by other microorganisms or conditions; a confirmed diagnosis should be based on mycobacteriologic examination from these sites. Diagnosis of TB in swine on a herd basis is important and usually depends on detection of infected lymph nodes from swine at slaughter. When TB has been confirmed by microscopic and

bacteriologic examinations, the potential sources of infection should be determined and the management practices to eliminate the source, if possible, should be altered (Thoen, 2013).

Table 1. Tuberculin test of domestic animals with different cut-off values

Species	Dose of PPDB	Injection site	DTH time	Interpretations		
				Positive	Negative	Reference
Bovine						
●Standard (Holstein)	0.1mL	MCT/CFT	72±6h	≥4mm	<2mm	OIE, 2009
●Severe (Zebu)	0.1mL	MCT/CFT	72±6h	>2mm	<2mm	Ameni *et al*., 2008
Caprine	0.1mL	CFT	72±6h	≥3.5mm	<3.5mm	Tafess *et al*., 2011
Ovine	0.1mL	CFT or Inside of the thigh	72±6h	≥5mm	<5mm	Radostits *et al*., 2007; CFIA, 2019
Camelids	0.1mL	PAT	72±6h	>1.5mm ≥3mm	<1.5mm < 3mm	CFIA, 2019 Jibril *et al*., 2018
Swine	0.1mL	BE	48h	>2mm	<2mm	CFIA, 2019 APHA, 2019

PPDB: Purified protein derivative of bovine antigen; DTH: Delayed type hypersensitivity reaction; CFT: Caudal fold test; MCT: Mid-cervical test; PAT: Postaxillary test; BE: Base of the ear test

The detection of TB in live swine has been a major hindrance in efforts to study swine TB. Clinical signs of diagnostic value are, of course, not present and the reliability of the tuberculin test has been questioned. Reported inaccuracies in tests may be due to a lack of cross-reactivity between the infecting *M. avium* serotype and the serotype used to prepare the skin test reagent or in some cases to improper administration of the tuberculin. Avian old tuberculin was used in all skin test procedures. The correlation established between proportions of swine reacting to tuberculin and swine containing lesions of TB at slaughter implies that this test may have use

epidemiologically if not diagnostically. Tuberculin skin tests performed on swine and then slaughtered showed a 97.1% correlation between the number of swine responding to tuberculin and the number found to have tuberculous lesions at slaughter. No correlation of skin test response and presence of lesions was possible for individual swine but these results imply that the test may have value when used to determine prevalence of the disease in a herd of swine (Songer *et al*., 1980).

Many suspicious reactions occur in swine because of the tendency of lesions to regress and the sensitivity to tuberculin to diminish. Maximum sensitivity is occurring 3-9 weeks after infection. A retest in 6-8 weeks should determine whether or not the disease is progressing. Although positive reactors may in time revert to a negative status, there may be macroscopic lesions in these animals at necropsy. However, viable organisms are not usually recoverable from the lesions because the infection is apparently having been overcome (Radostits *et al*., 2007).

The greater of the reactions to either PPDA or PPDB indicates the organism responsible for the sensitization. It is not uncommon to observe overlap of bTB and MAC positivity in cattle due to either antigenic cross reactivity and/or co-infection. There exists no clear evidence on the immunological relationship between these two but studies have shown that co-infection with MAC compromises bTB skin test results by negatively influencing the sensitivity of the tuberculin test. Cattle sensitized by MAC might conceal *M. bovis* for a period of time. However, it is not clear to what extent this disease could jeopardize the detection of bTB with skin test thus requiring further research (Mekonnen *et al*., 2019). The lesions of MAC at postmortem inspection are characteristic but culture and identification of the organism is required for confirmation. Growth is slow and PCR technologies offer faster diagnosis with some ability to differentiate between individual species and serovars. Smears of lesions associated with some of these agents do not stain positive with acid-fast stains (Radostits *et al*., 2007).

Few clinicopathological tests are carried out because of the universal dependence on the tuberculin test for diagnosis and the policy of slaughtering all positive reactors whether they are open cases or not. Sputum or discharges may be examined by inoculation into guinea-pigs but

improved cultural techniques and the use of nucleic acid probes make laboratory animal inoculation unnecessary. The basis of all TB eradication schemes to date is the tuberculin test and a knowledge of the various tests used, their deficiencies and advantages is essential. It should be remembered, however, that clinical examination is still of value particularly in seeking out the occasional advanced cases which do not give a positive reaction to a tuberculin test (Radostits *et al*., 2007).

Delayed type hypersensitivity reaction develops in swine inoculated with *M. bovis* orally or intradermally. Swine and humans have similar patterns of resistance and susceptibility to virulent mycobacteria and develop lesions with a similar histologic character (Bolin *et al*., 1997). Some decrease in skin sensitivity after parturition occurs in sows infected with *M. bovis* but may not occur when the infection is associated with *M. avium*. Comparative tests work efficiently in this species with little or no reaction to heterologous tuberculin (Radostits *et al*., 2007).

2. 10. 2. Postmortem inspection

Lesions occurring in swine naturally infected with *M. bovis* and *M. tuberculosis* are indistinguishable (Bolin *et al*., 1997). Detection of mycobacterial disease in a live animal is often very difficult; therefore, the presence of disease must be determined by postmortem examination (Thoen, 2013). Abattoir surveillance, cattle identification and tracing systems to attain an effective traceback investigation to the farm of infection origin in cases of animals with TB lesions detected at slaughterhouse inspection should be conducted (De Kantor *et al*., 2008).

Gross lesions: Granuloma is an organized pathological structure that consists of differentiated macrophages (epithelioid macrophages) with a characteristic morphology, T-lymphocytes, some B-lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components. The complex, dynamic interactions within granulomatous lesions reflect a composite of macrophage and helper-T cell function, cytokine production and mycobacterial activity that in turn influence the morphological appearance of granuloma. Lesion, necrosis, liquefaction, mineralization and regression represent some of the outcomes of these interactions that dictate lesion size, appearance and ultimately the presentation of disease in the host (Mamo, 2014).

Tuberculous granulomas in cattle usually have a yellowish appearance with caseous, caseo-calcareous or calcified in consistency and are sometimes encapsulated. Tuberculous granulomas or tubercles in cattle are commonly found in the lymph nodes particularly those of the head and thorax (bronchial, mediastinal, retropharyngeal and portal lymph nodes) along with tissue affected. Apart from this, tubercles are also common in the lung, spleen, liver and the surfaces of body cavities. However, in disseminated cases multiple small granulomas may be found in numerous organs such as female genitalia but are rare on the male genitalia (Radostits *et al*., 2007; Anon, 2009; Katale *et al*., 2012; Verma *et al*., 2014; Mamo, 2014). In countries with good control programmes infected cattle typically have few lesions at necropsy. Most of these lesions are found in the lymph nodes associated with the respiratory system. However, small lesions can often be discovered in the lungs of cattle if the tissues are sectioned (Anon, 2009).

Granulomas in swine were predominately observed in submandibular lymph nodes and to a lesser extent in other body locations. The submandibular lymph node was the organ in which MTBC was more frequently detected followed by the liver, lungs and spleen. Interestingly, MTBC was identified in a similar rate from both liver and lung samples with most of the granulomatous lesions belonging to stages III and IV. These results support the theory that both the respiratory and digestive routes of infection play an important role in swine. Several authors have suggested that generalized TB in swine is frequent whereas others have reported a restriction of TBL lesions to head lymph nodes or less frequently to the respiratory tract (Cardoso-Toset *et al*., 2015).

Miliary abscesses in the lung may extend to cause a suppurative bronchopneumonia. The pus has a characteristic cream to orange colour and varies in consistency from thick cream to crumbly cheese. Tuberculous nodules may appear on the pleura and peritoneum. All localized lesions of TB tend to stimulate an enveloping fibrous capsule but the degree of encapsulation varies with the rate of development of the lesion. Generalized cases are denoted by the presence of miliary TB with small, transparent, shot-like lesions in many organs or by pulmonary lesions which are not well-encapsulated and caseated. Generalized TB, with military tubercles in most organs, is

seen in swine but the common finding is localization in the tonsils, submaxillary, cervical, hepatic, bronchial, mediastinal and mesenteric lymph nodes. The nodes are markedly enlarged and consist of masses of white, caseous, sometimes calcified material surrounded by a strong, fibrous capsule and interlaced by strands of fibrous tissue (Radostits *et al*., 2007).

Chronic lesions are characteristically discrete and nodular, contain thick, yellow to orange, caseous material, often calcified and surrounded by a thick fibrous capsule. Although such lesions are less likely to cause heavy contamination of the environment than open lesions, affected animals are important as sources of infection. It should be noted that suspected cattle slaughtered as part of bTB eradication programmes may be culture-positive and yet have no typical gross or microscopic lesions (Radostits *et al*., 2007). A thorough postmortem examination is done in swine to detect the presence of macroscopic lesions (Bolin *et al*., 1997). Lesions were found only in lymph nodes associated with the digestive tract. Grossly, the lymph nodes varied in size from normal to slightly enlarged. Soft foci of caseous necrosis were usually present and varied in size from approximately 1mm to 1cm in diameter. Calcification was common, implying that lesions were old and that the infections had begun early in the lives of the animals. Gross lesions were found more frequently and were more extensive in mesenteric lymph nodes than in cervical lymph nodes. For example, during a three month period in 1977, 51% of the swine slaughtered had lesions only in the mesenteric lymph nodes whereas only 20% had lesions in cervical lymph nodes, either alone or accompanied by lesions in mesenteric lymph nodes (Songer *et al*., 1980).

The body regions and tissues with lesions are pooled as follows: head (tonsil, mandibular, parotid, retropharyngeal and deep cervical lymph nodes); thorax (lung, tracheobronchial and mediastinal lymph nodes); abdomen (spleen, liver, kidney, adrenal gland, mesenteric, hepatic and medial iliac lymph nodes) and peripheral lymph nodes (superficial cervical, subiliac and deep popliteal lymph nodes). Granulomas are detected at necropsy in the lungs, liver, spleen, peritoneum and multiple lymph nodes in swine that developed disseminated TB. Lung lesions varied from diffuse consolidation to multiple discrete granulomas in swine with disseminated disease or disease localized to the thorax. Fibrinous and granulomatous pleuritis are common.

The disseminated disease in swine is similar in lesion distribution and character to that seen in extrapulmonary or disseminated TB in humans. The presence of mycobacteria in tissues before development of detectable lesions is possible (Bolin *et al*., 1997). Granulomas, as the main lesions associated with TB, have been widely classified within different stages of development that may help in the interpretation of disease progression. A lower bacterial load has been associated with more advanced stages of granulomas established in their primary sites of infections (Cardoso-Toset *et al*., 2015). The stages of granuloma formation in all animals are described hereunder (Wangoo *et al*., 2005):

Stage I (initial) granulomas were characterized by accumulations of epithelioid macrophages admixed with low numbers of lymphocytes and neutrophils. Multinucleated giant cells were sometimes present but necrosis was absent. When present, AFB were seen within macrophages or multinucleated giant cells.

Stage II (solid) granulomas were characterized by accumulations of epithelioid macrophages surrounded by a thin and incomplete connective tissue capsule. Infiltrates of neutrophils, lymphocytes and multinucleated giant cells were sometimes present. Necrosis was centrally located and minimal to mild. When present, AFB were seen within macrophages or multinucleated giant cells.

Stage III (necrotic) granulomas were characterized by necrotic cores, some with small foci of dystrophic mineralization which is surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. As distance from the necrotic core increased so do the relative number of lymphocytes also increased but the number of epithelioid macrophages and multinucleated giant cells decreased. The entire granuloma was surrounded by a thin to moderate fibrous capsule. When present, AFB were seen within the necrotic core and, to a lesser extent, within macrophages or multinucleated giant cells.

Stage IV (necrotic and mineralized) granulomas were characterized by a variably thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores often with foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells

surrounded necrotic areas. These cellular infiltrates were bordered by a zone of large numbers of lymphocytes. AFB were most often present within the necrotic core.

2. 10. 3. Conventional techniques

Media: Many different media have been devised for cultivating tubercle bacilli and they are categorized into three main groups: egg-based media, agar-based media and liquid media. In addition, tubercle bacilli may also be grown on chick embryos and in tissue culture. Agar or liquid medium supplemented with serum or bovine albumin is also used to grow mycobacteria (Shimeles, 2008). A set of solid egg-enriched media such as Lowenstein-Jensen (LJ) containing glycerol/pyruvate and asparagines, Colestos base or Stonebrinks are most commonly used in veterinary mycobacteriology for primary isolation. These media should contain either pyruvate or glycerol (Verma *et al*., 2014). To date, the most frequently used media for isolation of *M. bovis* are LJ and Ogawa-medium (both contain eggs, phosphate and magnesium). LJ medium can be obtained commercially. An agar-based media such as middle brook, 7H10 and 7H11 or blood based agar medium may also be used. The media are prepared as solid slants in screw-capped bottles which are tightly closed to avoid desiccation. Malachite green dye (0.025g/100ml) is commonly used as selective agent. *M. tuberculosis*, *M. avium* and many of the atypical mycobacteria require glycerol for growth. However, glycerol is inhibitory to *M. bovis* while sodium pyruvate (0.4%) enhances its growth. Thus, the media with glycerol and without glycerol but with sodium pyruvate should be inoculated. The media can be made more selective by the addition of cycloheximide (400µg/ml), lincomycin (2µg/ml) and nalidixic acid (35µg/ml). Each new batch of culture medium should be inoculated with the stock strains of mycobacteria to ensure that the medium supports satisfactory growth (Bolin *et al*., 1997; Fentahun and Luke, 2012; Verma *et al*., 2014).

The traditional method of inoculating solid media such as LJ or 7H10/7H11 is slow and takes 6-8 weeks of incubation to diagnose the infection and further more time to determine the susceptibility patterns that results in delay in initiation of appropriate therapy (Birhanu *et al*., 2015). Growth usually becomes visible in 3-6 weeks (Anon, 2009).

Culture: Samples for culture at necropsy should be collected from abnormal lymph nodes and affected organs such as the lungs, liver and spleen. These samples should be collected into clean and preferably sterile containers (Anon, 2009; OIE, 2009).

The presence of *M. bovis* in clinical and tissue samples collected after postmortem examination may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism on primary isolation medium. *M. bovis* can be demonstrated microscopically on direct smears from clinical samples (blood stained purulent exudates i.e., cough sputum and pleural fluid) and on prepared tissue materials /lung biopsy/ (Habarugira *et al*., 2014; Verma *et al*., 2014). Preliminary examination of tissues suspected of being tuberculous should include the preparation of suitably stained smears. The Zeihl-Neelsen (ZN) method is commonly used to stain the mycobacteria. The kinyoum modification of the ZN stain is recommended because no heat is required. The bacteria in stained slides including *M. bovis* are examined under an ordinary light microscope for the presence of acid-fast bacilli (AFB) which appear as short red or pink rods, colloidal or bacillary cells 1-3µm in length occurring singly or in clumps (Fentahun and Luke, 2012; Birhanu *et al*., 2015). The acid fastness of *M. bovis* is normally demonstrated with the classic ZN stain. Moreover, a fluorescent acid-fast stain may also be used (Habarugira *et al*., 2014; Verma *et al*., 2014). The cell walls of acid-fast bacteria contain approximately equal amounts of polysaccharide. The high lipid content, which ranges from 20-40% of the dry cell weight, is largely responsible for the ability of these bacteria to resist decolourization with acidified organic solvents (Fentahun and Luke, 2012; Birhanu *et al*., 2015).

Specimens should be shipped to the laboratory quickly and prompt shipment maximizes the chance of isolating *M. bovis*. If shipping must be delayed, the samples can be refrigerated or frozen. If refrigeration or freezing is not feasible, 0.5% (w/v) boric acid may be added for periods of a week or less (Anon, 2009; OIE, 2009).

Mycobacteria grow slowly and cultures are incubated for a minimum of 8weeks, preferably for 10-12 weeks, and incubated at $37^{O}C$ with or without CO_2. *M. tuberculosis* and *M. avium* prefer

the caps on the culture media to be loose while *M. bovis* grows best in airtight containers. Moreover, the media should be in tightly closed tubes to avoid desiccation. Slants should be examined on a regular basis for presence of any colony growth. When colony growth is visible, smears are prepared and stained by using the ZN staining technique for microscopic examination to select AFB positive cultures (Verma *et al*., 2014). A non-contaminated sample is needed for examination because *M. avium* is non-chromogenic and non-photochromogenic, slow-growing and other bacteria can easily overgrow it (Songer *et al*., 1980).

The mycobacteria that produce yellowish-orange carotenoid pigments are called chromogenic. The term photochromogenic is applied to those mycobacteria that produce pigment only if exposed to light. The scotochromogenic mycobacteria produce pigment when incubated either in light or in the dark. Pigment formation is tested with young well-developed colonies on LJ medium. The cultures are exposed to a 100 watt clear electric light bulb at a distance of 50cm for at least an hour and then incubated again in darkness for a further 1-3 days. After this treatment the photochromogens will develop pigment. Older colonies of mycobacteria in the TB group often have a yellowish hue but they are described as non-chromogenic. *M. bovis* shows a dysgenic colony shape on LJ medium which is characterized by microaerophilic growth while *M. tuberculosis* shows eugenic colony shape and aerophilic growth. The *M. bovis* colony on glycerol containing media form small moist-sheen, flat, smooth, white colonies that break up easily when touched, has sparse and thin growth. However, *M. bovis* grow well on pyruvate containing media without glycerol. The luxuriant growth of *M. tuberculosis* on glycerol containing media is giving the characteristic rough, dry, tough, buff, raised, irregular and hard to break up easily (wrinkled) colonies while the growth of *M. avium* on media containing glycerol is also described as eugenic, whitish and stocky colonies that break up easily (Fentahun and Luke, 2012).

Culturing of *M. bovis* is difficult, time-consuming and poses a considerable public health risk (Radostits *et al*., 2007). It is a fastidious organism (Woyessa *et al*., 2014). Environmental mycobacteria grow more rapidly than *M. bovis* and contamination with these organisms can cause false negatives (Anon, 2009; OIE, 2009).

The colony growth time of *M. bovis* is ≥ 3 weeks but it is 10–14 days for *M. tuberculosis*. The identity of the organism can be confirmed with cultural characteristics or PCR assays. PCR can also detect *M. bovis* directly in clinical samples. Genetic fingerprinting techniques (e.g. spoligotyping) can differentiate the various strains of *M. bovis*. *M. tuberculosis* is a member of the slow-growing pathogenic mycobacterial species characterized by a 12-24hours division rate and prolonged culture period on agar upto 21 days. Why *M. tuberculosis* grows so slowly is not well understood. However, the proposed mechanisms include limitation of nutrient uptake through the highly impermeable cell wall and slow rates of RNA synthesis (Sakamoto, 2012). All procedures for mycobacterial culture should be done in a biological safety cabinet level III as the bacilli may survive in heat-fixed smears or become aerosolized during specimen preparation (Anon, 2009).

Gross Pathology and Histopathology: Tuberculous granulomas may be found in any of the lymph nodes. Microscopic appearance of a typical bovine tuberculous lesion consists of a peripheral zone of mononuclear cells, fibroblasts and giant cells with central caseous necrosis. The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions such as caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages. The presence of acid-fast organisms in histological sections may not be detected. Tentative diagnosis can be made by observing lesion and liquefaction including the aforementioned histological changes in the processed tissue samples. The regressions of affected tissues represent some of the outcomes of these interactions that dictate lesion size, appearance and ultimately the presentation of disease in the host (Quinn *et al*., 1999; Habarugira *et al*., 2014; Mamo *et al*., 2014; Verma *et al*., 2014).

Tubercles in swine are described as necrotic-calcified, proliferative or purulent gross lesions compatible with TB. Different body locations such as other lymph nodes and thoracic or abdominal organs can also be affected although TBL lesions in swine are frequently limited to the head lymph nodes. According to the cellular components, granulomatous and pyogranulomatous lesions can be identified in TBL lesions (Cardoso-Toset *et al*., 2015).

2. 10. 4. Molecular techniques

Nucleic acid recognition methods: PCR has been widely used for the detection of MTBC in clinical samples (mainly sputum) in human cases and has recently been used for the diagnosis of TB in animals. The real time PCR (RT-PCR) determines the status of infection in cattle for bTB as compared to the IFN-γ, mRNA in blood culture. Another useful diagnostic method for bTB in cattle is RT-qPCR (Verma *et al*., 2014). The sensitivity of PCR ranges from 70-90% compared to the results of culture and its specificity varies between 90-95%. In smear of positive cases, the sensitivity of PCR is greater than 95% but in smear of negative cases it is only 50-60%. Therefore, at present amplification methods should not replace diagnostic conventional culture (Fentahun and Luke, 2012).

The development and introduction of molecular genotyping methods for *M. tuberculosis* has significantly improved our knowledge of TB transmission, mycobacterial phylogeny and evolution. PCR-based genotyping methods require small amounts of non-purified DNA and produce results in a digital format enabling portability of results and creation of national and international databases for routine and research purposes. More recently, whole genome next generation sequencing (WGS) technologies became available providing even higher discriminatory power. Genotyping is currently recognized as an essential tool for tracing outbreaks, transmission and surveillance purposes to facilitate public health interventions and study *M. tuberculosis* population structure in diagnostic and research contexts (Nikolayevskyy *et al*., 2016).

Recent molecular studies with a limited number of isolates from birds, humans and other mammals clearly indicated that *M. avium* can be transmitted between birds and swine but the studies did not disclose a similar cross transmission between birds and humans for the isolates tested. It is generally accepted that swine, rabbits and mink are highly susceptible to *M. avium*; deer can also become infected. Dogs appear to be quite resistant to the avian type of TB (Roffe, nd).

Cultured slow-growing, cream coloured and cauliflower like colonies were confirmed to be *M. bovis* by genotypic methods (Ingram *et al*., 2010). A number of molecular based diagnostic methods are available to characterize and type mycobacterial isolates at a species and/or strain levels (Mamo, 2014). They are used as diagnostic and confirmatory tests for TB and are expected to detect as low as 1 to 10 organisms (Fentahun and Luke, 2012). The mycobacterial cell from culture colony is killed in $80^{O}C$ water bath for 1 hour. The most common ones used in molecular epidemiology of MTBC include multiplex polymerase chain reaction (mPCR), region of difference (RD) deletion typing, spoligotyping and MIRU-VNTR 24-loci typing (Mamo, 2014).

Multiplex Polymerase Chain Reaction (Genus typing): Multiplex PCR (mPCR) method utilizes the PCR targets on the sequence of the genus *Mycobacterium* within the 16S rRNA gene (G1, G2) hypervariable region that is known to be specific to *M. intracellularae* (MYCINT-F) and *M. avium* (MYCAV-R) and the MTB70 gene specific for MTBC (TB-1A, TB-1B). This method differentiates all members belonging to the genus *Mycobacterium* and furthermore characterizes the groups belonging to the MTBC and MAC. On the gel electrophoresis result all members of the genus *Mycobacterium* produce a band of 1030bp, members of MAC (*M. avium* subspecies Intracellularae) produces a band of 850bp, and *M. avium* subspecies Paratuberculosis produces a band of 180bp. Members of MTBC including *M. bovis* produces a band of 372bp (Ashenafi *et al*., 2013; Mamo, 2014).

