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ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

**THE EPIDEMIOLOGY AND PUBLIC HEALTH IMPLICATION OF BOVINE
TUBERCULOSIS IN ALAGE DAIRY FARM, SOUTHERN ETHIOPIA**



BY

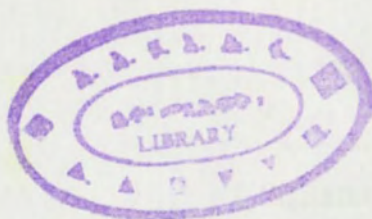
DAWIT SHIMELIS TAYE

JANUARY, 2009

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TUBERCULOSIS IN ALAGE DAIRY FARM, SOUTHERN ETHIOPIA**



A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in
Partial Fulfillment of the Requirements for the Degree of Masters of Veterinary Science
in Tropical Veterinary Public Health

BY

DAWIT SHIMELIS TAYE

THE EPIDEMIOLOGY AND PUBLIC HEALTH IMPLICATION OF BOVINE
TUBERCULOSIS IN ALAGE DAIRY FARM, SOUTHERN ETHIOPIA

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ABBREVIATIONS

AAU	Addis Ababa University
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
BCS	Body condition score
Bp	Base pairs
BTB	Bovine tuberculosis
CI	Confidence interval
DNA	Deoxyribonucleic acid
DR	Direct repeat
DVM	Doctor of Veterinary Medicine
EPTB	Extra-pulmonary tuberculosis
FAO	Food and Agriculture Organization
FVM	Faculty of Veterinary Medicine
HIV	Human immunodeficiency virus
IU	International unit
Lact. non-preg	Lactating non-pregnant
Lact. preg	Lactating pregnant
L-J	Löwenstein Jensen
MBTC	<i>Mycobacterium tuberculosis</i> complex
MSc	Masters of Science
Non-lact non-preg	Non-lactating non-pregnant
Non-lact. preg	Lactating non-pregnant
OIE	Office International des Epizooties
OR	Odds ratio
PCR	Polymerase chain reaction
PPD	Purified protein derivatives
PTB	Pulmonary tuberculosis
RD	Region of diversity
Rpm	Revolutions per minute



SIDCT	Single intradermal comparative test
SIDT	Single intradermal test
SPSS	Statistical Package for Social Science Students
TB	Tuberculosis
V	Volt
VLA	Veterinary Laboratory Agency
WHO	World Health Organization

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ABSTRACT

Across-sectional study was conducted on a total of 355 dairy cattle and 8 pulmonary tuberculosis patients between October 2007 and April 2008 to determine the prevalence, risk factors and