The primers used are MYCGEN-F, 5'-AGA GTT TGA TCC TGG CTC GA 3'; (35 ng/µL), MYCGEN-R, 5'-TGC ACA CAG GCC ACA AGG GA 3', (35 ng/µL); MYCAV-R, 5'-ACC AGA AGA CAT GCG TCT TG 3' (35 ng/µL); MYCINT-F, 5'-CCT TTA GGC GCA TGT CTT TA 3' (75 ng/µL); TB1-F, 5'-GAA CAA TCC GGA GTT GAC AA 3' (20 ng/ µL); TB1-R, 5'-AGC ACG CTG TCA ATC ATG TA 3' (20 ng/µL). The reaction is to be carried out using thermal cycler (Applied Biosystems, GeneAMP 9700) (Ashenafi *et al*., 2013).

Region of difference (RD) deletion typing (Speciation): RD deletion typing is a PCR-based typing method that makes use of the MTBC chromosomal regions of difference for deletion loci (Mamo, 2014). To discriminate isolates of the MTBC to the species level, RD typing can be

carried out with forward, reverse and internal primers for RD4, RD9 and RD10 (Ameni *et al*., 2011). The regions of difference represent the loss of genetic material that arise due to errors in DNA replication, movement of mobile genetic elements, mycobacteriophage-mediated transduction or recombination between adjacent homologous DNA fragments with loss of the intervening sequence. Some of these large sequence polymorphisms (LSPs) have been found to be restricted to one MTBC strain or subspecies while others appeared to be differentially distributed among the MTBC groupings. These data have been used to develop a PCR-based method for accurately differentiating several MTBC groupings. Several PCR primer pairs specific to the loci were used which include 16S rRNA, Rv0577, Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), Rv3120 (RD12), Rv2073 (RD9), Rv1257 (RD13), IS1561 (MiD3) and TbD1 (Mamo, 2014).

Selective amplification of the 16S rRNA gene was performed on several MTBC and NTM strains and the primers amplified a DNA fragment from all the tested mycobacteria. This gene was therefore chosen to provide the positive control when evaluating mycobacteria by PCR. The Rv0577 gene was found to be an MTBC restricted gene. Primers were designed that could specifically and consistently amplify the Rv0577 coding region and this could therefore be used as a genotypic marker for the MTBC and could be used to distinguish the MTBC species from NTM species. *IS*1561 (MiD3) a transposase pseudogene fragment was found to be positive for all MTBC isolates except *M. microti*. Its absence therefore could serve as a good indicator for *M. microti*. Deletion of a 12.7kb Rv1510 gene (RD4) could serve as an indicator for *M. bovis* while it is present in *M. tuberculosis*, *M. africanum* and *M. microti*. On gel electrophoresis, *M. bovis* shows a band of 446bp while *M. tuberculosis* shows a band of 335bp. The Rv3877 and Rv3878 (RD1 locus) was selectively absent in *M. bovis* BCG. In general, this MTBC PCR typing panel provides an advanced approach to determine the subspecies of MTBC isolates and to differentiate them from clinically important NTM species (Mamo, 2014).

Spacer oligonucleotide typing (spoligotyping): Spoligotyping is a PCR-based molecular method developed to detect and type species/strains of the MTBC. Spoligotype profiles are assigned according to the spoligotype database (SpolDB4) Mbovis.org. The genomes of MTBC carry a single region on the chromosome called the DR locus and DNA polymorphism on this DR locus

allows for strain typing. The DR locus spans up to 5kb and represents 0.1% of the MTBC genome. The MTBC DR region comprises multiple well-conserved 36bp DR regions interspersed with non-repetitive spacer sequences varying in length from 34-41bp. One DR and its neighbouring non-repetitive space are termed "Direct Variant Repeat (DVR)". *M. tuberculosis* strains vary in the number of DRs, in the presence or absence of particular spacers and the vast majority of the *M. tuberculosis* strains contain one or more *IS*6110 elements in the DR region (Fentahun and Luke, 2012; Mamo, 2014).

The spacers in contrast to the DRs are usually present once in the DR region and more than 100 different spacer sequences have been identified in the MTBC and 43 of them have been selected for use in spoligotyping. When the DR regions of several strains were compared, it was observed that the order of the spacers is about the same in all strains but deletions and/or insertions of spacers and DRs occur. This polymorphism at DVR has been exploited to type and distinguish MTBC strains for epidemiological studies. In this method, the whole DR region is amplified and labeled by PCR using DR-specific primers and the presence of any of a set of 43 different spacers is determined by hybridization of the amplified DNA to 43 spacer oligonucleotides which are covalently linked to a membrane. Later detection of hybridization signals is done by the enhanced chemiluminescence (ECL) detection system and a reaction resulting in the emission of light which can be detected by autoradiography of the membrane (Mamo, 2014).

Spoligotyping applied to culture is simple, robust and highly reproducible. Spoligotyping is a useful method for screening and epidemiologic control of TB dissemination, particularly when results are required quickly such as identification of outbreak, contact tracing of TB, prevent further spread of the disease or in the management of transmission of multidrug-resistant TB especially in restricted high-risk situations such as prisons, schools and hospitals (Mamo, 2014). The sensitivity and specificity of this technique has been found to be 96 and 98%, respectively with the clinical samples. Spoligotyping has the clearest advantages over *IS*6110-RFLP typing, in principle, it can be used simultaneously for the detection and typing of MTBC bacteria in one assay and requires viable organisms (Fentahun and Luke, 2012) being significantly less technical demanding than RFLP fingerprinting with a much shorter turnaround time. In addition, the

degree of differentiation achieved by spoligotyping is higher than that of *IS*6110-RFLP for *M. bovis* which usually contains one or two *IS*6110 copies. One of the major drawbacks to spoligotyping is that it can only identify polymorphisms within the DR cluster whereas RFLP typing can detect genetic differences arising from multiple loci (Mamo, 2014).

Molecular typing of the *M. bovis* strains isolated from the slaughtered animals in Ethiopia identified four different spoligotype patterns which previously have been shown as common in several regions of Ethiopia. Three of these four spoligotypes (SB1176, SB1477 and SB0133) carry a specific spoligotype feature (spacers 4–7 missing) typical for members of a clonal complex identified as *M. bovis* African 2 (Af2) so far only found in East Africa (Berg *et al*., 2011). The fourth spoligotype (SB0134) does not belong to the Af2 clonal complex and is therefore likely to have a different epidemiological history. *M. bovis* isolates with spoligotype SB0134 has been found also in Europe. However, the phylogenetic relationship between the African and European strains of SB0134 is not known. Future chromosomal sequencing may shed light over their relationship (Firdessa *et al*., 2012).

Proper epidemiologic and phylogenetic inferences are not always an easy task due to lack of understanding of the mechanisms behind the mutations leading to the polymorphism of these genomic targets. It was demonstrated that phylogenetically unrelated MTBC strains could be found with the same spoligotype pattern as a result of independent mutational events (homoplasy) which is an observation that corroborates the fact that spoligotyping is prone to homoplasy to a higher extent than the MIRU-VNTRs (Viegas, 2015).

Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing: MIRU-VNTR typing relies on PCR-amplification using primers specific for the flanking regions of the VNTRs and on the determination of the sizes of the amplicons after electrophoretic migration. As the length of the repeat units is known, these sizes reflect the numbers of the amplified MIRU-VNTR copies. The result is a numerical code corresponding to the repeat number in each VNTR locus. Such numerical genotypes are intrinsically portable and are thus particularly convenient for both intra- and inter-laboratory comparative studies. Initial

VNTR typing systems for MTBC strains made use of very limited sets of loci including exact tandem repeats (ETR), mycobacterial interspersed repetitive units (MIRUs) and sets of Queen's University Belfast (QUB) VNTRs. MIRU-VNTR 24-loci typing has been proposed for international standardization based on analysis of the clonal stability and evolutionary rates of MIRU-VNTR markers in the genetic lineage of tubercle bacilli collected worldwide. In addition, the method has been improved as high-speed automated genotyping system with the use of multiplex PCRs for the target MIRU-VNTR loci on a fluorescence-based DNA analyzer with computerized automation genotyping (Mamo. 2014).

Mycobacterial interspersed repetitive units-variable number tandem repeat typing utilizes variations in repetitive sequences which are not under selection pressure and evolve relatively rapidly making them suitable for prospective molecular epidemiological investigations and surveillance purposes. MIRU-VNTR genotyping using a standardized set of 24 loci has become an international standard and is currently in use for routine *M. tuberculosis* genotyping in many European countries and globally. The discriminatory power of MIRU-VNTR typing generally depends on the number and set of loci used and could be further improved by inclusion of hypervariable loci especially in settings where highly homogenous genotypes (East Asian lineage e. g. Beijing) prevail (Nikolayevskyy *et al*., 2016).

The *M. bovis* isolates were further discriminated by MIRU-VNTR typing which suggested low diversity within each isolated spoligotype. This is in agreement with other studies as in high prevalence settings the genetic diversity of sampled strains is usually low reflecting on-going high local transmission events/rates. However, in comparison with genotypes of *M. bovis* strains that were collected from other parts of Ethiopia about 2–3 years earlier some variations were observed and possible epidemiological links between specific regions of the country can be suggested. Two distinct MIRU-VNTR features were seen within spoligotype SB0133 of which the one identified in central Ethiopia was also seen in sites along the Rift Valley from Woldiya in the North to Negelle in the South. The other genotype of SB0133 was isolated from Jinka (Southwestern Ethiopia) and Ghimbi (West Ethiopia). Similarly, the single strain of type SB1176 collected in Jinka deviated in the MIRU-VNTR pattern from those seen in Woldiya and central

Ethiopia. However, no genotype diversity was seen between the one isolate of SB0134 from Gondar (Northwestern Ethiopia) when compared to *M. bovis* strains of SB0134 isolated in central Ethiopia (Firdessa *et al*., 2013).

Epidemiological links can be suggested for strains collected within the North/Central Ethiopian highlands and through the Rift Valley down to Negelle based on overall molecular typing results of *M. bovis* collection. While strains isolated from Jinka and Ghimbi in the South/West of Ethiopia diverged. This may reflect on past and ongoing cattle movements and trading patterns in Ethiopia. The low strain frequency from some collection sites means that, these results should be interpreted with care. Nevertheless, this genotype comparison gives an indication of the diversity and the epidemiology of *M. bovis* in Ethiopia. Future studies may give better clarification on this subject. The 24-loci MIRU-VNTR typing employed showed no diversity in at least 14 of the 24 loci (variation seen between spoligotypes). This is in agreement with other studies which have shown that VNTR typing of only a selective set of loci is informative for generating a better resolution between *M. bovis* isolates. This suggests that any future MIRU-VNTR typing of *M. bovis* strains from Ethiopia could consider including only loci that show diversity in its local strain population, however, a more comprehensive evaluation of what loci to select is recommended (Firdessa *et al*., 2012).

The lineages of MTBC are described here. A novel new lineage of *M. tuberculosis* (Lineage 7: East African 2, Woldiya lineage, Northern Ethiopia) which is strongly associated with the Horn of Africa in addition to the already existing six known lineages: Lineage 1: Indo-Oceanic lineage; Lineage 2: East Asian lineage; Lineage 3: East African-Indian (EAI) lineage; lineage 4: Euro American-African lineage; Lineage 5: West African-1 lineage; and Lineage 6: West African-2 lineage (Firdessa *et al*., 2013; Mamo, 2014; Viegas, 2015).

The genome sequence analysis in Ethiopia over the years 2006-2010 revealed the presence of lineages 1, 3, 4 and 7 (Firdessa *et al*., 2013). The most common spoligotype identified from farmers in Sellale, central Ethiopia was the T family and the predominant lineage was the Euro-American. Similarly, previous studies in Ethiopia showed that T and CAS genotypes were the

dominant families isolated from TB positive farmers (Ameni *et al*., 2013). The T, CAS and U families of *M. tuberculosis* isolates from pulmonary TB patients in Amhara Region of Northwestern Ethiopia were the most common sublineages reported (Yimer *et al*., 2013).

2. 11. Differential Diagnosis

Tuberculosis is difficult to diagnose on clinical examination alone because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection (Radostits *et al*., 2007). TBL lesions can be important causes of condemnation in cattle and swine at abattoir inspection representing significant important economic losses (Cardoso-Toset *et al*., 2015). Mycobacteriosis is associated with *M. tuberculosis*, the *M. avium*-intracellulare complex and atypical mycobacteria. In cattle, it should be differentiated from (Radostits *et al*., 2007; Anon, 2009):

► Contagious bovine pleuropneumonia (CBPP),
► *Pasteurella* or *Corynebacterium pyogenes* Pneumonia,
►Lung abscess due to aspiration pneumonia,
►Pleurisy and pericarditis following traumatic reticulitis,
►Chronic contagious bovine pleuropneumonia,
►Upper respiratory disease,
►Actinobacillosis,
►Bovine leukosis,
►Chronic aberrant liver fluke infestation,
►Lymphadenopathy/caseous lymphadenitis or melioidosis/, and
►Other causes of mastitis

Tuberculosis in swine is usually so benign that cases do not present themselves as clinical problems and are found only at necropsy (Radostits *et al*., 2007). MAC, MTBC and *Rhodococcus equi* have been reported as the species most frequently associated with TBL lesions and these infections typically result in indistinguishable gross lesions in swine. Other genera such as *Corynebacterium* spp., *Streptococcus* spp., or *Staphylococcus* spp. have also been

isolated in caseous lymphadenitis in swine, highlighting the potential diversity of pathogens that might be associated with TBL lesions in this species. This diversity of microorganisms together with the zoonotic nature of several of them are factors that should be considered by public health authorities (Radostits *et al*., 2007; Cardoso-Toset *et al*., 2015):

► *Mycobacterium avium* complex (MAC)

►*Mycobacterium tuberculosis* complex (MTBC)

►*Rhodococcus equi,*

►*Corynebacterium* spp.,

►*Streptococcus* spp., and

►*Staphylococcus* spp.

2. 12. Treatment

Treatment of infected animals is rarely attempted because of the high cost, lengthy time and the larger goal of eliminating the disease (OIE, 2009). Antimicrobial treatment has been attempted in some species but the treatment must be long term and clinical improvement can occur without bacteriological cure. The risk of shedding organisms, hazards to humans and potential for drug resistance make treatment controversial. In some countries it may be illegal (Anon, 2009).

The treatment of animals with TB has undergone some examination and claims have been made for the efficiency of long-term oral medication with isoniazid both as treatment and as prophylaxis because of the progress being made in the treatment of human TB with such drugs as isoniazid, combinations of streptomycin and para-aminosalicylic and other acids. It is not a favoured option in eradication-conscious countries (Radostits *et al*., 2007). The first line of anti-TB drugs in humans are isoniazid, rifampicin, pyrazinamide and ethambutol. In addition, the second injectable drugs are capreomycin, kanamycin, amikacin and any fluoroquinolone (Viegas, 2015).

2. 13. Control

Bovine TB remains prevalent in sub-Saharan African countries where national control strategies are often non-existent though it is eradicated or controlled in most parts of the developed world (Tschopp *et al.*, 2010; Muller *et al*., 2013). Control in a herd rests on removal of the infected animals, prevention of spread of infection and avoidance of further introduction of the disease (Radostits *et al*., 2007). The herd will be considered free of infection only when no TB has been found over a five-year period (Maas *et al*., 2002).

Retests of the herd should be carried out at intervals of 3-months until a negative test is obtained. A further test is conducted 6-months later and if the herd is again negative then it may be classed as free of the disease. Subsequent check tests should be carried out annually (Radostits *et al*., 2007). Over 2years period, 4-negative whole-herd tests will be required for the lifting of movement restrictions (2 consecutive tests with negative results in a year). A 5th tuberculin test will be done 5-8years later for a herd to become **"certified-free"**. Movement controls meant that stock from infected herds could only move to slaughter or to other infected herds. Controls will be imposed until the infection will be eradicated. Tail tags identified the owner and property of origin of cattle and enabled traceback of all cattle that were moved, sold or slaughtered (Australian Government, 2012).

The standard control measure applied to bTB is test-and-slaughter (De Kantor *et al*., 2008) and it can also be controlled by test-and-segregation method. However, some countries use test-and-segregation programmes during the early stages of eradication and switch to test-and-slaughter methods in the final stage (Anon, 2009). The test-and-slaughter policy, a disease control programme based on slaughter of positive reactor animals, is not properly implemented despite the knowledge that this policy has successfully reduced the prevalence of bTB. The lack of public finances is obstacles in the control of bTB in many countries. Bovine TB is prevalent due largely to absence of effective disease control methods including regular milk pasteurization and slaughterhouse meat inspection in most countries in Africa (Muller *et al*., 2013).

Proper meat inspection is not effectively carried out due to the inadequacy of the veterinary service sector although meat is recommended to be abattoir-inspected before entering markets (Katale *et al*., 2012). This situation is exacerbated by the presence of multiple additional risk factors such as **human behaviour** and the high prevalence of HIV infections. HIV/AIDS is thought to facilitate transmission and progression to active disease of any form of TB. Some studies showed a significantly increased proportion of *M. bovis* infections among HIV–co-infected TB patients compared with HIV-negative TB patients (Muller *et al*., 2013). It is only Algeria, Burkina Faso, Cameroon, Morocco, Namibia and South Africa out of 48 countries in Africa that apply a test-and-slaughter policy as a control measure and consider bTB as a **notifiable disease**. In Tanzania, lack of clear policies on how bTB can be controlled and the failure of health authorities to recognize *M. bovis* as cause of TB hinder the control of the disease (Katale *et al*., 2012). Bovine TB control and its eventual eradication will have a positive impact both on the economy and public health in countries where the disease prevails (De Kantor *et al*., 2008).

The time-temperature combinations necessary to destroy *M. avium* and *M. bovis* in certain meat products have to be determined. The survival of these mycobacteria had not yet been well studied. In the USA, most swine TB is caused by the MAC but most cases of bTB and some cases of swine TB are caused by *M. bovis. M. bovis* is destroyed at temperatures 6-7°C lower than those necessary to destroy the MAC. Above 60°C, no viable units remained after Vienna sausages had reached the designated temperatures. The time exposure was less than 10 minutes. Treatment with a 2% phenolic disinfectant (amphyl) followed by formaldehyde vapour was effective in disinfecting equipments contaminated by meat emulsions containing *M. bovis* (Kleeberg, 1984; Thoen, 2013).

Control of mycobacterial infection in swine is difficult since no vaccine is available (Thoen, 2013). *M. bovis* infection in swine usually results from the feeding of infected milk, skim milk or whey to swine or allowing cattle and swine to graze the same pasture. The first step in the control of TB in a swine herd is to remove the source of infection and then to test-and-slaughter the reacting animals. The non-progressive nature of the disease means that transmission between

swine is unlikely to occur to a significant extent except perhaps in breeding animals. Farm attendants should be checked as they may serve as a source of infection (Kleeberg; 1984; Radostits *et al*., 2007). Culling on the basis of skin sensitivity in swine herds with enzootic infection of MAC is usually not practical because of the high prevalence of infection and high environmental contamination. Control procedures concentrate on the reduction of environmental contamination by a change from bedding to solid or slatted floors, frequent washing and disinfection of pen floors, separate weaner and grower facilities and exclusion of wild birds from buildings and feed areas (Songer *et al*., 1980; Radostits *et al*., 2007).

The sources of infection were found to be infected wood shavings, sawdust or peat used for bedding especially in farrowing buildings based on tuberculin skin test responses and examinations of tissues collected at slaughter. Researchers examined some herds with a high incidence of swine TB and found that these animals were kept on sawdust or bark shavings. Other researchers had discovered that these materials frequently contain a high concentration of *M. avium*. When the bedding material was removed for a new group of swine then the incidence of swine TB in that herd dropped to zero. There are few options for eliminating TB from infected herds. Little is known about decontamination of infected soil since mycobacteria can survive in this environment for at least 4years. To avoid such problems, concrete lots should be used whenever possible. Concrete surfaces and equipments including farrowing crates and feeders must be disinfected. Mycobacterial infection will recur if the sources of infections cannot be effectively decontaminated or if replacement stock is not separated from the source (Songer *et al*., 1980; Weeks, 1985; Thoen, 2013).

Producers may choose to endure the 6-month period until all exposed swine have been slaughtered if the source of infection such as infected bedding (e.g., peat) can be determined and eliminated. Mycobacterial disease increases the need for mandatory identification of swine for slaughter. When TB is diagnosed, a producer is free to send the swine to slaughter through a public market and request that the packer and other producers share the economic loss. The ability to trace swine with mycobacterial infection to the herd of infection origin is useful to solve this problem (Thoen, 2013).

The current control measures with regard to bTB in swine involve compulsory slaughter of affected swine herds and in some cases at-risk neighbours. The earlier the disease is detected, the less spread will occur and the fewer holdings will be affected and require culling (White, 2016). Farmers are advised not to use sawdust or wood shavings as bedding material in the farrow house or where young swine are being raised. Further, it is recommended that facilities in which swine are housed are regularly disinfected and that contact between swine and poultry or wild birds be minimized (Weeks, 1985).

Sanitation and disinfection may reduce the spread of the agent within the herd. *M. bovis* is relatively resistant to disinfectants and requires long contact times for inactivation. Effective disinfectants include 5% phenol, iodine solutions with a high concentration of available iodine, glutaraldehyde and formaldehyde. In environments with low concentrations of organic material, 1% sodium hypochlorite with a long contact time is also effective. *M. bovis* is also susceptible to moist heat of 121°C (250°F) for a minimum of 15minutes (Anon, 2009).

Culling to reduce the population density can decrease transmission of TB. However, culling may have unanticipated effects such as increasing the dispersal of the remaining members of a species. **Barriers** can be used around hay storage areas to prevent wildlife access. In addition, **biosecurity measures** on farms decrease interactions between wildlife and domesticated animals (Anon, 2009; Muller *et al*., 2013).

Vaccination is practiced in human medicine but it is not widely used as a preventive measure in animals. The efficacy of existing animal vaccines is variable and it interferes with testing to eliminate the disease. A number of new candidate vaccines are currently being tested (OIE, 2009) though effective bTB vaccines are not currently available for cattle. However, new vaccines are being developed and tested particularly for wildlife reservoirs. Rodent control may also be advisable on affected farms; meadow voles and house mice can be infected experimentally and voles shed *M. bovis* in faeces (Anon, 2009).

2. 14. Prevention

Infection with *M. tuberculosis* may prevent super-infection with *M. bovis* since the first infection will give rise to a measure of immunity against the second when TB is widespread in a population (Kleeberg, 1984). **Hygienic measures** to prevent the spread of infection should be instituted as soon as the first group of reactors is removed. Feed troughs should be cleaned and thoroughly disinfected with hot 5% phenol or equivalent cresol disinfectant. Feed and water troughs should be emptied and similarly disinfected. Suspicious reactors are being held for retesting and should be isolated from the remainder of the herd. Separation of infected and susceptible animals by a **double fence** provides practical protection against spread of the disease. It is important that calves being reared as herd replacements be fed on TB-free milk either from known free cows or pasteurized. Rearing calves on skim milk from a communal source is a dangerous practice unless the skim milk is pasturized. All other classes of livestock on the farm should be examined for evidence of TB. Farm attendants should be checked as they may provide a source of infection. If a number of reactors are culled, attention must be given to the possibility of infection being reintroduced with replacements. Replacements should come from **accredited herds (those herds that their TB status is known)**. Failing this, the animals should be tested immediately, isolated and retested in 60 days. Infection from other herds should be addressed by preventing communal use of watering facilities or pasture and by maintaining adequate boundary fences. It is inadvisable to attempt a control programme until it can be guaranteed that all animals can be gathered, identified, tested and segregated which is a difficult proposition in cattle run on extensive range country with little manpower and few fences (Radostits *et al*., 2007).

The preventive use of anti–TB drugs in feed is either illegal or of unknown value. Preventing the disease in non-infected herds is more effective than trying to eliminate the disease from infected herds. It is important not to raise swine and poultry in close proximity on the same premises. Feeding uncooked garbage, unpasteurized milk or other materials that might contain viable mycobacteria to swine must be avoided. Breeding stock should be purchased from herds free of TB, i. e. those in which no lesions of TB are found at slaughter. Efforts should be made to

prevent all contact between swine and wild birds. The potential for transmission of MAC from infected wild birds to swine is probably low but must be considered. Swine should not be housed in old poultry buildings unless they have first been thoroughly cleaned and disinfected using **tuberculocidal disinfectants**. The use of wood shavings, sawdust or peat for bedding especially in farrowing buildings should be eliminated (Thoen, 2013).

2. 15. Eradication

Control and eradication programmes are targeting cattle population (Tschopp *et al*., 2011). Once eradication is nearly complete, postmortem meat inspection (slaughter surveillance with tracing of infected animals), intensive surveillance including on-farm visits, systematic individual testing of cattle and removal of infected and in-contact animals as well as movement controls may be a more efficient use of resources which have been very successful in reducing or eliminating TB (Anon, 2009). Postmortem meat inspection of animals looks for the tubercles in the lungs and lymph nodes. Detecting these infected animals prevents unsafe meat from entering the food chain and allows veterinary services to traceback to the herd of origin of the infected animal which can then be tested and eliminated if needed. A state of virtual eradication has been achieved in many countries for years but minor recrudescences occur (Radostits *et al*., 2007; OIE, 2009).

Pasteurization of milk of infected animals to a temperature sufficient to kill the bacteria has prevented the spread of TB to humans (OIE, 2009) because milk is a potent vehicle for disseminating *M. tuberculosis* and *M. bovis.* It also shows that in all countries which seriously intend eradicating TB, it is necessary to culture milk samples and TBLs more often. Lesions could also be sent to special laboratories for isolation and identification (Kleeberg, 1984).

Eradication programmes in developed countries have reduced or eliminated TB in cattle and human TB is now rare; however, the occurrence of *M. bovis* in wildlife reservoir hosts complicates eradication efforts and can make complete eradication difficult (Anon, 2009). The

methods used have depended on a number of factors but ultimately the test-and-slaughter policy has been the only one by which effective eradication had been achieved. In the final stages of an eradication programme, a number of problems attain much greater importance than in the early stages of the campaign. Eradication can commence in herds and areas which have a low incidence of TB. These will provide a nucleus of TB-free herd to supply replacements for further areas as they are brought into the eradication scheme. **When the overall incidence of TB is 5% or less, compulsory testing and the slaughter of reactors is the only satisfactory method of eradication**. A combination of lines of attack is usually employed (Radostits *et al*., 2007).

Recent epidemiological models suggest that once *M. bovis* is introduced, the probability of becoming established in a wildlife population is at least 10%. Furthermore, once established *M. bovis* can physically be impossible to eradicate. Recent analysis found that, to achieve eradication in Michigan for example would cost at least US $1.5 million annually over the next 30years (Miller and Sweeney, 2013).

3. MATERIALS AND METHODS

3. 1. Description of the Study Areas

The study *Woredas* (Districts) in North Shoa Administrative Zone of Amhara Region were purposively selected based on their potential of dairy cattle which consist of Zebu (Z), Holstein Friesian (HF), crosses of Z and HF, Horro and Jersey breeds. The *Woredas* were *Angolellana-Tera, Basona-Werana* and *Debre Berhan*. The current study was conducted with the aim to estimate the prevalence of bTB in dairy cattle originated from 96 dairy farms (n = 96). The three *Woredas* represent a milkshed of North Shoa Administrative Zone of Amhara Region in central Ethiopia and hence collectively called Debre Berhan milkshed. Next to *Woredas*, there are *Kebeles* in decreasing order of administrative importance. *Kebele* here refers to the smallest administrative unit in Ethiopia. The rural *Kebeles* are called Peasant Associations (PAs). There are sub-peasnat associations within PAs. The study duration was six months that extended from July to December 2018.

Debre Berhan is the capital, administrative and economic center of North Shoa Administrative Zone. The study dairy farms were located in *Debre Berhan* (n = 55), *Angolellana-Tera* (n = 16), *Basona-Werana* (n = 24) and Bishoftu (n = 1). Dairy farms included in the study originated from 10 *Kebeles*: seven *Kebeles* from *Debre Berhan* (01, 02, 04, 06, 07, 08 and 09) and one sub-peasant association representing 38 households, two *Kebeles* from *Angolellana-Tera* (2 large farms and one sub-peasant association representing 14 households) and one *Kebele* from *Basona-Werana* (one sub-peasant association representing 24 households).

The areas to study swine TB were purposively selected because they are the sources of swine production for different regions of Ethiopia. The study to estimate the prevalence of bTB in swine was conducted in nine (n = 9 farms) farmed swine of Alage Agricultural, Technical and Vocational Education and Training (ATVET) College (n = 1 farm) and Bishoftu, East Shoa

Zone, Oromia Region (n = 5 farms), and Akaki Kality subcity in Addis Ababa (n = 3 farms), central Ethiopia. There was one dairy farm in Bishoftu where dairy cattle and swine were farmed together. Moreover, an abattoir based study using postmortem examination of suspected TB lesions was conducted on those farmed swine which were bovine PPD reactors in SICT test (n =5) and those brought to Addis Ababa Abattoirs Enterprise (AAAE) for slaughter for pork (n = 635). The study duration was from September 2016 to December 2017.

Debre Berhan milkshed: Debre Berhan milkshed is located in the Northeast direction of Addis Ababa at a distance of 130km. Based on the 2007 national census conducted by the Central Statistical Agency of Ethiopia (CSA, 2007), *Debre Berhan*, *Angolellana-Tera* and *Basona-Werana Woredas* have got a total human population size of 268, 510 of whom 133, 069 are women and 135, 441 are men. Geographically, the milkshed is located at 9°41'N latitude and 39°32'E longitude at an altitudinal range of 2600-2840 meters above sea level (masl). It is one of the coolest places located at sub-tropical zone of Ethiopia. All weather concrete road starts in Addis Ababa, passes through Debre Berhan and extends as far as Dessie and Tigray. The households experienced mixed crop-livestock production system within an average small private landholding of 1ha per family. Over 90% of the agricultural output is still produced by individual subsistence smallholders who have "farming rights" over the land they till (Gryseels and Anderson, 1983).

The soil types are mainly red and dark-brown. The rainfall distribution in the study areas is typically bimodal characterized by long and short rainy seasons extending from June to September and February to May, respectively. The average annual rainfall ranges from 900 to 1150mm with 70% falling between July and September. The minimum, maximum and average air temperatures are 16^0C, 27.5^0C and 21.75^0C, respectively. The mean relative humidity of the study areas is in the range of 35-68%. Relative humidity is the lowest from November to March (Gryseels and Anderson, 1983).

The milkshed is known for its potential of local Zebu cattle, sheep, goats, equines, poultry, honey bee colonies and few fishery resources. Grazing on communal lands and fallow plots constitute

the main sources of livestock feed. It is supplemented with straws, crop residues and stubble grazing. Work oxen and cows in milk are supplemented with hay in the dry season and straw in the wet season. Small amount of concentrate is provided to the milking cows on daily basis. The major crops grown are teff (*Eragrostis tef*), wheat, barley, beans, peas, chick peas, lentils, oil crops, pulses, root crops and few highland fruit and vegetable varieties (Gryseels and Anderson, 1983).

***Addis Ababa Abattoirs Enterprise /AAAE/*:** It is a company located in the heart of Addis Ababa City Administration, the capital of Ethiopia. The company is mainly working in slaughtering of cattle, sheep, goats, camel and swine. It is located at *Kera* (*Kera* means a place for animal slaughter), *Kebele*/*Woreda* 08/09 of Kirkos subcity which has got one satellite abattoir in Akaki Kality subcity. There are 33 meat inspectors and nearly 1000 labourers to ensure the distribution of wholesome beef, mutton and meat to residences of the city as well as pork for foreigners **(**Kirkos Office of Urban Agriculture, 2019).

Alage Agricultural, Technical and Vocational Education and Training /ATVET/: It is situated at 217 kilometers Southwest of Addis Ababa in the vicinity of the Abijata and Shala lakes of the Ethiopian Rift Valley**.** Its GPS location is $7^{O}30'$ N latitude and 38°30' E longitude. The area covers 4200ha of land with an altitude of 1600 masl characterized by mild subtropical weather with minimum and maximum temperatures of $11^{O}C$ and $29^{O}C$, respectively. The area experiences bimodal rainfall distribution with an annual average range of 700-900mm. The three defined seasons based on rainfall distribution are short rainy season extending from March to April, long rainy season extending from June to September and long dry season extending from October to January. The dominant soil type is black clay soil (vertisol) and sand silt clay with a pH of 7.9 (ATVET Office, 2019).

Akaki Kality in Addis Ababa: It is a large subcity among the 10 subcities of Addis Ababa City Administration. It is situated to the South part of Addis Ababa. As of 2019 its human population is estimated to be 220,740 of which 114,095 are females and 106,645 males. It is one of the most densely populated subcities. Most of the *Kebeles*/*Woredas* are found at the out skirts of the city. *Akaki Kality* is located in GPS coordinates of 8° 53' 45" N latitude and 38° 47' 21" E longitude at an elevation of 2140 masl. It is 20 kilometers far from the city's center. Many industries are

found in this subcity and it is the industrial zone of Addis Ababa as well as the country. The daily average temperature ranges from 9.9-24.6°C and the annual mean rainfall is 1254mm. There are two distinct seasonal patterns namely the main wet season locally known as *Kiremt* that extends from June to September contributing about 70% of the total annual rainfall. The minor rainy season locally known as *Belg* contributes moisture to the region from mid-February to mid-April. The remaining five months are dry season (Akaki Kaliti Office of Urban Agriculture, 2019).

Bishoftu: It is located at 47 kilometers Southeast of Addis Ababa in a geo-reference coordinates of 8°44'5" N latitude and 39°0'31" E longitude. It is situated at an altitude of 1878masl. The national census by the Central Statistical Agency (CSA, 2007) of Ethiopia reported a total population size of 99,928 of whom 52,068 were women and 47,860 were men. The average annual temperature is 18.7^{O}C with average annual minimum and maximum air temperatures of 14°C and 26°C, respectively. Its average annual rainfall is 866mm. Livestock production is an integral part of the system. Cattle, small ruminant, poultry, swine and equines are the major livestock species kept with fast growing smallholder dairy production. Bishoftu is known for its lakes including Lake Bishoftu, Lake Hora, Lake Koriftu to mention but few (Bishoftu Office of Urban Agriculture, 2019).

The following figures (8 & 9) indicated the current study areas:

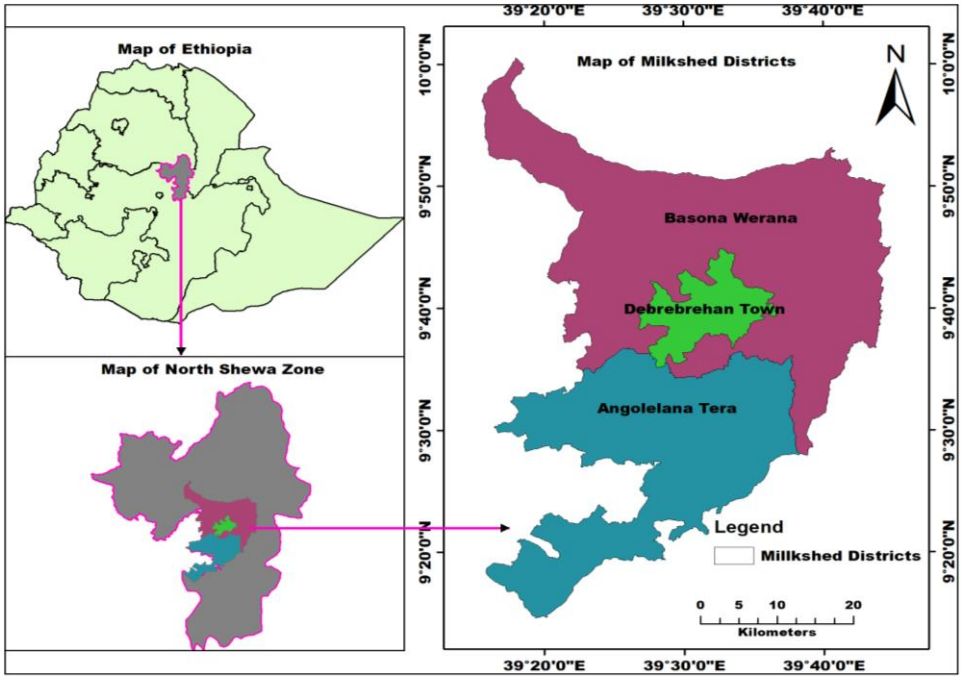

Figure 8. Map of Debre Berhan Milkshed to study bTB (ArcGIS, 2021)

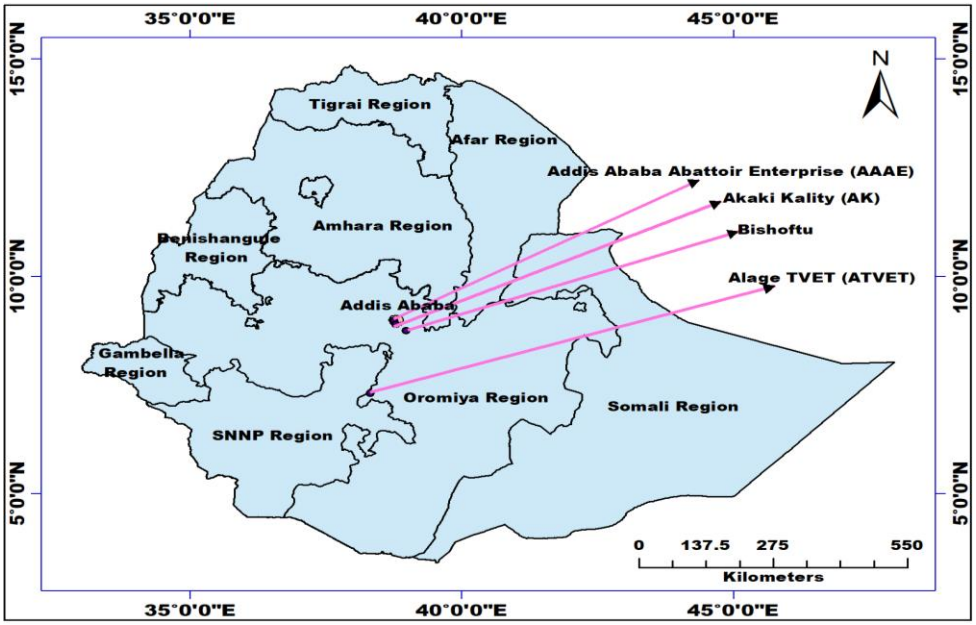

Figure 9. Map of the study areas of swine TB in central Ethiopia (ArcGIS, 2021)

3. 2. Study Population

The recruitment of dairy herds within the study areas was based on the existence of high numbers of Holstein-Friesian and their crossbred cattle as well as variation in herd size with the latter being categorized into three groups: small [1–10 cattle], medium [11–50 cattle] and large [>50 cattle] herds based on the method followed by Firdessa *et al*. (2012). In general, the sample dairy farms were categorized into small (85%: 82/96), medium (13%: 12/96) and large (2%: 2/96) based on the herd size they possessed. The study was carried out on 625 heads of dairy cattle originated from 96 herds. There were 13 women (14%: 13/96) and 83 men (86%: 83/96) household heads who participated in this study.

Working definitions for farm characteristics (sub-categorization):

- **Households** in the context of this study refer to dairy farmers managing their dairy cattle within their own individual residential compound.
- **Dairy farms** in the context of this study refer to those farms managed on the land allocated for dairy cattle farming purpose.

Households (n = 78) resided in three sub-peasant associations namely *Angolella_Seminesh* (*Basona-Werana*), *Kormargefiya* (*Angolelana-Tera*) and *Zanjira* (*Debre Berhan*) participated in this study. Dairy cattle involved in this study were 256 heads categorized into small (99%: 77/78) and medium (1%: 1/78) herd sizes.

Dairy farms (n = 18) included in the present study were found in Bishoftu town, *Debre Berhan* and *Angolelana-Tera* districts. Dairy cattle involved in this study were 369 heads categorized into small (28%: 5/18), medium (61%: 11/18) and large (11%: 2/18) herd sizes.

Dairy cattle herd owners (n =96), swine herd owners and their neighbours (n =50) and human health professionals (n = 19) were also involved for face-to-face interview. Dairy farmers and their family (n = 100) resided in Debre Berhan milkshed who manifested the constitutional signs of TB and referred to the main catchment Debre Berhan Zonal Referral Hospital (DBZRH) were

the study population in the epidemiological/zoonotic link of this study targeting to isolate *M. bovis* from their sputa and hypothesized its direction of transmission.

The sizes of swine herds were categorized into small (≤20 swine), medium (21-50 swine) and large (>50 swine) based on the methodology followed by Goraga *et al*. (2017). Hence, the swine herds recruited in the present study were categorized into small (22%: 2/9), medium (33%: 3/9) and large (45%: 4/9). However, the swine breeds in Ethiopia are not characterized yet.

The husbandry practices of cattle and swine in the study areas were characterized as semi-intensive and intensive systems of management.

3. 3. Sample Size Determination

A cluster in this study had an equivalent contextual meaning to a farm, a herd or a household. The sample size for both dairy cattle and swine skin test was determined by **o**ne-stage cluster sampling considering an individual cluster as a unit of sampling. The first step in determining sample size in one-stage cluster sampling is prediction of the average number of animals per cluster. The appropriate formula to calculate the sample size for a 95% confidence interval and 5% precision by Thrusfield (2007) was adopted:

$$g = \underline{1.96^2\{(nVc) + Pexp\ (1 - exp)\}}/nd^2$$

Where, g = number of clusters to be sampled; 1.96 = multiplier of the 95% confidence interval (CI); n = predicted average number of animals per cluster; Vc = between-cluster variance; Pexp = expected prevalence and d = desired absolute precision

Sample size to dairy cattle: n = the average number of dairy cattle per cluster = 4; Pexp= expected prevalence = 2.7% (Shimeles, 2008); d= desired absolute precision = 5% and Vc = assumed between-cluster variance = 0.02 (previous experience in bTB studies of central Ethiopia). Then, the numbers of clusters were computed as follows:

$$g = \underline{1.96^2\{(4 \times 0.02) + 0.027\ (1 - 0.027)\}}/4 \times (0.05)^2$$

***Given values*:** g = 41 clusters per site x 3 *Woredas* = 123 clusters. The number of animals to be tested were from 123 clusters x 4 heads of dairy cattle = 492 heads of dairy cattle. In total, 96 herds representing 625 heads of dairy cattle were included in the study.

Sample size to swine: n = the average number of swine per cluster = 25; Pexp= expected prevalence = 50% (since this study is the first of its kind in Ethiopia); d= desired absolute precision = 5% and Vc = assumed between-cluster variance = 0.02 (previous experience in bTB studies of central Ethiopia). Then, the numbers of clusters were computed as follows:

$$g = \underline{1.96^2 \{ (25 \times 0.02) + 0.5 (1-0.5)}\} / 25 \times (0.05)^2$$

Accordingly, the calculated numbers of clusters to be sampled were nearly 46 representing 1150 heads of swine. However, the actual number of clusters representing the study population were 12 and the number of swine in the current study sites were small (G = 464). Hence, the sample size needed an adjustment (Adj) as follows:

$$Adjn = G \times g / G + g = 12 \times 9 / 12 + 9 = 6 \text{ clusters}$$

The required number of swine to be PPD tested was not restricted to 6 clusters. Therefore, 9 out of 12 clusters representing 329 heads of swine that fulfilled the inclusion criteria were tuberculin skin tested. Those swine (n = 135) that were under 3 months of age, late pregnant and near to farrow were not PPD tested.

Necropsy study: Five strong PPD reactors' swine at > 2 mm cut-off value which were herded with dairy cattle (4 heads) and alone (1 head) in Bishoftu were purchased, slaughtered and inspected in detail at necropsy facility of the College of Veterinary Medicine and Agriculture of Addis Ababa University (CVMA, AAU), Bishoftu, Ethiopia. In addition, 635 heads of swine which were presented for slaughter at AAAE were eligible for both detailed antemortem examination and necropsy. In total, 640 heads of swine were used for the current necropsy study. Antemortem examination and necropsy were conducted from July 2015 to March 2016 for the duration of seven months following the procedure described in FAO (1994).

Human tuberculosis study: This study was carried out by making use of three approaches described hereunder. Generally, 265 subjects consisting of 19 human health professionals who

served as key informants, active TB suspected 100 human patients, 96 dairy herd owners, 50 swine herd owners and their neighbours were participated. Face-to-face interview was presented to 165 participants (19 professionals, 96 dairy herd owners and 50 swine herd owners and their neighbours). The study was carried out by making use of a semi-structured and open-ended questionnaire which was pretested and adjusted for clarity.

The first approach was to conduct key informant interview of human health professionals (n = 19) working with different levels of specializations in different health facilities (hospitals and clinics). Professionals were interviewed to express their opinions and experiences about the current overall status of human TB in Debre Berhan milkshed. The protocol adopted by WHO (2017) was used as the national TB control policy and algorithm by the Ministry of Health (MOH) in Ethiopia. This protocol was followed by all professionals in different health institutions. This survey was helpful to generate general baseline data about human TB to conduct the following subsequent studies.

The second approach was to assess the awareness level of farm owners/attendants about the zoonotic transmission of cattle and swine TB to humans. The sample size for this survey was determined using the formula recommended by Arsham (2006) with the assumption of 5% standard error (SE) at 95% confidence level:

$$n = 0.25/ SE^2$$

Where, n = sample size and SE= standard error (5%); substituting the value in the formula:

$$n= 0.25/ (0.05)^2 = 100$$

Dairy farm owners/attendants (n = 96), however, were interviewed at the time when dairy cattle were tuberculin skin tested. On the other hand, 100 participants pertaining to swine farms would have been interviewed. However, there were few swine farm owners in the study areas in which all of them were participated in this study. Moreover, livestock farmers who resided around the swine farms (neighbours) were also interviewed. In total, 50 volunteer participants from both the farms and their surroundings were interviewed.

The third approach was to point out an epidemiological or zoonotic link study of bTB. Sputa from active TB suspected 100 human patients who were referred to DBZRH from Debre Berhan milkshed were purposively and carefully collected following the protocol /algorithm/ adopted by

WHO (2017). The objective of sputa collection and laboratory analysis was to isolate, type and hypothesize the direction of transmission of *M. bovis* to humans. Sputa were collected by laboratory technologists of the hospital. The inclusion criteria to collect human sputa were cough for two weeks or more, inappetance, weight loss, night sweat, chest pain and haemoptysis (the coughing up of blood). Patients on medication were excluded from the study. It was safely transported in an ice box packed with ice to the TB laboratory at Aklilu Lemma Institute of Pathobiology of Addis Ababa University (ALIPB, AAU).

3. 4. Study Design and Sampling Strategy

A cross-sectional study design was employed to estimate the prevalence of bTB in both dairy cattle and swine together with the associated risk factors precipitating the disease. In addition, this design was also employed for questionnaire survey and sputa collection. Multistage purposive sampling was employed to select the study areas up to *Kebele* /PA level.

The sampling frame containing lists of clusters was established for each study area by the help of local livestock development agents. An individual cluster was taken as a unit of sampling. Clusters were selected by simple random sampling using random numbers. All animals in all herds that fulfilled the inclusion criteria were then included in the study. Before the tuberculin skin test, animals were identified by their ear tags or their names in each of the farms. Temporary ID numbers were written on the back of animals for those without ear tags using an indelible ink that could not be erased at least for a week. All data were recorded in a LogBook before skin test.

Keen observation and discussion using semi-structured and open-ended questionnaire with each farm owner/manager were carried out during tuberculin testing to know basic animal related risk factors such as age, sex, body condition score (BCS), breed (local or exotic), physiological state (open or pregnant), lactation status (dry or lactating), parity (no parity or number of calving),

management (semi-intensive or intensive), herd size (small, medium or large) and farm hygiene. The data collected from farm records/owners were registered in a LogBook before the skin test.

The hygienic status of each farm was judged as poor, medium or good based on aspects such as odour, waste drainage, cleanness of floor and the animals, barn ventilation and light source as well as animal stocking density (herd size) based on the objective criteria adopted by FAO and IDF (2011). Data on animal feed sources and feeding practices were also collected and recorded.

The ages of cattle were categorized into ≤5 and >5 years based on the dentition formula adopted by Mississippi State University (MSU, 2013). BCS of cattle was categorized into poor, medium or good following the guidelines given in Nicholson and Butterworth (1986) for local breeds and Kellogg (2009) for exotic breeds.

The ages of swine were categorized into <2 years as young and ≥ 2 years as adult based on the dentition formula adopted by USDA (2018). BCS of swine was categorized into poor, medium or good following the guideline given in CFSPH (2011). The age, sex, origin and body condition scores of apparently healthy swine brought for slaughter to AAAE were also recorded.

The following inclusion and exclusion criteria were employed before dairy cattle and swine were tuberculin tested (Radostits *et al*., 2007; Strain *et al*., 2011):

Inclusion criteria: Cattle/swine in all herds that were 7 weeks of prepartum and postpartum (due to immunological hyporeactivity occurring in association with birth) and calves/piglets with the age of above 3 months (calves/piglets drinking colostrum from infected cows/sows give positive reactions for up to 3 weeks after birth even though they may not be infected) were included in the tuberculin skin test. All apparently healthy swine brought for slaughtering to AAAE during the study period were also included in necropsy study.

Exclusion criteria: Animals within 6 weeks before and after giving birth (recently gave birth/periparturient cows/sows) and younger than 3 months of age were excluded from the study.

3. 5. Skin Testing

All owners of dairy cattle and swine gave their informed oral consent and assent to have their animals PPD tested (Ereqat *et al*., 2013). All animals were restrained. In each of the farms, animals were identified by their tags or their names. Before the animals were tuberculin tested, they were subjected to clinical assessment for any disease conditions based on the guideline given in Radostits *et al*. (2007). A fold of skin within each shaved area was measured with 0.01 graduated callipers. The same set of callipers was used to measure the skin thickness before and after tuberculin testing. The site was marked with an indelible ink prior to any PPD injection. Here, tuberculin and PPD had a similar meaning. All animals in the selected clusters were tuberculin tested. A correct injection was confirmed by palpating the intradermally injected tuberculin into the raised fold, which was lodged, so that a small pea-like swelling (induration) at each site of injection was palpable. The same person had conducted the skin test and measured the skin fold thickness before and after PPD injection. A herd with at least one positive reactor to bovine PPD in a farm was considered as positive (OIE, 2009; APHA, 2019).

3. 5. 1. SICCT test in dairy cattle

Tuberculin testing of dairy cattle was carried out by a single intradermal comparative cervical tuberculin (SICCT) test following an already established global protocol (OIE, 2009). Two sites on lateral side of the mid-neck at 12-15cm approximate distance were delineated, shaved and cleaned. The injection sites were positioned on the same side of the neck in a horizontal direction. A fold of skin was picked up, measured and recorded before any PPD injection (B0: at time 0). Aliquots of 0.1ml of 3000 IU/mL bovine PPD and 0.1ml of 2500 IU/mL avian PPD (PRiONiCS, Lelystad, The Netherlands) were injected intradermally into the respective shaved sites. The area near to the head of the animal was injected with avian PPD and the other near to prescapular lymph node was injected with bovine PPD using 1mL separate preset sterile insulin syringes fitted with short sterile needles separately. The skin-fold thickness of each injection site was re-measured 72 hours after injection (B72: at 72h). The skin test responses to bovine PPD were interpreted following two cut-off values namely the standard (OIE, 2009) and severe (Ameni *et al*., 2008):

Standard: The interpretation of the result was made based on the difference in skin thickness at the bovine and avian PPD injection sites. The animal was considered as positive for bTB if the skin thickness at bovine PPD injection site minus the skin thickness at avian PPD injection site was $\Delta B\text{-}\Delta A \geq 4$mm, inconclusive/doubtful if $\Delta B\text{-}\Delta A > 2$mm -< 4mm and negative if $\Delta B\text{-}\Delta A$ <2mm. A positive reaction to *M. avium* was interpreted if $\Delta A > 4$mm and negative if $\Delta A < 4$mm. Doubtful reactors dairy cattle to bovine PPD were included with positive reactors to avoid them from being the sources of zoonotic TB to humans.

Severe: The same skin test results after injection of bovine PPD were reinterpreted. Accordingly, the skin test result was considered positive if $\Delta B\text{-}\Delta A > 2$mm and negative if $\Delta B\text{-}\Delta A < 2$mm.

3. 5. 2. SICT test in swine

Tuberculin testing of swine was carried out by the single intradermal comparative tuberculin (SICT) test following already established protocol (Songer *et al*., 1980; CFIA, 2019; APHA, 2019). Before injection of any PPD, the hair immediately caudal to the right and left base of the ears was shaved at one site on both sides of the neck (approximately 4cm square). The shaved sites were not disinfected with alcohol but washed with clean ground water if there was dirt and awaited until dry. The thickness of skin at each injection site was measured before injection of any PPD. The right side was injected with bovine PPD (0.1ml of 3000 IU/mL) while the left side was injected with avian PPD (0.1ml of 2500 IU/mL) intradermally (PRiONiCS, Lelystad, The Netherlands) with 1mL sterile insulin syringe fitted with needle separately into the respective shaved sites (Songer *et al*., 1980; APHA, 2019).

The tuberculin skin test result was read and the thickness of the skin at each injection site was remeasured again after 48 hours of PPD injection (Songer *et al*., 1980; Merck, 2006; Radostits *et al*., 2007; APHA, 2019). The test results were interpreted based on the guidelines provided by CFIA (2019) and APHA (2019) as follows:

■ there was visible or palpable induration in tissue at the site of injection. A reaction to the bovine or avian tuberculin was considered **positive** when an increase in skin-fold thickness of more than 2mm (>2mm) and/or oedema can be observed after the injection of tuberculins.

■ there was no visible or palpable change in tissue at the site of injection and a decrease in skin fold thickness of less than 2mm (<2mm)) with no oedema can be observed after the injection of tuberculins indicate a **negative** reaction.

Swine reacted to either bovine or avian PPD were obtained using the formula:
[(Bov48−Bov0) − (Av48−Av0)] > 2mm to bovine PPD and [Av48−Av0) >2mm to avian PPD. Bov0 and Av0 indicated skin thickness before injecting bovine and avian tuberculins, respectively. On the other hand, Bov48 and Av48 were the corresponding skin fold thickness 48h post injection of bovine and avian tuberculins, respectively (OIE, 2009).

Cattle-swine interspecies transmission study: Dairy cattle which were 28 in number (26 females and 2 males) and herded with swine were tuberculin skin tested following OIE (2009) protocol with the aim to hypothesize interspecies transmission of bTB between cattle and swine. The test results were read after 72h and interpreted using ≥4mm (OIE, 2009) and >2mm (Ameni *et al*., 2008) cut-off values.

3. 6. Assessment of Risk Factors

Questionnaire survey geared to know the awareness level of farm owners/attendants towards the zoonotic transmission of both bovine and swine TB to human beings was conducted based on a pretested semi-structured and open-ended questionnaire. The questionnaire was designed with closed and open ended questions to collect data on the risk factors precipitating the occurrence of TB in animals and human beings. The owner or manager of each farm was interviewed with the local language during the time of tuberculin testing. Data related to the animal farms such as herd size, different farm structures including their locations, management practices, purpose of establishment, movement of animals and contact with other animals and wildlife were collected and recorded.

Data on animal barn related environmental risk factors such as temperature, darkness, moistness, direct sunlight, pathogen protection scheme (farm biosecurity) and type of housing were collected and recorded. During data collection, attention was also given to the people's milk consumption habits, know-how on the zoonotic transmission of bTB, socio-economic status, educational background, family history of TB infection and treatment, proximity to livestock and human to assess the awareness level of farm owners/attendants towards zoonotic transmission of bTB. The data were collected and recorded in a LogBook.

3. 7. Biological Samples

The sample containers were labelled, biological samples were aseptically collected, cooled, carefully transported to TB laboratory at ALIPB and frozen until mycobacterial culturing.

Gross lesions of swine tissues: Whenever gross lesions suggestive of TB were detected in any of the tissues (tissues here represent lymph nodes and tissues from the lungs, kidneys, liver and spleen) then the tissues were classified as having lesions. In general, TBL lesions (necropsy) were collected from 276 swine tissues including strong bovine PPD reactors and slaughtered swine at farm as well as those apparently healthy swine that were brought to AAAE to be slaughtered for pork. Necropsy and gross pathological examination were conducted following established protocol in FAO (1994). Each lobe of the lung was inspected externally and then sliced into 2cm-thick slices to facilitate detection of any typical TBL lesions. Similarly, liver, spleen and kidneys as well as other lymph nodes (submandibular, lateral and medial retropharyngeal, bronchial, mediastinal and mesenteric) were sliced into thin sections and inspected for the presence of typical TBL lesions. Each lymph node was sliced into 2mm-slices to detect TBL lesions. Furthermore, lesion type (caseous or calcified) and TB stage (localized or generalized) were identified and recorded. TBL lesions for histopathology were preserved in 10% neutral buffered formalin and transported to histopathological examination laboratory at the National Animal Health Diagnostics and Investigation Center (NAHDIC) found in Sebeta, Ethiopia.

Collection of tissue specimens was carried out using a different set of disposable and sterile surgical instruments (forceps, scalpel blades and short sleeve hand gloves) for each animal and kept in Falcon® tubes (5ml; Becton Dickinson Labware, Franklin Lakes, New Jersey, USA). Upon postmortem examination, visible TBLs were collected per animal in individual 5ml sterile universal tubes containing sterile 0.9% phosphate-buffered saline (PBS) adjusted at pH 7.2.

Histopathological examination of swine tissues: Fat and other tissues were trimmed from TBL lesions and collected during necropsy examination. The tissue processing for histopathological examination was performed according to Santos *et al*. (2010) protocol. Briefly, the tissues were dehydrated in different grades of ethanol (70%, 95% and 100%), cleared in xylene and refixed with formalin in an automatic tissue processing machine. The tissues were then embedded in paraffin using an embedding machine and cut into thin sections of 4-5µm using a microtome. The tissue sections were subsequently stained with haematoxylin and eosin and prepared for microscopic examination following the established procedures (Bancroft and Cook, 1994).

Human sputa: Sputa samples were collected from all individuals who were suspected patients of active TB and referred to DBZRH. Sputum (~5mL) from each patient (on spot and at once) was collected and kept in 5ml sterile universal tube using sterile leak proof disposable plastic material. The samples were collected by laboratory personnels under sterile conditions and kept in sterile 0.9% phosphate-buffered saline (PBS) adjusted at pH 7.2. They were transported to the TB laboratory of ALIPB and being frozen at -20°C until processing. The samples were then processed for mycobacterial culturing following the national TB control policy and algorithm adopted by WHO (2017) and implemented by the MOH of Ethiopia (Ameni *et al*., 2013; Pai *et al*., 2014; WHO, 2017).

3. 8. Transport and Storage of Samples

Each sample collected in the field was kept in a cooler box packed with ice and transported to the TB laboratory at ALIPB in Addis Ababa, Ethiopia for mycobacteriological analysis. The samples

were stored at +4°C when they were processed within 24-48 hours or kept at -20°C/-80°C if processed later than two days after sample collection.

3. 9. Sample Processing and Laboratory Analysis

3. 9. 1. Culturing

Tissue samples were sectioned using sterile scalpel blades, minced with scissors and homogenized with a sterile mortar and pestle under a biological safety cabinet level III following the global procedure adopted by OIE (2009). Moreover, sputa samples were processed based on the global protocol adopted by WHO (2017).

The homogenates were decontaminated by adding an equal volume of 4% NaOH for 15min in order to remove contaminants (3ml of 0.9% saline should be added to the solution to revitalize the mycobacteria). Then, the samples were transferred into a sterile falcon tubes and centrifuged at 3,000 revolutions per minute (rpm) for 15min to concentrate the mycobacteria. The supernatant was discarded and 2ml of the sediment was neutralized by 1% (0.1 N) HCL using phenol red as an indicator. Neutralization was achieved when the colour of the solution changed from purple to yellow. Then, 0.1ml of the sediment of mycobacteria was heavily inoculated/seeded onto a slant of two different Loewenstein-Jensen (LJ) media supplemented either with glycerol or 0.4% pyruvate optimized for the culture of *M. bovis* and its primary isolation (Elias *et al*., 2008; OIE, 2009; Mamo *et al*., 2011; Ameni *et al*., 2013; Pai *et al*., 2014; WHO, 2017).

The tubes with cultures were incubated aerobically at 37°C at an angle for the first week (with daily observation) and in the upright position for up to 8-12 weeks. Growth of mycobacteria was monitored every week for up to 8 weeks with weekly observation for visible bacterial growth and morphology of the colonies (Elias *et al*., 2008). Cultures were considered negative for AFB if no colony growth was detected after 8-12 weeks. Cultures that showed colony growth were stained with ZN staining to assess AFB isolates. Positive cultures were preserved with freezing

media. The colonies were heat killed in water bath at 80°C for 60 minutes. The frozen and heat killed isolates were stored at -20°C for further molecular analysis (Mamo *et al*., 2011).

3. 9. 2. Molecular typing

Region of difference (RD9) based PCR: Identification of *M. tuberculosis* from the other members of the MTBC species was conducted using RD9-based PCR. RD9-based PCR was performed on the DNA of heat-killed cells to confirm the presence or absence of RD9 following the bench protocol described by Brosch *et al*. (2002) using three primers: RD9flankF, 5′-GTG TAG GTC AGC CCCATC C-3′; RD9intR, 5′-CTG GAC CTC GAT GAC CAC TC-3′; and RD9flankR, 5′-GCC CAA CAG CTCGAC ATC-3′. The PCR amplification was conducted using a standard thermocycler (VWR Thermocycler, VWR International, East Grinstead, UK). The volume of the reaction mixture was 20µl, comprising of 10µl HotStarTaq Master Mix (Qiagen, Crawley, UK), 7.1µl distilled water, 0.3µl each of the three primers (100 mM) and 2µl DNA template. The reaction was heated for 10 minutes at 95ºC for enzyme activation following which 35 cycles of 1 min of denaturation at 95 ºC, 0.5 min of annealing at 61 ºC and 2 min of extension at 72 ºC were run and then a final extension was made at 72ºC for 10 min. After removing the amplicon product from the Thermocycler, it was then subjected to agarose gel electrophoresis. For gel electrophoresis, 8µl PCR products were mixed with 2µl loading dye, loaded onto 1.5% agarose gel and electrophoresed at 100V and 500mA for 45 min. The gel was then visualized using a computerized Multi-Image Light Cabinet (VWR). For a band size of 396bp, an isolate was considered as *M. tuberculosis* whereas a band size of 575bp was considered to correspond to either *M. bovis* or *M. africanum*. *M. tuberculosis* H37Rv and *M. bovis* BCG were used as a positive control whereas Qiagen distilled water was used as a negative control (Parsons *et al*., 2002).

3. 10. Storage of Stock Cultures

Acid fast bacilli positive cultures were prepared as 20% (v/v) glycerol stocks and stored at -80°C for future use. The *M. tuberculosis* isolated was pooled to the isolate bank of TB laboratory at ALIPB (Abayneh, 2013).

3. 11. Ethical Clearance

The present study was carried out in strict accordance with the recommendations in the guide for the care and use of animals for research. The protocol was approved by the Animal Research Review Committee of CVMA, AAU (Ethical Clearance Certificate Ref. No. to swine: VM/ERC/007/03/09/2016 and Ethical Clearance Certificate Ref. No. to cattle: VM/ERC/02/08/11/2018). The research proposal was submitted to the institution, evaluated and approved by the Ethical Review Committee. The objective of the study and its non-invasive nature were told to both dairy and swine farm owners/managers. All owners gave their informed oral consent and assent to have their animals tuberculin tested. Moreover, they had the liberty either to proceed with the study or discontinue at any stage of the research. Free medication, consultation and follow up were provided by members of the study team and local veterinarians. After permission was obtained from both dairy and swine farm owners, all efforts were made to ameliorate the suffering of dairy cattle and swine by gentle restraining, avoidance of disturbing noise and unnecessary movement.

The human research ethical clearance was obtained from Institutional Review Board (IRB) at ALIPB with Ethical Clearance Certificate Ref. No.: ALIPB IRB/023/2011/2019. The research proposal was submitted to IRB, evaluated, approved and permitted.

3. 12. Dataset Management and Statistical Analysis

The data collected and recorded during field tuberculin test, necropsy study, collection of biological samples and laboratory analysis were entered and stored in separate MS-Excel 2010 spread sheet, thoroughly screened for errors, coded, imported and analyzed in Stata Version 13.0 for Windows (Stata, 2013).

Descriptive statistics was used to summarize and present data. The individual animal level prevalence of bTB was calculated as the number of tuberculin tested positive cattle and swine to

the total number of tuberculin inoculated expressed in percent. The herd prevalence of bTB in cattle and swine was also calculated as the number of herd/s tested positive to the total number of herds tested expressed in percent. Doubtful reactors were included in positive reactors in statistical analysis.

The overall abattoir TBL lesion prevalence in swine was calculated as the number of slaughtered swine which had TBL lesions to the total number slaughtered expressed in percent.

The association of putative risk factors to bovine and swine TB was determined using odds ratio (OR) and chi-square ($\chi 2$) test of independence in univariable logistic regression. The strength of association of the potential risk factors to bovine and swine TB were indicated by the OR in multivariable logistic regression. The 95% CI did not include one among its values. Statistical significance was set at $p<0.05$. Cattle/swine herd was considered as a random effect and risk factors were considered as fixed effects. Variables in the univariable logistic regression analysis were selected for multivariable logistic regression analysis when p value was <0.25. Variables were fitted separately to the final multivariable logistic regression model. A variable was considered to be a confounder and included in the model if its inclusion altered the OR of the estimated risk by 25% or more (Stata, 2013).

4. RESULTS

4. 1. Bovine Tuberculosis

4. 1. 1. Prevalence and risk factors

The purpose of keeping dairy cattle in Debre Berhan milkshed is predominantly indispensable for milk production for sale. The SICCT test was carried out on 625 heads of dairy cattle in the milkshed in which majority of them were female animals accounting for 88% (549/625). On the other hand, 12% (76/625) of them were males. The apparent individual animal level prevalence of bTB in dairy cattle was 17% (106/625; 95% CI: 14.2-20.2) at ≥4mm cut-off value and 18.4% (115/625; 95% CI: 15.5- 21.7) at >2mm cut-off value. The herd prevalence was 16.7% (16/96; 95% CI: 10.1-26) at ≥4mm cut-off value and 22.9% (22/96; 95% CI: 15-33) at >2mm cut-off value in 96 dairy herds tested. Of the tested dairy cattle, 11.4% (71/625; 95% CI: 9-14) reacted to only avian PPD and 5.6% (35/625; 95% CI: 4-8) to both avian and bovine PPDs. Also, 1.1% (7/625; 95%CI: 0.5-2.4) of dairy cattle were doubtful reactors and included with positive reactors because they are zoonotic risks to humans. All the results of bovine PPD reactors were interepreted at ≥4mm cut-off value.

Univariable logistic regression analysis to see the association of host risk factors with tuberculin test positivity of dairy cattle to bovine PPD in Debre Berhan milkshed was carried out on data generated at ≥4mm cut-off value. Hence, sex was associated with bovine tuberculin positivity with statistical marginal significance (p = 0.060). The association of poor body condition, open physiological state, large herd size, poor farm hygiene, exotic breed and intensive management with tuberculin positivity was statistically highly significant (p<0.05). However, the association of age, lactation and parity with tuberculin positivity was not statistically significant (p>0.05) (Table 2).

Table 2. Univariable logistic regression analysis of predictors with the outcome (n = 625)

Variable	Category	Number Examined	Number (%) Positive	95% CI	COR (95% CI)	*p*-value
Sex	Male	76	7 (9.2)	(4.1, 18.6)	1	
	Female	549	99 (18.0)	(15.0, 21.6)	2.2 (1.0, 4.9)	0.060
Age	≤ 5 yrs	378	58 (15.3)	(12.0, 19.5)	1	
	>5 yrs	247	48 (19.4)	(14.8, 25.0)	1.3 (0.9, 2.0)	0.184
BCS	Good	218	33 (15.1)	(10.8, 20.8)	1	
	Medium	352	52 (14.8)	(11.4, 19.0)	1.0 (0.6, 1.6)	0.905
	Poor	55	21 (38.2)	(25.7, 52.3)	3.5 (1.8, 6.7)	0.000
Physiol. state	Open	313	68 (21.7)	(17.4, 26.8)	1	
	Pregnant	236	31 (13.1)	(9.2, 18.3)	1.8 (1.2, 2.9)	0.011
Lactation	Dry	254	47 (18.5)	(14.1, 24.0)	1	
	Lactating	295	52 (17.6)	(13.6, 22.6)	0.9 (0.6, 1.5)	0.807
Parity	Heifer	182	32 (17.6)	(12.5, 24.1)	1	
	[1-3]	275	53 (19.3)	(14.9, 24.5)	1.1 (0.7, 1.8)	0.630
	≥4	92	14 (15.2)	(8.9, 24.6)	0.8 (0.4, 1.7)	0.635
Herd size	Small	273	7 (2.6)	(1.2, 5.4)	1	
	Medium	95	5 (5.3)	(2.0, 12.4)	2.1 (0.7, 6.8)	0.212
	Large	257	94 (36.6)	(30.7, 42.8)	21.9 (9.9, 48.4)	0.000
F. hygiene	Good	192	66 (34.4)	(27.8, 41.6)	1	
	Medium	101	7 (6.9)	(3.1, 14.2)	0.7 (0.3, 1.6)	0.363
	Poor	332	33 (9.9)	(7.0, 13.8)	4.7 (3.0, 7.6)	0.000
Breed	Local	230	6 (2.6)	(1.1, 5.9)	1	
	Exotic	395	100 (25.3)	(21.2, 30.0)	12.7 (5.5, 29.4)	0.000
Management	S. intensive	263	8 (3.0)	(1.4, 6.1)	1	
	Intensive	362	98 (27.1)	(22.6, 32.0)	11.8 (5.6, 24.8)	0.000

BCS: Body condition score; Physiol. State: Physiological state; F. hygiene: Farm hygiene; COR: Crude Odds Ratio; CI: Confidence interval; Number 1: Refers to reference; S. intensive: Semi-intensive

Multivariable logistic regression analysis at ≥4mm cut-off value revealed dairy cattle with poor body condition (AOR= 3.7; 95% CI: 1.6-8.4; p = 0.002), large herd size (AOR = 29.5; 95% CI: 5.6-154.1; p = 0.000) and exotic breed (AOR = 3.7; 95% CI: 1.3-10.7; p = 0.018) had 4, 30 and 4 times the odds of being tuberculin reactors compared to their counterparts with statistical significance, respectively. However, the association of age, sex, physiological state and management with tuberculin test positivity was not statistically significant (p>0.05) (Table 3).

Table 3. Multivariable logistic regression analysis of predictors with the outcome (n = 625)

Variable	Category	Number Examined	Number (%) Positive	95% CI	AOR (95% CI)	*p*-value
Sex	Male	76	7 (9.2)	(4.4, 18.2)	1	
	Female	549	99 (18.3)	(15.0, 21.5)	0.8 (0.2, 2.8)	0.983
Age	≤ 5 yrs	378	58 (15.3)	(12.0, 19.4)	1	
	>5 yrs	247	48 (19.4)	(14.9, 24.9)	1.4 (0.8, 2.4)	0.215
BCS	Good	218	33 (15.1)	(11.0, 20.6)	1	
	Medium	352	52 (14.8)	(11.4, 18.9)	1.2 (0.6, 2.2)	0.908
	Poor	55	21 (38.2)	(26.3, 51.7)	3.7 (1.6, 8.4)	0.002
Physiol. state	Open	313	68 (21.7)	(17.4, 26.6)	1	
	Pregnant	236	31 (13.1)	(9.2, 18.3)	1.8 (1.0, 3.4)	0.696
Herd size	Small	273	7 (2.6)	(1.2, 5.3)	1	
	Medium	95	5 (5.3)	(2.2, 12.1)	2.5 (0.6, 10.1)	0.278
	Large	257	94 (36.6)	(30.9, 42.7)	29.5 (5.6,154.1)	0.000
Breed	Local	230	6 (2.6)	(1.2, 5.7)	1	
	Exotic	395	100 (25.3)	(21.3, 30.0)	3.7 (1.3, 10.7)	0.018
Management	S. intensive	263	8 (3.0)	(1.5, 6.0)	1	
	Intensive	362	98 (27.1)	(22.7, 31.9)	0.3 (0.1, 1.7)	0.191

AOR: Adjusted Odds Ratio; Farm hygiene was not considered in multivariable logistic regression model due to collinearity with breed, thus not presented in the table; to detect collinearity, the gamma statistics reference value is > 0.6 and the finding of this study was 0.8804

4. 2. Swine Tuberculosis

4. 2. 1. Prevalence and risk factors

The purpose of swine production in central Ethiopia is mainly for pork production for foreign consumers. The SICT test was conducted on 329 heads of swine. The individual level apparent prevalence of bTB in swine was 3% (10/329; 95% CI: 2-6) at >2mm cut-off value. In the total 9 clusters tested, the herd prevalence was 11% (1/9; 95% CI: 1-49) at >2mm cut-off value. In addition, 2.7% (9/329; 95% CI: 6-13) of swine reacted to only avian PPD and 1.5% (5/329; 95% CI: 3-8) reacted to both avian and bovine PPDs at >2mm cut-off value.

Dairy cattle herded with swine in one farm were tuberculin skin tested by using SICCT test. Of the total 28 dairy cattle tuberculin tested, 29% (8/28; 95% CI: 2-27) of them reacted to only avian PPD, 71% (20/28; 95% CI: 11-44) reacted to only bovine PPD and 14% (4/28; 95% CI: 0.5-22) reacted to both avian and bovine PPDs at ≥4mm cut-off value. All the dairy cattle were strong tuberculin reactors and no negative as well as inconclusive results were obtained.

Female swine were more reactive than males. In addition, swine in the age category of ≥ 2 years were more reactive than those of <2 years old. Swine with poor body condition were also more reactive than that of medium body condition. None of the swine with good body condition score were reacted (Table 4).

Univariable logistic regression analysis of host risk factors revealed body condition (p = 0.000) and parity (p = 0.026) of swine were significantly associated with bovine tuberculin test positivity. Moreover, physiological state of swine was associated to bovine tuberculin test positivity with marginal statistical significance (p = 0.058) (Table 4).

Table 4. The associations of different risk factors to swine skin test positivity to post bovine PPD injection and interpreted at >2mm cut-off value

Variable	No. tested	Results			
		PPDB positive	%	χ^2	*p*-value
Sex	329	10	3	1.96	0.199
Male	133	2	1.5		
Female	196	8	4.1		
Age	329	10	3	0.00	-
<2 years	261	0	0		
≥2 years	68	10	14.7		
BCS	329	10	3	17.29	0.000
≥5: good	105	0	0		
[3-4]: Medium	178	2	1.1		
[1-2]: Poor	46	8	17.4		
Physiological state	196	8	4.1	3.74	0.058
Dry	137	3	2.2		
Pregnant	59	5	8.5		
Lactation	196	8	4.1	0.00	0.973
Non-lactating	146	6	4.1		
Lactating	50	2	4		
Parity	196	8	4.1	7.57	0.026
No parity	111	1	0.9		
[1-3] parity	64	6	9.4		
[4-6] parity	21	1	4.8		

Multivariable logistic regression analysis revealed poor body condition in swine had a protective exposure effect to bovine tuberculin test positivity (AOR = 0.06; 95% CI: 0.0-1.1) with statistical marginal significance (p = 0.055). On the contrary, the association of pregnancy and parity to bovine tuberculin positivity of swine was not statistically significant (p>0.05) (Table 5).

Table 5. Multivariable logistic regression analysis of tuberculin reactors' swine to bovine PPD with the associated risk factors

Variable	No. tested	No. positives	COR (95% CI)	AOR (95% CI)	*p*-value to AOR
BCS					
≥5: Good	105	0	-	-	
[3-4]: Medium	178	2	1	1	
[1-2]: Poor	4 6	8	0.05 (0.01-0.26)	0.06 (0.00-1.07)	0.055
Physiol. state					
Open	137	3	1	1	
Pregnant	59	5	0.24 (0.06-1.05)	1.17 (0.06-22.45)	0.917
Parity					
No parity	111	1	1	1	
[1-3] parity	64	6	11.38 (1.3-96.79)	3.80 (0.08-185.02)	0.501
[4-6] parity	21	1	5.5 (0.3-91.58)	1.54 (0.05-45.23)	0.803

Age and sex were not considered in multivariable logistic regression model due to collinearity issues thus not presented in the table; Physiol.state: Physiological state; Open: dry or non-pregnant

The gross pathological changes observed on the skin in some of tuberculin tested positive swine were swelling characterized by firm and erythematic nodules. A positive reaction at the injection site constitutes a diffuse swelling, skin thickening, superficial necrosis and sloughing (Figure 10).

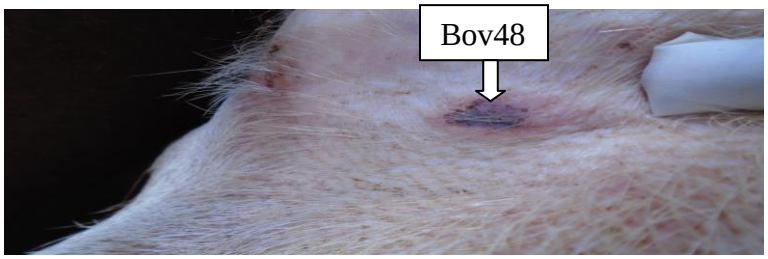

Figure 10. Skin test response of swine to bovine PPD (Photo: Kassa, 2016):

Firm and erythematic swelling of the skin near to the right ear base of swine after 48hours of bovine PPD injection

4. 2. 2. Abattoir lesion prevalence

The abattoir lesion prevalence in swine was 4.1% (26/640; 95% CI: 2.8-6.0). Basic host related risk factors such as sex, age, BCS and origin were not statistically associated with the occurrence of TBL lesions (p>0.05) as it can be depicted by (Table 6).

Table 6. Association of host related risk factors to TBL lesions in swine

Variable	No. inspd	No. positive	Prevalence (%)	χ^2	COR	95% CI	*p*-value
Sex	640	26	4.1	0.85	1.46	0. 64-3.33	0.365
Female	277	9	3.2				
Male	363	17	4.7				
Age	640	26	4.1	2.21	1.81	0.83-4.00	0.137
<2 years	386	12	3.1				
≥2 years	254	4	1.6				
BCS	640	26	4.1	0.82	1.16	0.45-3.00	0.753
Poor	206	7	3.4				
Medium	305	12	3.9				
Good	129	7	5.4				
Origin	640	26	4.1	0.51	1.29	0.55-3.03	0.558
AA	262	9	3.4				
Bishoftu	319	14	4.4				
Others	59	3	5.1				

No. inspd: Total number of swine postmortem inspected; AA: Addis Ababa; Others: refer to names of zonal towns such as Bahr Dar, Debre Berhan, Dessie and Gondar

4. 2. 3. Gross pathology and histopathology

Gross pathological characterization of 58 (58/276) TBL lesions was carried out from the total of 276 samples collected. Of these, abattoir postmortem inspection at AAAE (26/276) and necropsy examination at CVMA-AAU (32/276) revealed enlarged, cheesy and caseous lesions in submandibular lymph nodes. Lesions in the retropharyngeal lymph nodes were characterized as purulent, early caseation and caseous. The mesenteric lymph nodes were enlarged. Pus and caseous lesions were observed in the spleen. Focal calcification was observed in the liver. White spots, focal calcification and caseous lesions were seen in the lungs. Gross TBL lesions in the present study were repeatedly encountered in retropharyngeal (27%: 7/26), submandibular (12%:

3/26) and mesenteric (15%: 4/26) lymph nodes as well as 4% (1/26) in each of the lungs, spleen and liver (Figure 11).

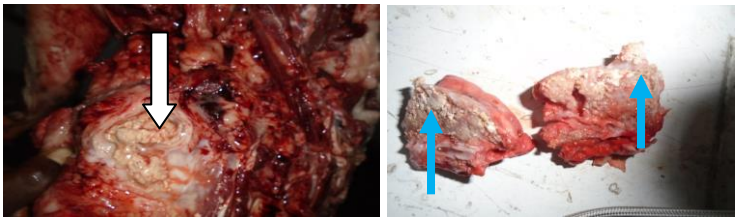

Figure 11. Lesion in submandibular lymph nodes of swine (Photo: Kassa, 2016): Tubercular granulomas characterized by caseous lesions, enlarged and cheesy in consistency

Histopathological analysis of the 32 necropsy samples (32/276) collected from five strong PPD reactors swine revealed granulomas with central necrosis and calcification. The histological arrangement from the center to outer was made up of lymphocytes, macrophages and epithelioid macrophages distributed under connective tissue layers. Moreover, the central area is made of necrotic cellular debris, calcium deposits and connective tissue capsule walled off the granulomas from the surrounding tissue. The presence of concomitant pyogranulomatous and granulomatous lesions in different organs were also observed. Some granulomas were characterized by necrotic foci, intense calcification and fibrosis with absence of epithelioid cells. Multiple small granulomas in the lymph node with less dense lymphocyte at periphery and epithelioid cells surrounding the deep outer lymphatic layer of the granulomas were observed (Figure 12).

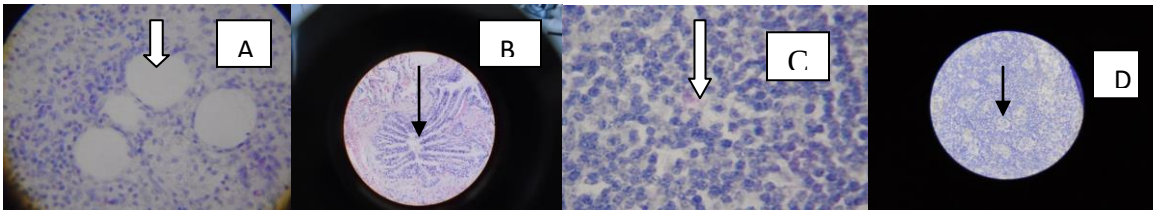

Figure 8. Granulomatous lesions of cervical lymph nodes (Hematoxylin and eosin, 40X):

A/Tubercular granuloma of submandibular lymph node characterized by lymphoid depletion with central necrosis, epithelioid cells and peripheral inflammatory cells (mononuclear lymphocytes and macrophages); and

B/ Tubercular granuloma of retrophryngeal lymph node with central necrosis and peripheral inflammatory cells

Macrophages in submandibular lymph node (Hematoxylin and eosin, 40X):

C/ Granulomas with central necrosis, calcification and eosinophilic lymphadenities; and

D/ Clustered epithelioid macrophages surrounded by lymphocytes

4. 2. 4. Culturing and molecular typing

Mycobacteriological culturing of 276 TBL lesions collected from a total of 640 slaughtered swine (276/640) showed only 1.1% (3/276) growth of presumptive TB colonies on solid slant LJ media which were enriched with either glycerol or pyruvate (Figure 13). Presumptive colonial morphology on solid LJ primary media revealed rough, dry, raised, irregular colonies which are hard to break up easily.

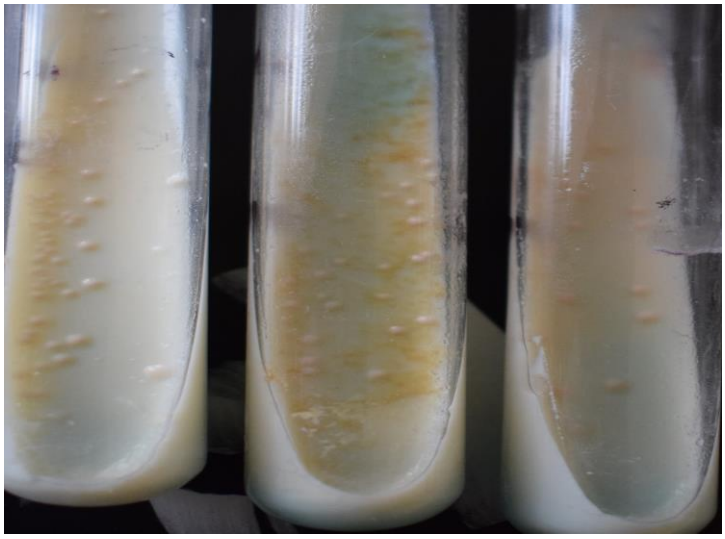

Figure 13. Colony growth from swine tissues on primary solid slant LJ media (Photo: Kassa, 2018)

Molecular typing of two isolates from cultured tissues of swine revealed intact RD9 and 396bp. The isolates were thus confirmed to be *M. tuberculosis* (Figure 14).

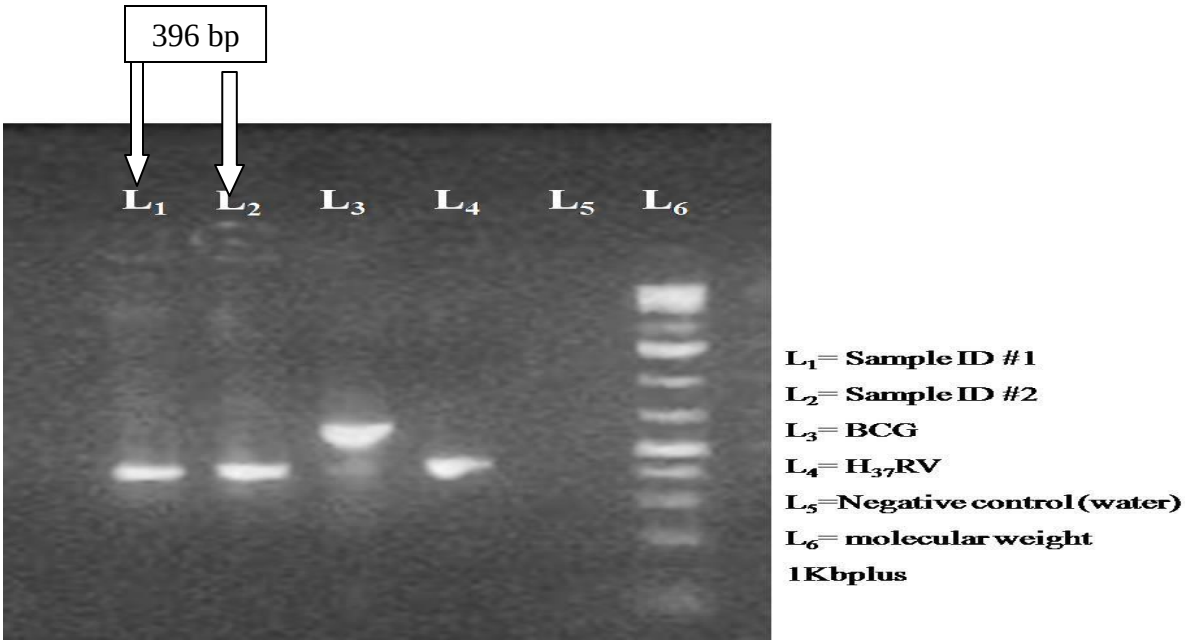

Figure 14. Deletion typing of swine isolates: Gel electrophoretic separation of PCR products obtained by region of difference-9 (RD9) deletion typing of genomic DNAs of *M. tuberculosis* isolated from swine tissue. The DNA for each lane is as follows: lanes 1-2 were 100bp DNA ladder (swine tissue samples); lane 3 was *M. bovis* (BCG) (Positive control); lane 4 was *M. tuberculosis* (H37Rv) (Positive control); lane 5 was water (blank as negative control); lane 6 was molecular-weight marker representing DNA fragments (BioRad) (Brosch *et al*., 2002; Parsons *et al*., 2002)

4. 3. Human Tuberculosis

4. 3. 1. Culturing and molecular typing

The sources of sputa for culturing were active TB suspected 100 human patients who resided within Debre Berhan milkshed and refered to DBZRH. The sputa were cultured on solid slant LJ media which were enriched with either glycerol or pyruvate. Colony growth was detected in 4%

(4/100) of the sputa cultured. The cultural characteristics of the colonies were rough, dry, raised, irregular and hard to break up easily.

Molecular typing of the four isolates from human sputa revealed intact RD9 and 396bp. The isolates were thus confirmed to be *M. tuberculosis* (Figure 15).

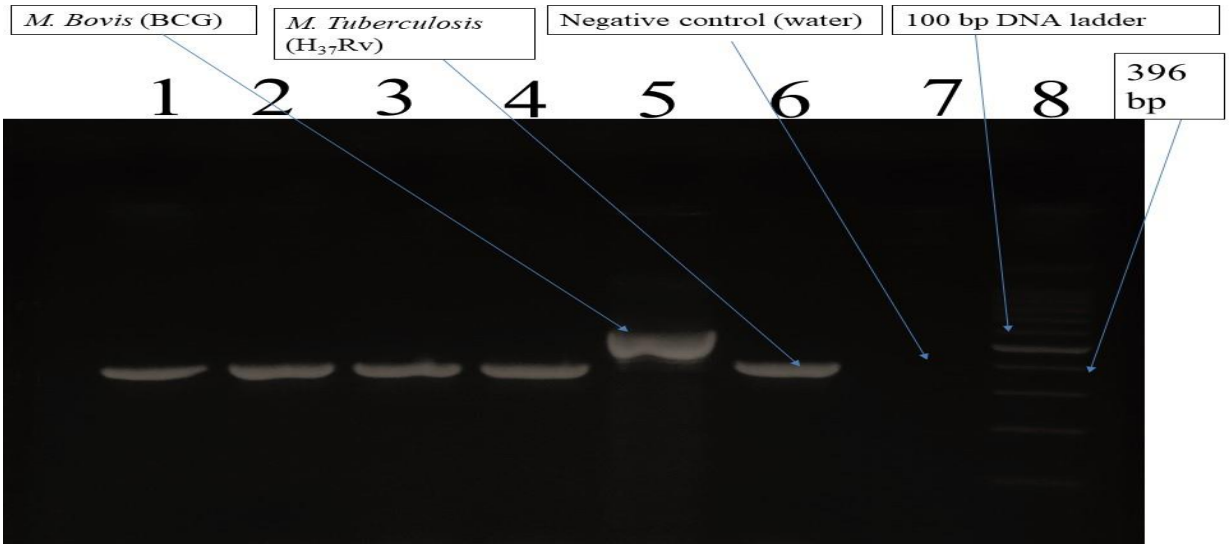

Figure 15. Deletion typing of human isolates: Gel electrophoretic separation of PCR products obtained by region of difference-9 (RD9) deletion typing of genomic DNAs of *M. tuberculosis* isolated from human sputa. The DNA for each lane is as follows: lanes 1-4, molecular-weight markers representing DNA fragments (BioRad); lane 5, *M. bovis* (BCG) (Positive control); lane 6, *M. tuberculosis* (H37Rv) (Positive control); lane 7, Water (Blank as negative control); lane 8, 100bp DNA ladder (human sputa samples) (Brosch *et al*., 2002; Parsons *et al*., 2002)

4. 3. 2. Questionnaire

1. *Key informants*: The key informants (n = 19) were human health professionals in different levels of specialisation. Majority of them indicated kids and old age groups (74%: 14/19) were more affected segments of the community than others. On the other hand, few of them indicated the equal affection of all age groups (26%: 5/19). Weakened immunity (100%: 19/19) was indicated as one of the predisposing factors to TB infection. Moreover, immunocompromised persons and those with chronic illnesses (42%: 8/19) were seen to be more affected by TB due to weakened immunity. In addition, persons in high risk situations such as prisoners, hospital

admitted, school children and persons in overcrowded conditions were more exposed to TB than others.

Periodic screening of sputa, follow up, counseling and HIV testing, personal protection, early treatment, patient provision of high protein diet, adherence to the anti-TB drugs and public education were pointed out as parts of the routine jobs and remedial actions undertaken in TB control programme following the national TB control policy and algorithm. An enquiry made to know exposed professionals while on duty also revealed that 68% (13/19) of the respondents had experienced the exposure of some professionals to TB via aerosolisation from active TB patients while 32% (6/19) of others had not.

The respondents (100%: 19/19) had seen the frequent presentation of HIV/TB co-infection in individual patients and weakened immunity was blamed to be its reason.

2. *Dairy herds and human:* The herds of dairy cattle were categorized into small, medium and large herd sizes. All the interviewees (n = 96) were owners of dairy farms that possessed dairy cattle. They were categorized by sex and age (Table 7).

Table 7. Dairy cattle herd size, sex and age of participants (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Dairy cattle herd size	**96**	**100**
Small	82	85
Medium	12	13
Large	2	2
Sex of participated household heads	**96**	**100**
Female	13	14
Male	83	86
Age of participants	**96**	**100**
20-30 years	12	12
31-41 years	41	43
≥42 years	43	45

The educational background of the respondents were illiterate, basic education, grades 1-8, grades 9-12, degree and veterinary professionals (Table 8).

Table 8. Educational background of participants (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Educational background	**96**	**100**
Illiterate	10	10
Basic education (write and read)	21	22
Grades 1-8	44	46
Grades 9-12	18	19
Degree	1	1
Veterinary professionals	2	2

Some dairy farm owners had pointed out their experience of observing wasted and infected dairy cattle. However, they would not differentiate bTB from the effect of gastrointestinal parasites. Few farmers recall the previous history of their dairy cattle tuberculin skin tested. Farmers unanimously indicated by saying doing nothing to their skin tested positive animals (Table 9).

Table 9. Different experiences of dairy farm owners (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Experienced wasted animals	**96**	**100**
Yes	21	22
No	60	62
No recall	15	16
Recall the previous history of dairy cattle tuberculin skin tested	**96**	**100**
Yes	3	3
No	93	97
Decision on skin tested positive dairy cattle	**96**	**100**
Doing nothing	96	100

Some dairy farm owners indicated that few family members had fallen sick of TB though the source as a cause was not described. However, they were treated with anti-TB drugs and recovered (Table 10).

Table 10. Consumption habits, awareness of dairy farmers and their family about zoonotic TB (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Consumption habit of cows' milk and its products	**96**	**100**
Consumed raw	65	68
Consumed boiled	31	32
Awareness to the zoonotic transmission of bTB from cattle to humans	**96**	**100**
Unaware	75	78
Aware	21	22
Previous history of any family member infected by TB	**96**	**100**
Yes	8	8
No	88	92

Dairy herd related variables were described subsequently as follows.The years of establishment of dairy farms were indicated by (Table 11).

Table 11. Years of dairy farm establishments (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Establishment years	**96**	**100**
Since 1-5 years ago	40	42
Since 6-10 years ago	25	26
Since 11-15 years ago	17	18
Since >15 years ago	13	13
No recall	1	1

The establishment history of dairy farms and their sources of dairy cattle were indicated by (Table 12).

Table 12. Establishment history of dairy farms and sources of dairy cattle (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Establishment history of dairy farms	**96**	**100**
New and family gift	11	12
New and homebred	8	8
New and purchased from local farms and markets	68	71
New and purchased from Addis Ababa, Bishoftu, Holeta and NGOs	7	7
No recall	2	2

The types of dairy barns were closed without entrance of sunlight (61%: 59/96) and loose (39%: 37/96) (Figure 16).

Figure 16. Completely closed barn for dairy cattle (Photo: Kassa, 2018)

Dairy farmers shared shelter with their animals (39%: 37/96) but 61% (59/96) of them did not (Figure 17).

Figure 17. Shelter sharing of humans with dairy animals (Photo: Kassa, 2018)

The dairy farms were grouped as follows based on their hygienic status (Table 13).

Table 13. The hygienic status of dairy farms (n = 96)

Characteristics	**Total No. Interviewed**	**Percent (%)**
Hygiene	**96**	**100**
Poor	84	87.5
Medium	6	6.25
Good	6	6.25

It was very difficult to identify the breeds of dairy cattle in Debre Berhan milkshed due to absence of pedigree record and published breed characterization references in the study areas. However, based on owners' information and support from livestock professionals in the study areas the dairy animals were categorized into local and exotic breeds. Observation of different dairy farms, discussion with dairy farmers and livestock professionals to identify the husbandry practices implemented in Debre Berhan milkshed revealed intensive and semi-intensive systems of management (Table 14).

Table 14. Breeds of dairy cattle with their systems of management (n = 625)

Characteristics	Total No. Interviewed	Percent (%)
Breed	**625**	**100**
Local	230	36.8
Exotic	395	63.2
Management	**625**	**100**
Intensive	362	57.92
Semi-intensive	263	42.08

The feed types for dairy cattle were assessed and seen to be small quantities of roughages (hay and straw from teff, barley and wheat) and trace amount of brewery by-product and concentrate in majority of the households. Medium and large farms were not also seen to feed sufficient quantities of hay and concentrate to their dairy animals due to expensive cost. Therefore, the body conditions of the animals in many cases were poor.

The ventilation conditions of dairy farms were suffocated due to closedness (with only poorly opened doors), overstocking and poor construction design. However, some farms were ventilated. The dairy farms were at close proximity to humans and other domestic animals. The

farms were poorly managed (bad odour due to poor waste drainage system, muddy and moist floor, buildup of ammonia and no light in majority of the cases). There was herd contact in communal watering points and pasture land. But some herds did not have any contact (Table 15).

Table 15. Status of dairy farms (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Ventilation	**96**	**100**
Suffocated	73	76
Ventilated	23	24
Herd contact	**96**	**100**
Yes	84	88
No	12	12

There were no farm biosecurity measures at all which were attested by frequent coming of visitors without hygienic measures, introduction of newly purchased animals into the existing herds and absence of pathogen protection scheme due to lack of disinfectant at farm gate entrance. Generally, there was no disinfectant use at all in small, medium and large farms.

The dairy farms were predominantly established to produce milk for sale to cafes, restaurants, collection centers, dairy cooperatives and residents. However, there was little home consumption. The major reasons of cow culling were old age and drop in milk yield, diseases and infertility (Table 16).

Table 16. Purpose of dairy farms established for and reasons of cow culling (n = 96)

Characteristics	Total No. Interviewed	Percent (%)
Purpose	**96**	**100**
Milk production for sale	95	99
Home consumption	1	1
Cow culling reasons	**96**	**100**
Old age and drop in milk yield	83	87
Diseases	10	10
Infertility	1	1
No recall	2	2

3. *Swine herds and human:* Keen observation and face-to-face interview in different swine farms and nearby residences were carried out with the aim to know the awareness of farm owners/attendants about the zoonotic transmission of swine TB to man. In total, 50 voluntary participants were interviewed and the following variables were identified.

Participants described their consumption habits of raw cows' milk and beef. Majority of them (94%: 47/50) consumed milk and beef raw but few of them (6%: 3/50) cooked before use.

Livestock and people live in close proximity. Many farmers (60%: 30/50) shared shelter with their livestock but some of them (40%: 20/50) did not. Observation revealed the sharing of same shelter between swine and humans in few farms (Figure 18).

Figure 18. Human bed room within the swine barn (Photo: Kassa, 2016)

Swine and dairy cattle shared same farms. Swine usually feed on thermally untreated leftover feed items from human beings and milk as well as its products from dairy animals.

The owners of swine farms were categorized based on their socio-economic status. Majority of the owners were poor (92%: 46/50) due to low income whereas few of them were relatively medium (4%: 2/50) and rich (4%: 2/50) in their income status. Swine farm owners were also categorized into illiterate (84%: 42/50) and literate (16%: 8/50) based on their educational background. Farmers explained the history of TB infection and treatment in their families. A person in a family (2%; 1/50) was exposed to TB though the source was not described. However, the patient was treated and recovered. This was encountered only in one swine farm herded with bTB infected dairy cattle. On the other hand, many of them (98%: 49/50) were not exposed at all. The awareness of participants about the zoonotic transmission of swine TB from swine to human beings was very poor (94%: 47/50) indicating that many farmers are at risk. However, few of them (6%: 3/50) were aware of it. The swine herds were categorized into small, medium and large (Table 17). Swine herd related variables were also described subsequently as follows.

Table 17. Swine herd sizes and structure of swine barns (n = 9)

Characteristics	Total No. Interviewed	Percent (%)
Herd sizes	**9**	**100**
Small	2	22
Medium	3	33
Large	4	45
Barn structure	**9**	**100**
Poorly constructed	8	89
Good	1	11

Observation and discussion with swine owners to know the feed sources for swine revealed spoiled vegetables from supermarkets, spoiled milk and its products from dairy farms and processing centres and spoiled food from restaurants and hospitals. Generally, the swine were poorly fed in majority of the cases and their body conditions were wasted. Swine were frequently seen of feeding on their own manure in majority of the cases due to shortage of feed (Figures 19-21).

Figure 19. Poor body conditioned swine due to poor feeding (Photo: Kassa, 2016)

Figure 20. Poorly constructed swine barns (Photo: Kassa, 2016)

Figure 21. Feed for swine collected from supermarkets (spoiled fruit), restaurants and lounge (spoiled human food) and body of cooked dead birds from poultry farms due to unknown cause (cooker and cooked body of poultry) (Photo: Kassa, 2016)

Participants indicated the locations of the swine farms as 96% (48/50) at close proximity to both humans and other domestic animals but 4% (2/50) of them were isolated.

The swine farm management practices in majority of the cases were poor. The swine barns were completely closed without ventilation in many cases but a few of the barns were one side opened and well ventilated. Many swine farms had got hot room temperature with a smell of ammonia. On the other hand, a few of the barns were loose, well ventilated except their coldness to swine during the night and with relatively low sensible level of ammonia (Table 18 and Figure 22).

Table 18. Swine barn management practices (n = 50)

Characteristics	Total No. Interviewed	Percent (%)
Farm management	**50**	**100**
Poor	40	80
Good	10	20
Swine barn types	**50**	**100**
Completely closed	43	86
One side opened	7	14
Barn room condition	**50**	**100**
Hot room temperature with a smell of ammonia	39	78
Loose and well ventilated	11	22

Figure 22. Completely closed swine barn without ventilation (Photo: Kassa, 2016)

Swine in different farms were overstocked and their space requirements in many cases were not considered at all. However, moderate crowding was seen in a few farms. There was poor waste drainage system in many of the swine farms which is evidenced by piledup of manure in front of the doors of swine barns with a resultant buildup of ammonia. As a result, there was bad odour in many cases. On the other hand, good waste management was observed in few swine farms. The

floors of the swine barns were muddy and moist (92%: 46/50). As a result, soiled skin swine was the frequent observation. On the other hand, the floors of few barns (8%: 4/50) were relatively less muddy. The swine barns were with no light at all (dark) in many cases (88%: 44/50) (Table 19 and Figure 23).

Table 19. Sanitation status, stocking density and waste drainage (n = 50)

Characteristics	Total No. Interviewed	Percent (%)
Sanitation	**50**	**100**
Poor	47	94
Medium	2	4
Good	1	2
Swine stocking density	**50**	**100**
Overstocked	47	94
Moderate crowding	3	6
Waste drainage	**50**	**100**
Manure piledup	47	94
Good cleaning	3	6

Figure 23. Overstocked and soiled swine due to muddy and moist floor (Photo: Kassa, 2016)

The purposes of swine farms established for by farmers were predominantly to sale them alive to supermarkets through **''farm swine collector merchants''**. These swine farms were big farms whose owners were running pork business. These swine were then slaughtered at AAAE for pork sale by the supermarkets themselves for interested foreigners. On the other hand, there were several backyard slaughters in many of the small scale swine production system which were favoured by increasing demand for pork by Chinese people dwelling in Addis Ababa and its suburbs. There were Chinese informal pork traders and distributors to those Chinese who are currently working in different industries including mega projects (Table 20).

Table 20. Establishment purpose and swine herd contact (n =50)

Characteristics	Total No. Interviewed	Percent (%)
Purpose of swine farms established for	**50**	**100**
To sale alive to supermarkets	9	18
To backyard slaughter	41	82
Swine herd contact in	**50**	**100**
Small herd	40	80
Medium herd	6	12
Large herd	4	8

Majority of the swine from small herds wandered around and entered domestic animals' barns and residences of human beings. On the other hand, poultry were wandering in swine barns. Swine from medium and large herds wandered also outside of their barns (Figure 24).

Figure 24. Wandering of swine and chicken (Photo: Kassa, 2016)

Different swine farms did not have pathogen protection schemes. An assessment to know the swine farm biosecurity measures revealed the presence of herd contact and frequent coming of swine traders to the farms without use of farm gate disinfectant in majority of the cases (78%: 7/9). However, two large swine farms (22%: 2/9) usually use farm gate disinfectant at their point of entrance (Figure 25).

Figure 25. A large swine farm gate disinfectant for humans and vehicles (Photo: Kassa, 2016)

There was no any wildlife (90%: 45/50) nearby the swine farms. However, the nearby presence of hyena (10%: 5/50) to the farms was indicated. Dogs and chickens were wandering around swine farms in search of their feed (Figure 26).

Figure 26. Dogs and chicken near to swine farm in search of their feed (Photo: Kassa, 2016)

5. DISCUSSION

5. 1. Bovine Tuberculosis

5. 1. 1. Prevalence

The apparent animal level prevalence of bTB obtained in this study at standard (17%) and severe (18.4%) cut-off values were moderately high. Some of the tuberculin tested dairy cattle were reactive to both avian and bovine PPDs (5.6%) indicating the presence of mixed infection.

The endemic nature of bTB has been elucidated in Ethiopia since 1967 (Ameni *et al*., 2007). A pooled prevalence estimate of bTB in Ethiopia based on systematic review and meta-analysis of research results published from January 2000 to December 2016 was 5.8% (Sibhat *et al*., 2017). SICCT test based studies in cattle in Ethiopia reported at an animal level prevalence of 13.5% (732/5424) in the central highlands of Ethiopia (Ameni *et al*., 2007), 2.7% (14/524) in Debre Berhan and Basona Werana (Shimeles, 2008), 6.4% (27/425) in Arsi-Negele (Amenu *et al*., 2010), 1.8% (36/2033) in Sellale (Ameni *et al*., 2013), 11.4% (98/858) in Sululta (Biru *et al*., 2014), 2.1% (10/481) in North Gondar and Wollo (Mengistu *et al*., 2015), 1.3% (10/788) in and around Bahir Dar (Nuru *et al*., 2015), 4.4% (22/501) in and around Adama (Woldemariam *et al*., 2016), and 5.2% (143/2654) in emerging milksheds of regional cities such as Gondar, Hawassa and Mekelle (Mekonnen *et al*., 2019) at ≥4mm cut-off value were lower than the current finding. On the other hand, an animal level prevalence of 34.1% (386/1132) in five sub-cities of Addis Ababa City Administration (Tsegaye *et al*., 2010), and 30% (887/2956) in six selected sites in major dairying areas of central Ethiopia (Firdessa *et al.*, 2012) at ≥4mm cut-off value were higher than the finding in this study. It was similar to 17% (95/558) at ≥4mm cut-off value in Bishoftu (Bekele *et al*., 2016).

The sources of variation in tuberculin test result might be related to differences in the extent of study area coverage and duration of cattle to stay in the households (Mengistu *et al*., 2015).

Ameni *et al*. (2000) described that early tuberculous infections cannot be detected by traditional tuberculin testing but can be identified by the interferon-gamma (IFN-γ) test. The major epidemiological factors which might favour transmission among animals in this study were poor body condition, large herd size, poor farm hygiene, exotic breed, communal watering and grazing points, herd contact, incompatible spacing to the number of dairy animals (overstocking), and lack of awareness of dairy farmers about the transmission of bTB. Poorly ventilated housing to animals predisposes to bTB in that, the closer the animals are in contact the greater is the chance that bTB will be transmitted. Inadequate ventilation results in insufficient dilution of bacilli or insufficient removal of infectious droplet nuclei (Radostits *et al*., 2007).

5. 1. 2. Animal risk factors

The current study described that female animals were relatively more affected than males though it was not statistically significant. Mamo *et al*. (2013) and Terefe (2014) indicated that cows were observed to be more tuberculin reactors than bulls as bulls are sold or slaughtered in their early age. In addition, adult female breeding animals had longer and repeated chance of exposure to mycobacterial infection during their life time due to physiological stressor factors such as lactation, pregnancy and repeated calving (number of parity) which all cause low protein reserve that in turn suppress the immune responsiveness (Ameni *et al*., 2007; Elias *et al*., 2008; Mamo *et al*, 2013; Terefe, 2014; Bekele *et al*., 2016). The immunological hyporeactivity that occurs in association with pregnancy and parturition makes female breeding animals susceptible to the disease (Radostits *et al*., 2007).

The age of dairy cattle in this study was not found to be an important predictor of tuberculin test positivity. Dairy animals >5 years of age were relatively more tuberculin reactors than those of ≤5 years which could contaminate the dairy barns and be sources of infection to other members of the herds especially in poor hygienic and poorly ventilated farms which were frequently observed in the current study. Ameni *et al*. (2007) described that animals in [5-9] years of age were at a higher risk of infection with TB than those of 2 years of age or below. Similarly, Elias *et al*. (2008) and Mamo *et al*. (2013) indicated the increased in prevalence of bTB parallel to the increasing age of animals with possible reasons that older animals had relatively longer duration

and repeated chance of exposure to mycobacterial infection during their life time. Elias *et al*. (2008) and Ameni *et al*. (2013) described that, the increased incidence of bTB in older animals was experimentally confirmed in the murine system by a waning of protective immunity. The differences among cattle of different ages in reactivity to bovine tuberculin could also be a result of the slow progression of the disease to a detectable level (Ameni *et al*., 2007). Alelign *et al*. (2019) also indicated cattle in the age group of [5-10] years were more tuberculin reactors than younger age groups.

Stress caused by poor nutrition in animals could subject to severe diseases like TB (Ameni *et al*., 2006). Higher prevalence of bTB was reported in animals with poor body condition compared to those with good body condition score which conforms well to the established fact that animals' resistance to TB is reduced by a shortage of feed and/or unbalanced diet attributable to a deficiency of proteins, minerals and vitamins in the diet (Elias *et al*., 2008). Poor body conditioned animals in this study were associated with the increased risk of bovine tuberculin positivity as compared to their counterparts which is congruent to Ameni *et al*. (2006) and Elias *et al*. (2008). Nuru *et al*. (2015) also indicated poorly conditioned animals to be susceptible to bTB infection due to weak immunological responses.

The present study demonstrated more tuberculin reactivity in exotic dairy cattle breeds which is in agreement with the findings of many researchers (Cosivi *et al*., 1998; Ameni *et al*., 2006; Radostits *et al*., 2007; Elias *et al*., 2008) who concluded that exotic breeds of cattle may be less resistant to bTB compared with the autochthonous cattle breeds in the tropics. However, the exact cause of susceptibility differences to bTB between local and exotic breeds is a major subject of future genomic research.

Large herds in this study were observed to be more tuberculin reactors like to the finding of Elias *et al*. (2008) who indicated an increment in herd positivity parallel to increasing herd size. Factors significantly associated with an increased risk of tuberculin reaction are herd size, poor housing condition, age and poor body condition score (Elias *et al*. 2008). Increased inter-herd

and intra-herd contacts in larger herds favour lateral spread of infection making the prevalence of the disease greater than in small herds (Ameni *et al*., 2006; Elias *et al*., 2008).

5. 2. Swine Tuberculosis

5. 2. 1. Prevalence

The current study of swine TB by making use of SICT test is the first of its kind executed in Ethiopia. Tuberculin skin test and necropsy were employed to investigate the epidemiology of swine TB in central Ethiopia. Skin test (3%) and necropsy (4.1%) studies had confirmed the occurrence of swine TB at low prevalence. The lower relative prevalence of TB in swine is due to the tendency of the disease to remain localized in this species and the early age of slaughter (Radostits *et al*., 2007).

Many suspicious reactions occur in swine because of the tendency of lesions to regress and the sensitivity to tuberculin to diminish in which maximum sensitivity occurs 3-9 weeks after infection. Although positive reactors may in time revert to a negative status, there may be macroscopic lesion in these animals at necropsy. However, viable organisms are not usually recoverable from the lesion and the infection apparently having been overcome. Comparative tests work efficiently in this species with little or no reaction to heterologous tuberculin (Radostits *et al*., 2007). Reported inaccuracies in tests may be due to a lack of cross-reactivity between the infecting *M. avium* serotype and the serotype used to prepare the skin test reagent (Songer *et al*., 1980).

The greater of the reactions to either avian PPD or bovine PPD indicates the organism responsible for sensitization. However, several of the animals had strong cross-reactivity to avian and bovine PPDs. It is not uncommon to observe overlap of bTB and MAC positivity in cattle due to either antigenic cross reactivity and/or co-infection. There exists no clear evidence on the immunological relationship between these two but studies have shown that co-infection with MAC compromises bTB skin test results by negatively influencing the sensitivity of the

tuberculin test. Cattle sensitized by MAC might conceal *M. bovis* for a period of time. However, it is not clear to what extent this disease could jeopardize the detection of bTB with skin test thus requiring further research (Mekonnen *et al*., 2019). Some decrease in skin sensitivity after parturition occurs in sows infected with *M. bovis* but may not occur when the infection is associated with *M. avium* (Radostits *et al*., 2007).

SICCT test in this study revealed 71% of dairy cattle herded with swine reacted to bovine PPD. Cattle served as sources of infection to swine in bTB endemic farms (Arega *et al*., 2013). When the disease is common among dairy cattle in an area, 10-20% of the local swine are likely to be infected due to interspecies transmission from bovine to swine. Uninfected swine can easily get bacilli from their contaminated bedding, feed and drinking water (Radostits *et al*., 2007). Inhalation of infected aerosols or feeding on contaminated feed or water in the current study might be hypothesized to be sources of mycobacterial infection to swine. The predominant reaction of swine and bovine to bovine PPD in the current study has indicated its zoonotic risk to human beings in the commonly experienced shelter sharing of swine with human beings and herding together different species of livestock in Ethiopia.

The tuberculin skin test was evaluated in Arizona as an epidemiological tool for measuring prevalence of TB infection in swine herd and 63.3% (69/109) of swine revealed positive responses. At slaughter, 61.5% (67/109) of the swine were shown to have TBL lesions in one or more lymph nodes of the digestive tract. A good correlation (97.1%) exists between skin test reactors and swine with lesions (Matlova *et al.*, 2004).

5. 2. 2. Animal risk factors

Female swine in this study were more reactive to bovine PPD than males due to their longer duration in the farms and repeated chance of exposure to mycobacterial infection due to factors such as lactation, pregnancy and parturition which all are physiological stressors that suppress the immune responsiveness (Ameni *et al*., 2007; Elias *et al*., 2008; Mamo *et al*., 2013; Terefe, 2014; Bekele *et al*., 2016) and might cause infection via endogenous reactivation/exogenous

infection of baciili. The immunological hyporeactivity that occurs in association with pregnancy and parturition generally makes pregnant sows susceptible to the disease (Radostits *et al.*, 2007).

Swine with poor body condition were more affected in this study. Field observation also revealed poor feeding regimen. Stress caused by poor feeding in animals could subject to severe diseases like TB (Ameni *et al.*, 2006). Significantly lower numbers of lymphocyte subpopulations in nutritionally deficient cattle were demonstrated (Ameni *et al*., 2007).

5. 2. 3. Necropsy

Necropsy in the current study revealed low prevalence of TBL lesions in slaughtered swine. Necropsy has low sensitivity of lesion detection (Shitaye *et al*., 2007) due to lack of visible lesions in tuberculin reactors in the early stage of infection (Tsegaye *et al*., 2010). However, some dairy cattle without visible lesions were culture positive which may be due to prior spread of the bacilli in the tissues during an early stage of infection but yet to result in lesion (Berg *et al*., 2009). The lesion prevalence in the present study is higher than 0.009% (Shitaye *et al*., 2006), 0.02–1.83% (Shitaye *et al*., 2007) and 1.48% (Bogale *et al*., 2004) but slightly lower than 5.16% (Ameni and Wudie, 2003) and 5.8% (Arega *et al*., 2013). The severity of swine TB is increasing overtime compared to the retrospective report (Shitaye *et al*., 2006). Arega *et al*. (2013) experienced 67% (563/841) of the swine brought for slaughter were less than one year of age. Experience indicated that swine are slaughtered at their early age for digestible pork.

Significantly higher number of swine >1year of age were found to be lesioned than those of ≤1 year (Arega *et al*., 2013) which supported the frequent observation of gross TBL lesions in swine of ≥2years of age in this study. Gross TBL lesions in the present study were repeatedly encountered in lymph nodes of the gastrointestinal tract. The percentage of gross TBL lesions was the highest (29%) in submandibular lymph node and the lowest (6%) in mediastinal lymph node (Arega *et al*., 2013) which is congruent to this finding. Majority of TBL lesions were detected in swine where localization as non-progressive abscesses occurs in the lymph nodes of the head and neck (Radostits *et al*., 2007; Ameni *et al*., 2013).

The study in the Czech Republic detected TBL lesions in 3.6% of the slaughtered swine (Matlova *et al*., 2004). Among the predominant localization of TBL lesions in mesenteric and cervical lymph nodes in slaughtered swine, it could be suspected that animals were primarily infected by the ingestion of mycobacteria (Songer *et al*., 1980; Matlova *et al*., 2004). Study on environmental source of mycobacteriosis in swine which were exposed to dirt pens and then slaughtered in California revealed an abattoir TBL lesion prevalence of 9.4% (Gardener and Hird, 1989).

Haematoxylin-eosin (H-E) stained TBL lesions in histological analysis in this study revealed the presence of lymphocytes, lymphoid depletion, epithelioid cells, necrotic cellular debris, fibrosis, calcification and necrosis. Parallel to this, Ameni *et al*. (2000) indicated the presence of cellular infiltration, macrophages, giant cells, necrosis or calcification in H-E stained tissues in dairy cattle. Moreover, soft foci of caseous necrosis were present upon gross and microscopic examination (Songer *et al*., 1980) which is in line with the present finding.

5. 2. 4. Culturing and molecular typing

Culturing remains the gold standard method for definitive diagnosis of mycobacteria though it has low isolation sensitivity (Ereqat *et al*., 2013). Many swine tissue samples (276), in this study, were cultured on both solid LJ slants enriched either with glycerol or pyruvate. Only three visible colony growths (3/276) were demonstrated. Poor colony growth (1.1%) was observed in this study and the possible reasons indicated by Radostits *et al*. (2007) were the tendency of TB to remain localized in swine and the early age of slaughter of swine before acquiring the disease. Moreover, viable organisms are not usually recoverable from the lesion due to calcification and its consequent debridment of nutrients to bacilli (Radistits *et al*., 2007).

Speciation of MTBC isolates by RD deletion typing revealed a PCR product sizes of 335 bp (for RD4) and 396 bp (for RD9) in all the isolates and thus confirming that they are *M. tuberculosis* (Arega *et al*., 2013). Similarly, molecular typing using RD9 of isolates from swine tissues in the current study revealed an intact RD9 and the isolate was thus confirmed to be *M. tuberculosis* (396 bp).

Lower rate of mycobacterial growth from tissues with gross TBL lesions have been reported from Czech Republic (Pavlik *et al*., 2005), Egypt (Mohamed *et al*., 2009), Uganda (Muwonge *et al*., 2010), and many other countries (Thoen, 2006). Failure to demonstrate tubercle bacilli may be due to the occurrence of healed processes that contain no longer viable tubercle bacilli, or microorganisms other than tubercle bacilli such as *Nocardia farcinicus*, *Rhodococcus equi*, or *Rhodococcus sputi*, or *Corynebacterium pyogenes* that may cause the lesions, or inadequacy of the methods used for isolating tubercle bacilli. In addition, it could also be due to subjective differences in identifying TBL lesions (Araujo *et al*., 2014).

The isolation of *M. tuberculosis* from animal tissues by molecular typing has been reported by many authors. For instance, Cadmus *et al*. (2006) reported the finding of *M. tuberculosis* from cattle in Nigeria. Moreover, nested PCR (N-PCR) and culture revealed 15%–28% (n = 52) of the animals were infected with *M. tuberculosis* in India (Prasad *et al*., 2005).

Real-time PCR is faster and can detect MTBC directly from biological samples in humans although it has been very limited to detect MTBC in cattle tissue homogenates. The main constraints are cattle with PPD positive reactions do not always exhibit visible lesions during slaughter in the abattoir due to recent infection with a low mycobacterial load; the concentration of viable mycobacterial DNA is usually low when compared to the concentration of host DNA and the difficulty in extracting mycobacterial DNA from cattle tissues, since they have few bacteria and the structure of the biological sample itself exhibits **strong fibrosis** and **calcification**, which hinder the DNA detection process. Prolonged storage of samples in the deep freezer (-80°C) and the freeze–thaw cycles that occurred during electric power interruption may contribute to the low isolation rate of mycobacteria (Araujo *et al*., 2014).

Previous studies suggested interspecies transmission of mycobacteria in Ethiopia (Arega *et al*., 2013). The isolation of *M. tuberculosis* from swine suggests its transmission between human and swine, which could occur as a result of close contact with *M. tuberculosis* infected humans, feeding of undercooked garbage contaminated by sputum or body secretions from infected humans (Thoen, 2006).

5. 3. Human Tuberculosis

5. 3. 1. Culturing and molecular typing

Culturing in this study had demonstrated only four visible colony growth (4/100). Molecular typing using RD9 of four isolates from human sputa revealed an intact RD9 and the isolate was thus confirmed to be *M. tuberculosis*.

M. tuberculosis was also isolated from TBL lesions collected from cattle slaughtered at Debre Berhan municipality abattoir (Shimeles, 2008). This urges the integrated work of medical and veterinary personnels in Debre Berhan milkshed in order to create public awareness about the cyclic transmission of TB between human and animals as well as its public health implications.

Shimeles (2008) indicated that bTB is generally a neglected disease in Debre Berhan and Basona Werana districts and furthermore pointed out a need for molecular characterization of clinical isolates to ensure that correct estimates are made of the true burden of infection due to *M. bovis*. Hence, the identification of *M. bovis* as a cause of human TB in Debre Berhan will have great contribution to the existing TB control campaign (Shimeles, 2008).

5. 3. 2. Questionnaire

***Key informants*:** Human health professionals in different levels of medical services and health facilities in Debre Berhan milkshed were participated as key informant interviewees of the current study. With regard to this, field observation has proven that they demonstrated an all rounded commitment of serving the community in terms of TB diagnosis and awareness creation. However, some professionals were exposed to TB infection. Hence, workplace professionals' biosecurity measures such as mouth masks, hand gloves and well-fitted gowns should be in place to safeguard themselves.

The key informants indicated that kids and old age segments of the community were more affected due to under developed and debilitated immunity, respectively. The case of HIV/TB co-

infection was also frequently presented. It was also indicated that, other immunosuppressive disorders such as diabetes and cancer were incriminated as conditions of predisposition to TB. Professionals experienced people crowding sites such as prisons, schools, hospitals and market places could facilitate the transmission of TB. Generally, adherence to anti-TB treatment, medical follow up and good care with nutrition are the best remedial actions to resolve TB infection. To realize this, employing the algorithm adopted by WHO (2017) and implemented by Ethiopian MOH is of vital importance.

***Dairy farm owners and herd risk factors*:** Questionnaire was administered in a form of face-to-face interview to each household during dairy cattle were PPD tested. It was indicated that, the primary objective of the small herd dairy production system in Debre Berhan milkshed is to sale milk as a means of additional cash income to support the livelihood of smallholder dairy farmers. Moreover, dairy farm owners who possessed medium and large herds were targeted to run dairy business. Debre Berhan milkshed is a part of the national dairybelt of the country, one of the major dairying areas in the central highland of Ethiopia and major supplier of milk to residents of Debre Berhan and Addis Ababa.

Ameni *et al*. (2007) indicated the central highlands are the major dairying areas and the main sources of milk to Addis Ababa and main urban population center where 8% of its inhabitants live in. Ameni *et al*. (2007) also described that farmers within this dairybelt are conscious of the milk market and produce milk for commercial sale unlike the majority of Ethiopian farmers who produce milk for home consumption. The same observation pointed out that farmers keep high yielding crossbred (Zebu X Holstein) and Holstein dairy cattle mainly for milk production alongside native Zebu breeds which is parallel to our observation.

The prevalence of bTB is increasing parallel to an increase in dairy operations. A considerable number of infected cows secrete *M. bovis* and related species in milk. The problem is further compounded by the practice of pooling milk which was also frequently observed in this study, either in the farms or at milk collection centres, signifying a considerable health hazard to milk

consumers. This surely indicates that if measures are not taken promptly, the impact on the economy and public health could be enormous (Elias *et al*., 2008).

The houses of dairy cattle in smallholder system in Debre Berhan milkshed were predominantly closed type, with no light source in majority of the cases and with poor ventilation. Some of the smallholder dairy farmers experienced shared shelter (39%) with their animals. In addition, there was inter-herd contact (88%) in this study at pasture and watering points. *M. bovis* is readily destroyed by direct sunlight unless it is in a moist environment. However, it may remain viable for weeks in warm, moist and protected houses of dairy cattle (Radostits *et al.*, 2007). In sub-Saharan Africa, humans and animals share the same microenvironment and water holes (especially during droughts and the dry season) thereby potentially promoting the transmission of *M. bovis* from animals to humans (Ameni *et al*., 2007). Ameni *et al*. (2006) and Elias *et al*. (2008) indicated poor housing under poor management conditions, poor barn ventilation and close physical contact due to overcrowding facilitate the transmission of infective aerosols among animals. Furthermore, Ameni *et al*. (2006) speculated that overcrowding is a source of stress lowering the immune system of animals and consequently exposing them to TB.

Dairy farmers in the present study indicated old age and drop in milk yield were the major reasons of cow culling. Farmers were not aware of the clinical manifestations of bTB such as progressive emaciation, rough hair coat, exercise intolerance and sluggish movement (Radostits *et al*., 2007) if it occurs in their animals. Majority of the dairy farmers in the current study were not aware of (78%) bTB itself and its mode of transmission from cattle to human beings which is evidenced by human-animal proximity as well as consumption of raw milk and its products. The culture of raw milk consumption with corresponding large number of reactor cows to bovine PPD in the current study is the most important factor to the zoonotic risk of bTB to the dairy farming community in Debre Berhan milkshed which is supported by Elias *et al*. (2008) and Mamo *et al*. (2013). Different researchers isolated both *M. bovis* and *M. tuberculosis* from cows' milk including Elias *et al*. (2008). It is estimated that 82% of the milk is supplied unpasteurized by intra-urban and peri-urban producers to consumers which endangers the public to the zoonotic

risk of bTB. On the other hand, only 18% is supplied by dairy enterprises in pasteurized form (Ameni *et al*., 2007).

Many dairy farmers lacked the experience of observing bTB infected cattle in their herds in this study. Many farmers did not also have a recent memory of their herds tuberculin tested or not. Though few farmers recall the skin testing history of their herds against bTB, doing nothing strategy was the only alternative they had to their tested positive animals. Since bTB persists in a farm unless mitigating measures are put in place, cattle movements without preliminary test and certification could have spread the infection from infected focal farms to newly established ones (Elias *et al*., 2008).

Cattle owners' awareness study of bTB across different agro-ecological settings of Zambia revealed that 60.4% (64/106) of them had not heard of bTB in animals and 92.9% (39/42) had no basic knowledge of its mode of spread indicating low levels of awareness on bTB among cattle owners (Munyeme *et al*., 2010). Nuru *et al*. (2015) indicated low level of awareness (25%: 48/192) and knowledge about the zoonotic transmission of bTB among livestock owners in Bahir Dar and its surroundings. According to Alelign *et al*. (2019), 67% (74/111) of livestock owners in South Gondar did not know about the transmission of bTB from cattle to human. Same authors indicated 69% (77/111) of the respondents had the habit of consuming raw milk and other uncooked dairy products which is congruent to the current finding. The zoonotic risk of bTB is often associated with consumption of untreated milk and meat products as well as via aerosol in proximity to livestock (Cosivi *et al.*, 1998; Wilkins *et al.*, 2008).

Farmers and swine herd risk factors: Farmed swine included in small herds were characterized by uncontrolled movement and contact with livestock as well as human beings which is in agreement to Arega *et al*. (2013). Arega *et al*. (2013) indicated close contact of swine with animals and humans. Arega *et al*. (2013) also indicated the traditional small scale swine production system is the predominant system in Ethiopia characterised by absence or minimal health care, supplementary feeding and proper housing. Current observation revealed that, the swine farms were poorly constructed and the swine themselves were poorly managed (soiled

swine). The waste drainage system of the swine farms was severely poor in majority of the cases. Majority of the farms have no light source at all.

Poor sanitation, poor ventilation due to complete closure, buildup of ammonia, poor feeding conditions, overstocking and hot room temperature are all stressors to swine that can suppress their immune system and consequently predisposing them to various diseases including TB. Dairy farm-based study by Mekonnen *et al*. (2019) demonstrated herd size, farm hygiene, feeding condition and biosecurity as significant predictors for herd bTB positivity. Moreover, overstocking contributes to density dependent transmission of bTB (Ameni *et al*., 2006). Mekonnen *et al*. (2019) indicated stressful conditions showed higher odds of bTB positivity than herds with less stressed conditions implicating the deleterious effect of stress on the animals' resistance to the disease.

The swine in majority of the cases in the current study were seen to feed on untreated and spoiled (fishy) milk and cheese collected from different milk collection centres. They were also seen to feed on cooked body of dead birds, abattoir offals including poultry offals, garbage, stomach contents of slaughtered animals, spoiled beef and vegetables as well as human leftovers. Spoiled vegetables were collected from different fruit selling centres whereas human food leftovers including spoiled beef were collected from different restuarants. Both the quality and quantity of feed items provided to swine were poor and hence they were generally seen to be poorly fed on regular basis. Mekonnen *et al*. (2019) observed herds fed with poor to moderate levels were at higher risk of being bTB positive compared to well-nourished herds. In addition, same authors reviewed the feeding practice of supplement feeding diminished the risk of transient bTB outbreaks in UK. Arega *et al*. (2013) indicated swine that were kept on free grazing and fed with one or more of swill, poultry offal or left to roam for garbage harboured mycobacterial infections twice than those fed on commercial mixed feed. Same authors reviewed the association of *M. tuberculosis* outbreaks in swine fed on uncooked garbage from hospitals or residences housing human TB cases (Arega *et al*., 2013).

Observation in this study revealed the absence of all biosecurity measures which is evidenced by low level of awareness, herd contact, frequent coming of unregulated farm visitors and absence of disinfectant use except at two big farms. According to Mekonnen *et al*. (2019), farm biosecurity practices were measured by level of awareness, access to wildlife, mixing with neighbouring herds and farm enclosure. Biosecurity practices in farm management are important to prevent the introduction of bTB into a herd. In addition, farms practicing poor biosecurity measures showed higher likelihood of herd bTB positivity compared to those which practiced modest levels of biosecurity (Mekonnen *et al*., 2019).

Awareness related issues presented to participants revealed that majority of them were not aware (94%) of the zoonotic transmission of swine TB to human beings. This was evidenced by sharing shelter (60%) with livestock that can expose the community to zoonotic TB.

5. 4. Limitations and Future Areas of Research

The current study findings of bTB in Debre Berhan milkshed might not be inferable to the target population because dairy cattle within inaccessible areas of the milkshed were not tuberculin tested. There were also lack of interest and cooperation in some of the farm owners and development agents. In addition, the study lacked laboratory works pertaining to isolation, identification and molecular typing of the causative agents. This is so because there was lack of an ear-marked budget allocated to purchase at least reasonable number of bovine tuberculin tested reactor cattle. However, collection of TBL lesions from cattle slaughtered at Debre Berhan municipality abattoir was discontinued after a month of postmortem inspection because all the slaughtered animals were Zebu breed and their origin was predominantly out of Debre Berhan milkshed. Furthermore, there was no single opportunity of getting data to traceback the animals to their herds of origin. Shimeles (2008) who conducted a lesion prevalence study of bTB at Debre Berhan municipality abattoir concluded that, the origin of animals slaughtered could not exactly be traced back.

Discussion made with butchermen indicated the origins of purchased cattle for slaughter was from livestock markets of *Karra* in Addis Ababa, *Kotu-gebeya*, *Shewarobit*, *Debre Sina*, South Wollo and other distant places which are difficult to correlate the agent/s to the milkshed. Furthermore, both cattle and swine farm owners including their family were not tuberculin tested (Mantoux test) due to absence of human tuberculin skin test (TST) antigen.

There was no established cut-off value by OIE to interpret the swine tuberculin test result. The OIE (2009) manual for terrestrial animals clearly indicated that tuberculin test has not been well validated in most non-bovid and non-cervid species. However, Canadian Food Inspection Agency (CFIA, 2019) employed severe interpretation cut-off value which is only performed to test live swine targeted for semen production under export programmes. Therefore, the present tuberculin test results in swine were interpreted based on CFIA's (2019) guideline.

Alternative media other than the conventional solid LJ-media optimized to culture TB suspected tissue samples from swine were not available. There was poor colony growth even though as many tissue necropsies as possible were collected from AAAE which is the only abattoir providing swine slaughter services in Ethiopia. Of many samples cultured, only three demonstrated colony growth. The plan before commencement of the research was to test as many swine as possible, purchase and slaughter strong test positive ones. However, many swine farm owners (small to large farms) were not cooperative without logical reasons. Moreover, there were generally few large swine farms in the study areas. Hence, few numbers of swine compared to the target population from small to large farms were tuberculin tested based on voluntary basis. Therefore, the tuberculin test results of swine in the present study may not be inferable to the target population in central Ethiopia.

Gaps	Research demanded
Human skin test (Mantoux test)	Human skin test antigen should be made available and the occurrence of TB in human need to be elucidated
Cut-off value in swine skin test	There should be interpretation appraisal study of swine TB in Ethiopia so as to establish a cut-off value
Culturing media	There should be study on alternative media to culture mycobacteria
Transporting and freezing media	There is a need to study and made available mycobacterial transporting and freezing media to keep baccilli alive for culturing

6. CONCLUSION AND RECOMMENDATIONS

6. 1. Conclusion

The prevalence of bTB in dairy cattle in Debre Berhan milkshed was moderately high in both animal and herd levels. On the other hand, the prevalence of bTB in swine was low at animal level. However, the swine herd level prevalence was moderately high. In addition, both gross pathology and histopathological studies revealed presumptive TBL lesions in swine. *M. tuberculosis* was isolated from TBL lesions in swine and human sputa. Poor body condition, large herd size and exotic dairy cattle breeds were important predictors of bovine tuberculin test positivity in dairy cattle.

The occurrence of bTB, low public awareness, absence of farm biosecurtiy measures, complete absence of policy and strategy to control the disease nationally as well as locally, lack of government attention, increased demand for milk and improved breeds, consuming animals' products raw, poor health extension services and poor management practices are contributors to the occurrence of zoonotic TB in humans. Therefore, further in-depth epidemiological studies of bTB together with public awareness campaign in order to establish sound epidemiological control programmes on zoonotic TB are of critical importance and should be the first priority to intervene in the study areas.

6. 2. Recommendations

Based on the above conclusion the following practical and feasible recommendations were forwarded:

1. Community awareness creation about the zoonotic importance of bTB should be made.
2. Animal farm biosecurity and movement control for animals and humans should be strict.

3. Mixed species farming should be avoided.

4. Detailed postmortem inspection of the slaughtered animals should be made to look for TBL lesions.

5. Animal attendants should be screened for TB.

6. Control priority of bTB should be targeted from high to low prevalence settings.

7. Animals with TBL lesions should be traced back to their herds of origin.

8. Incentive mechanisms (certificate of recognition or bonuses or reasonable superior price of saling milk/beef) for bTB-free herds or bTB free milk or bTB free beef need to be established.

9. Farmers need to improve animal feeding, optimize herd size and increase the number of their crossbred animals.

10. Consumers at household level need to cook livestock products before use.

11. Dairy plant owners should be encouraged to pasteurize milk before reach to consumers.

12. The Government of Ethiopia needs to have policy and strategy to control bTB.

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8. APPENDICES

8. 1. Questionnaire Survey

Questionnaire to assess the risk factors that precipitate cattle/swine and human TB in central Ethiopia for a study duration of 2016-2018

Questionnaire Number: _____ Date of interview: _____

General Information

Name of interviewer and educational background: _____

Name of dairy/swine farm: _____Age of the farm: _____

Name of the owner (or animal attendant working in the farm not less than one year):

_____

Age (years): _____ Sex:_____ Address: Region / city: _____

Zone / sub-city: _____Woreda:_____Kebele:_____

House Number: _____Cellphone:_____Office telephone:_____

Home telephone: _____

Educational status: 1. illiterate 2. basic writing and reading (adult education) 3. Primary (Grade 1 to 6) 4. junior secondary (grade 7 to 8) 5. secondary (Grade 9 to 12) 6. 12+2 7. 12+4

Number of people who are living or working on the farm: _____

How the farm was started? Multiple options possible:

a. bought the enterprise: 1. Yes ___ 2. No ___

b. bought cattle/swine from other known dairy/swine farms: 1. Yes __ 2. No ___

c. bought cattle/swine from market without knowing their origin: 1. Yes __2. No ___

d. gift: 1. Yes ___ 2. No ____

e. other: 1. Yes ___ 2. No ___; If yes, specify: _____

A. Environmental / Host Risk Factors

1. Type of house/barn: _____1. indoor 2. outdoor 3. none, but fenced

2. Do cattle/swine share shelters with their owners / family members?

_____

3. What are the sanitary conditions of the house/barn in relation to odour, waste drainage, cleanness of floor and animals, light source, and animal stocking? Rate by:

1. Poor 2. Medium (satisfactory condition) 3. Excellent

---

4. How the ventilation status of the house / barn is expressed? Rate by:

1. Poor 2. Medium (satisfactory ventilation) 3. Excellent

---

5. What is the purpose of dairy/swine farm?

5. 1. To produce milk for sale ____and home consumption____

5. 2. To sale live swine for pork ____and backyard slaughter on farm for pork sale____

6. To whom the swine / pork produced in your farm is sold (multiple options possible)?

6. 1. to middle level cater: _____

6. 2. to supermarkets: _____

6. 3. other: _____

7. To whom the milk produced in your dairy farm is sold?

7. 1. To individuals: _____

7. 2. To cafes and restaurants: _____

7. 3. To milk collection centers: _____

7. 4. To dairy cooperatives: _____

7. 5. To milk processing agro-industries: _____

8. How can you get replacement stock? Multiple options possible:

8. 1. From own farm by natural mating___

8. 2. By purchasing from different sources such as another farm / market / gift___

8. 3. Other, specify: _____

9. From where have you purchased cattle/swine?

Specify location: farm, market, gift, Woreda, etc. _____

10. To what distance and area coverage have you sold cattle/swine?

Specify location: farm, market, gift, Woreda, etc. _____

11. What type of cattle/swine do you sell from your farm? Multiple options possible:

11. 1. Weak / poor body condition: 1. Yes ___ 2. No ____

11. 2. Diseased: 1. Yes ____2. No ____

11. 3. With low productive performances: 1. Yes ___2. No ____

11. 4. With high productive performances: 1. Yes ____ 2. No ___

11. 5. Other, specify:_____

12. Are cattle/swine in your farm mixed with cattle/swine of some other farms? Yes __ 2. No __

13. Have you had any cattle/swine in your herd with loss of appetite, weight-loss/chronic body wastage, weakness, a mild (a low–grade) fluctuating fever, intermittent hacking/moist cough, progressive emaciation, diarrhoea, large prominent lymph nodes, anorexia and induration of udder, chronic cough in the last six months? 1. Yes ____ 2. No____

14. Have cattle/swine on your farm been tuberculin / PPD tested before? Yes ___2. No ___

If yes, what happened to cattle/ swine tested positive? Multiple options possible:

14. 1. Remained at the farm: 1. Yes ____ 2. No _____

14. 2. Slaughtered: 1. Yes ____2. No ____

14. 3. Sold: 1. Yes _____ 2. No ____

15. Are there wildlife species nearby your farm and how is the wildlife densities expressed?

Yes: ___No: ___

If yes, name at least one / many: _____

Express the density_____

B. Awareness on Zoonotic Importance (Behavioural)

16. Do members of your family/farm drink raw milk from cattle regularly or occasionally (once per month or more)? 1. Yes ____ 2. No____

17. Do you know that bTB is primarily a disease of dairy cattle? Yes ___ 2. No ___

18. Do you know that bTB can be transmitted to man through consumption of raw milk / milk products obtained from infected cattle? 1. Yes ___ 2. No ____

19. Do you know that bTB can be transmitted to man through consumption of raw meat obtained from infected cattle? 1. Yes ____ 2. No ____

20. Have you ever informed that any of the people living / working on your farm had TB in the last two years or less than two years? 1. Yes ____ 2. No_____

If anyone has had TB on your farm, did he/she drink raw milk / milk products continuously for long period of time?

1. Yes ____2. No____: Mention the time: _____

If anyone has had TB on your farm, did he/she eat raw meat / meat products continuously for long period of time? 1. Yes ___2. No ___: Mention the time: _____

What are the presenting clinical signs? 1. Cervical lymphadenopathy (scrofula) 2. Chronic skin disease (lupus vulgaris) 3. Localized skin disease ("butcher's wart") 4. Fever, poor appetite, loss of weight, weakness, cough, night sweat, chest pain, cavitation and haemoptysis

System more frequently affected: _____

C. Farm Biosecurity Issues (Pathogen Risk Factors)

_____

D. Animal Feeding Regimen and Body Conditions (Host Risk Factors)

_____

E. For Health Professionals

21. Which age group of the community is more affected by TB and Why?

_____

22. What are the professional activities undertaken to control TB?

_____

23. Is there any national TB control programme either by the Government or NGO?

_____

24. Have you had an incidence of TB yourself due to professional exposure? Means of prevention: _____

25. What is your professional advice to either control or prevent TB?

_____

F. HIV/TB Co-infection

26. Which segment of the community is more affected by TB? Why?

Member of the community more affected: _____

Logical reason: _____

8. 2. Body Condition Scores (BCS) in Cattle

Body Condition Scores for cattle on individual farm relative to their TB status were recorded. For each animal, an assessment of the body condition was made and a score given. Farm animals were scored using a basic scoring system derived for zebu– like cattle after Nicholson and Butterworth (1986) and interpreted as:

- **BCS 1**: Marked emaciation (animal would be condemned at antemortem examination).
- **BCS 2**: Transverse processes project prominently and neural spines appear sharply.
- **BCS 3**: Individual dorsal spines are pointed to the touch. Hips, pins, tail-head and ribs are prominent. Transverse processes visible usually individually.
- **BCS 4**: Ribs, hips and pins clearly visible. Muscle mass between hooks and pins slightly concave. Slightly more flesh above the transverse processes than in BCS 3.
- **BCS 5**: Ribs are usually visible, little fat cover and dorsal spines barely visible.
- **BCS 6**: Animals are smooth and well covered: dorsal spines cannot be seen but are easily felt.
- **BCS 7**: Animals are smooth and well covered but fat deposits are not marked. Dorsal spines can be felt with firm pressure but feel rounded rather than sharp.
- **BCS 8**: Fat cover in critical areas can be seen easily and felt: transverse processes cannot be seen or felt.
- **BCS 9**: Heavy deposits of fat clearly visible on tail-head, brisket and cod: dorsal spines, ribs, hooks and pins fully covered and cannot be felt even with firm pressure.

Body Condition Scoring is an important part of modern dairy management. BCS after Kellogg (2009) was used for Holstein-Friesian cows, heifers and their crosses and interpreted as follows:

- **BCS 1**: Deep cavity around tail-head. Bones of pelvis and short ribs are sharp and easily felt. No fatty tissue in pelvic or loin area. Deep depression in loin.
- **BCS 2**: Shallow cavity around tail-head with some fatty tissue lining it and covering pin bones and pelvis easily felt. Ends of short ribs feel rounded and upper surfaces can be felt with slight pressure. Depression visible in loin area.
- **BCS 3**: No cavity around tail-head and fatty tissue easily felt over whole area. Pelvis can be felt with slight pressure. Thick layer of tissue covering top of short ribs which can be still be felt with pressure. Slight depression in loin area.
- **BCS 4**: Folds of fatty tissue are seen around tail-head with patches of fat covering pin bones. Pelvis can be felt with firm pressure. Short ribs can no longer be felt. No depression in loin area.
- **BSC 5**: Tail-head is buried in thick layer of fatty tissue. Pelvic bones cannot be felt even with firm pressure. Short ribs covered with thick layer of fatty tissue.

Appendix Table 1. Ranges of ideal body condition scores for cattle

Stage of Lactation	**Score**
Drying-off	3.5 - 4.0
Calving (older cows)	3.5 - 4.0
One-month postpartum	2.5 - 3.0
Mid-lactation	3.0
Late lactation	3.25 - 3.75
Calving (first lactation)	3.5

Source: Kellogg (2009)

Heifer Body Condition Scoring: Heifer body condition scoring can also be a useful tool for monitoring the energy status of heifers. Heifers that are too fat deposit and fat in the udder might later inhibit formation of milk secreting cells. If heifers get too fat, they may accumulate fat in their reproductive tract which will decrease fertility and increase the likelihood of dystocia. Older heifers that get too fat are more prone to have the same metabolic problems as lactating cows at the time of calving. Heifers that are too thin will have decreased fertility and other health problems compared to heifers that are thrifty and growing well. Generally, heifers will have slightly lower body condition scores than cows. For heifers less than six months old, their body condition score should range from 2.0 to 3.0. Usually heifers should not exceed 3.5 in body condition score. It is recommended that older heifers freshen at a 3.5 body condition score. A body condition score of 2.5 to 3.0 is desirable for heifers from six months old up to breeding age. At breeding, and shortly thereafter, their body condition scores may gradually increase from 3.0 to 3.5. Use caution in adding extra flesh to heifers in late gestation since the extra feed may contribute to large calves and thus calving problems (Kellogg, 2009).

8. 3. Cattle Aging

Appendix Table 2. Typical cattle ages when permanent teeth erupt, develop and wear

Teeth	Cattle age at occurrence		
	Eruption	Full development	Wear
Incisors			
Pinchers	18 to 24 months	24 months	Leveled at 5 to 6 years, noticeable wear at 7 to 8 years
1st intermediate pair	24 to 30 months	36 months	Leveled at 6 to 7 years, noticeable wear at 8 to 9 years
2nd intermediate pair	36 months	48 months	Leveled at 7 to 8 years, noticeable wear at 9 to 10 years
Corners	42 to 48 months	60 months	Leveled at 9 years, noticeable wear at 10 years
Premolars			
1st cheek tooth pair	24 to 30 months		
2nd cheek tooth pair	18 to 30 months		
3rd cheek tooth pair	30 to 36 months		
Molars			
4th cheek tooth pair	5 to 6 months		
5th cheek tooth pair	12 to 18 months		
6th cheek tooth pair	24 to 30 months		

Source: MSU (2013)

8. 4. Glossary

The following information was accessed on 21 March 2016 and retrieved from http://www.righteousbacon.com/when-a-pig-is-a-hog-and-when-its-not/:

These days many pork producers are both—even if their pigs and hogs are raised in separate facilities (and most are)—but the two are not necessarily one and the same.

The difference between a pig and a hog is mostly size and by extension age. But that's far from where the distinctions end. There are also weaners, feeders, shoats, gilts, sows, boars, barrows,

and a small dictionary full of other bits of swine-specific vocabulary to go along with the industry. A short list of those used most commonly is outlined below:

Swine – A generic term for all pigs, hogs, etc.

Gilt – A young female swine, generally under 12 months of age, who has not yet farrowed.

Sow – A mature female swine, generally 10+ months of age, who has farrowed at least one litter.

Boar – An intact male swine.

Barrow – A castrated male swine.

Pig – A very young swine. In layman's terms this would be a "piglet" but that term is rarely used by producers. Can also be a term of endearment for older swine.

Hog – An older swine, usually over about 120 pounds live weight.

Shoat – A young swine, usually between weaning and about 120 pounds live weight.

Weaner – A young swine at and during the point of weaning.

Feeder – A young swine usually between 40 and 70 pounds live weight that is being sold, bought, or held to be fed out to market weight.

Finisher – An older swine, usually over about 150 pounds live weight; one that is in the finishing stage of its growth, nearing market weight.

Market Hog – A hog that's ready to be processed into pork or sent "to market". The ideal market weight for hogs changes with pork demand and industry technology among other things. Usually market hogs weigh between 230 and 270 pounds live.

Bred–Pregnant. Usually used to preface the appropriate word for a particular female swine. "Bred Gilt" or " Bred Sow" depending on her age and stage of life.

Open – Used to preface the appropriate term to refer to a female swine that is not currently bred. For example "Open Gilt" or "Open Sow".

Farrow –As a noun it refers to a litter of newborn pigs, as a verb it is used to describe the act of giving birth.

8. 5. Body Condition Scores (BCS) in Swine

Appendix Table 3. Assessment of BCS of swine

Scores	Body Conditions
1 (Emaciated)	Landmark bones are prominent even without palpation. Considered unfit to travel
2 (Thin)	Bones can be easily felt with slight pressure
3 (Ideal)	The pig's bones are barely felt when palpating with firm pressure
4 (Fat)	Bones of the pig are undetectable with palpation
5 (Overly Fat)	A body score of 5 has the same palpation characteristics as a body score of 4. However, this animal is excessively overweight

Source: CFSPH (2011)

8. 6. Swine Aging

Appendix Table 4. Age classification in swine

Age Interval	Age	Defining Characteristics
1	0 to 8 weeks	i2 and p2 absent
2	8 to 20 weeks	i2 intact (fully erupted) or erupting AND/OR p2 intact or erupting
3	20 to 30 weeks	P1 intact or erupting AND/OR M1 intact or erupting
4	30 to 51 weeks	C intact or erupting AND/OR I3 intact or erupting
5	12 to 18 months	M2 intact or erupting AND/OR I1 intact or erupting AND/OR P2, P3, P4 intact or erupting AND i2 present
6	18 to 26 months	I2 intact or erupting AND/OR Lower M3 intact or erupting
7	26 to 36 months	Upper M3 erupting
8	36 to 48 months	M3s intact
9	48+ months	Visible wear on M3s All other teeth show visible wear Some teeth may be missing
Note: Capital and lowercase letters depict permanent and deciduous teeth, respectively. I — Incisor P — Premolar C — Canine M — Molar		

Source: USDA (2018)

8. 7. Hygiene Assessment of Dairy and Swine Farms

The sanitation status of farms was judged as poor, medium (satisfactory) or good based on aspects such as odour, waste drainage, cleanness of floor and animals, barn ventilation and light source and animal stocking based on the objective criteria developed by FAO and IDF (2011) and described hereunder:

►Poor farm hygiene was judged by poor drainage, smells after manure, poor ventilation, poor illumination and haphazard placement of animals in the barn (no regular site of tie). Moreover, it failed to apply the principles of good personal hygiene (GPH), good agricultural practices (GAP) and good manufacturing practices (GMP).

►Medium (satisfactory) farm hygiene was judged by good drainage inside the barn but the waste is deposited nearby the barn, regular manure cleaning from the barn but deposited nearby the barn, partial ventilation, partial illumination, regular placement site for each animal in the barn and application of farm production principles in modalities of on-and-off basis.

►Good farm hygiene was judged by:

● good drainage inside and outside the barns, good manure disposal to a reasonable distance to avoid spoilage of the dairy farm environment, good ventilation, good illumination, proper stock density in relation to barn size and application of farm management principles, animal contact and isolation and environmental cleaning,

● the biggest potential source of environmental damage is from pollution caused by manures, slurry, silage liquor, etc. The suggested good agricultural practices for the environment are characterized by an appropriate waste management system and ensure farming practices do not have an adverse impact on the local environment, and

● the guiding objectives for good farming practices are animal health, hygiene, animal feeding and watering, animal welfare and environment.

Appendix Table 5. Cattle and swine skin test biodata recording format

Farm	ID	Sex	Age	BCS	Physiol. state	Lactation stage	Parity	B1	B2	▲B	A1	A2	▲A

Appendix Table 6. Swine necropsy samples collection and recording format at AAAE

Number	ID	Date	Sex	Age	BCS	Origin	Type of specimen

Appendix Table 7. Recording format for collection of human sputa at DBZRH

Number	ID	Date	Sex	Age	Origin	Constitutional signs of TB seen during sample collection

Appendix Table 8. Host related risk factors and skin test positivity to different PPD antigens in dairy cattle in Debre Berhan milkshed

Variables	N	Results							
		PPDA+	%	PPDB+	%	Doubtful to PPDB	%	Mixed (+)	%
Sex	**625**	**71**	**11.4**	**99**	**16**	**7**	**1.1**	**35**	**5.6**
Male	75	7	9	7	9	0	0	3	4
Female	550	64	12	92	17	7	1.3	32	5.8
Age	**625**	**71**	**11**	**99**	**16**	**7**	**1.1**	**35**	**5.6**
≤5 years	378	34	9	55	15	3	0.8	19	5
[6-10] years	223	34	15	39	17.5	4	1.8	15	6.7
>10years	24	3	12.5	5	20.8	0	0	1	4.2
BCS	**625**	**71**	**11**	**99**	**16**	**7**	**1.1**	**35**	**5.6**
≥5: Good	218	23	10.6	32	14.7	1	0.5	10	4.6
[3-4]: Medium	352	39	11.1	47	13.4	5	1.4	19	5.4
[1-2]: Poor	55	9	16.4	20	36.4	1	1.8	6	10.9
Breed	**625**	**71**	**11**	**99**	**16**	**7**	**1.1**	**35**	**5.6**
Local	230	15	6.5	3	1.3	3	1.3	3	1.3
Exotic	395	56	14.2	96	24.3	4	1	32	8.1
Management	**625**	**71**	**11**	**99**	**16**	**7**	**1.1**	**35**	**5.6**
Semi-intensiv.	263	19	7.2	5	1.9	3	1.1	3	1.1
Intensive	362	52	14.4	94	26	4	1.1	32	8.8
Physiol. state	**550**	**64**	**11.6**	**92**	**16.7**	**7**	**1.3**	**32**	**5.8**
Open	314	37	11.8	67	21.3	1	0.3	22	7
Pregnant	236	27	11.4	25	10.6	6	2.5	10	4.2
Lactation	**550**	**64**	**11.6**	**92**	**16.7**	**7**	**1.3**	**32**	**5.8**
Non-lactating	255	29	11.4	44	17.3	3	1.2	15	5.9
Lactating	295	35	11.9	48	16.3	4	1.4	17	5.8
Parity	**550**	**64**	**11.6**	**92**	**16.7**	**7**	**1.3**	**32**	**5.8**
No parity	183	15	8.2	31	16.9	1	0.5	10	0.5
[1-3] parity	275	40	14.5	48	17.5	5	1.8	18	6.5
[4-6] parity	82	7	8.5	10	12.2	1	1.2	3	3.7
≥7 parity	10	2	20	3	30	0	0	1	10

N: Total number of dairy cattle skin tested; BCS: Body condition score; PPDA+: Positive to avian purified protein derivative antigen; PPDB+: Positive to bovine purified protein derivative antigen; Mixed infection: the same animals reacted to both avian and bovine PPD antigens at the same time; Semi-intensiv: Semi-intensive; Physiol.state: Physiological state; Open: Dry or non-pregnant

Appendix Table 9. Host related risk factors and skin test positivity to bovine PPD antigen at two different cut-off values in dairy cattle in Debre Berhan milkshed

Variables	N	At ≥4mm cutoff		At >2mm cutoff	
		PPDB+	%	PPDB+	%
Sex	**625**	**99**	**16**	**106**	**17**
Male	75	7	9.3	7	9.3
Female	550	92	17	99	18
Age	**625**	**99**	**16**	**106**	**17**
≤5 years	378	55	15	58	15.3
[6-10] years	223	39	17.5	43	19.3
>10 years	24	5	20.8	5	20.8
BCS	**625**	**99**	**16**	**106**	**17**
≥5: Good	218	32	14.7	33	15
[3-4]: Medium	352	47	13.4	52	14.8
[1-2]: Poor	55	20	36.4	21	38.2
Breed	**625**	**99**	**16**	**106**	**17**
Local	230	3	1.3	6	2.6
Exotic	395	96	24.3	100	25.3
Management	**625**	**99**	**16**	**106**	**17**
Semi-intensive	263	5	1.9	8	3
Intensive	362	94	26	98	27.1
Physiological state	**550**	**92**	**16.7**	**99**	**18**
Open	314	67	21.3	68	21.7
Pregnant	236	25	10.6	31	13
Lactation	**550**	**92**	**16.7**	**99**	**18**
Non-lactating	255	44	17.3	43	16.9
Lactating	295	48	16.3	52	17.6
Parity	**550**	**92**	**16.7**	**99**	**18**
No parity	183	31	16.9	32	17.5
[1-3] parity	275	48	17.5	53	19.3
[4-6] parity	82	10	12.2	11	13.4
≥7 parity	10	3	30	3	30
Herd size	**96**	**16**	**16.7**	**22**	**22.9**
Small	82	6	7.3	7	8.5
Medium	7	3	42.9	5	71.4
Large	7	7	100	10	143
Farm hygiene	**96**	**16**	**16.7**	**17**	**17.7**
Poor	84	7	8.3	8	9.5
Medium	6	4	66.7	4	66.7
Good	6	5	83.3	5	83.3

Appendix Table 10. Number and percent of swine positive to tuberculin skin test at >2mm value to different antigens by host related factors

Variables	No. tested	Results					
		PPDA positive	%	PPDB positive	%	Mixed positive	%
Sex	**329**	**9**	**2.7**	**10**	**3**	**5**	**1.5**
Male	133	1	0.8	2	1.5	1	0.8
Female	196	8	4.1	8	4.1	4	2
Age	**329**	**9**	**2.7**	**10**	**3**	**5**	**1.5**
<2 years	261	2	0.8	0	0	0	0
≥2 years	68	7	10.3	10	14.7	5	7.4
BCS	**329**	**9**	**2.7**	**10**	**3**	**5**	**1.5**
≥5: Good	105	0	0	0	0	0	0
[3-4]: Medium	178	3	1.7	2	1.1	1	0.6
[1-2]: Poor	46	6	13	8	17.4	4	8.7
Physiological state	**196**	**8**	**4.1**	**8**	**4.1**	**4**	**2**
Open	137	5	3.6	3	2.2	1	0.7
Pregnant	59	3	5.1	5	8.5	3	5.1
Lactation	**196**	**8**	**4.1**	**8**	**4.1**	**4**	**2**
Non-lactating	146	7	4.8	6	4.1	3	2.1
Lactating	50	1	2	2	4	1	2
Parity	**196**	**8**	**4.1**	**8**	**4.1**	**4**	**2**
No parity	111	3	2.7	1	0.9	4	3.6
[1-3] parity	64	5	7.8	6	9.4	0	0
[4-6] parity	21	0	0	1	4.8	0	0

No.: Total number of swine tuberculin skin tested; BCS: Body condition score; PPDA: Avian purified protein derivative antigen; PPDB: Bovine purified protein derivative antigen; Open: dry or non-pregnant

8. 8. Certificates of Research Ethical Clearance

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የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ/ደብረ ዘይት

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/007/03/09/2016

Name of Applicant: **Dr Kassa Demissie (PhD)**

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: **Swine tuberculosis:** ***Epidemiology, Public health significance and Molecular characterization of its causative agents in central Ethiopia"***

Date of application:	**June 2016**
Nature of the project:	**On farm and abattoir investigation**
Target animal species:	**Swine**
Number of animals involved:	**No animals 1033**
Study area:	**Central Ethiopia**

Minutes No. and date of review: **VM/ERC/003/9/016, 08/12/2016**

The above indicated research project is acceptable from animal research ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

The applicant is advised to obtain additional Ethical clearance for Human aspect

Dr Getachew Terefe
Chairman

Signature

መልሱን በሚጽፉልን ጊዜ አባክዎ የእኛን ደብዳቤ ቁጥር ይጥቀሱልን
Please quote Our Ref. No. When replying
ፋክስ} ስልክ } ፖ.ሣ.ቁ
Fax 251-11-4339933 Tel. +251 114338450 P.O.Box 34
ቢሾፍቱ/ደብረዘይት፤ ኢትዮጵያ
Bishoftu/Debre Zeit, Ethiopia

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/08/11/2018

Name of Applicant: Kassa Demissie (DVM, MSc, PhD fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Bovine tuberculosis: Epidemiology, public Health significance and Molecular characterization of its causative agents in milk sheds in three districts of North Showa, Central Ethiopia*

Date of application: **25/10/2018**

Nature of the project: **non-invasive**
Target animal species: **cattle**
Number of animals involved: **610**
Study area: **North Showa, Ethiopia**

Minutes No. and date of review: **VM/ERC/08/11/018, 21/12/2018**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary
3. A separate clearance is required for works on human subjects

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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አዲስ አበባ ፣ ኢትዮጵያ
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ADDIS ABABA UNIVERSITY
Aklilu Lemma Institute of Pathobiology
Addis Ababa, ETHIOPIA
☎ **00251-1-276-30-91/13-57-25**
e-mail: aau-ipb@telecom.net.et

Aklilu Lemma Institute of Pathobiology Institutional Review Board

Ethical Clearance Certificate

Ref. No.: ALIPB IRB/023/2011/2019
Date: Thursday, 23 May. 2019

Title of the Project: 'Bovine TB: Epidemiology, public health significance and molecular characterization of its causative agents in dairy cattle of central Ethiopia'

PI: Kassa Demissie

Recommendation of the ALIPB Institutional Review Board

Dear Kassa,

Congratulations, the ALIPB IRB has reviewed the above mentioned PhD Research Proposal and noted its scientific merit. The IRB would like to remind the student to submit progress reports of the work every 6 months and the final report upon completion of the study. Furthermore, the student is expected to notify the ALIPB/IRB ahead of time any amendments or modifications in the protocol or premature suspension or termination of the study.

STATUS: APPROVED

IRB Chairperson: **Prof. Tilahun Teklehaymanot**
Signature: _____

IRB Secretary: **Dr Lemu Golassa**
Signature: _____ 23 05 2019

Approval
Name: **Prof. Mirutse Giday**
Signature: _____
Date: 23/05/2019

CC// ALIUPB IRB office

ALIPB IRB Notes

8. 9. List of Publications

Paper 1. Kassa Demissie, Jirata Shiferaw, Girmay Medhin, Aboma Zewude, Asegedech Sirak, Takele Abayneh, Gezahegne Mamo, Gobena Ameni (2020). Prevalence and risk factors of swine tuberculosis in central Ethiopia. *Ethiop. Vet. J.*, **24**: 16-34. DOI https://dx.doi.org/10.4314/evj.v24i2.2.

Paper 2. Kassa Demissie, Gezahegne Mamo, Musse Girma, Balako Gumi, Takele Abayneh, Gobena Ameni (2022). Prevalence and risk factors associated with bovine tuberculosis in local and crossbred dairy cattle in Debre Berhan milkshed, central Ethiopia. *Ethiop. Vet. J.*, **26**: 49-65. DOI https://dx.doi.org/10.4314/evj.v26i1.4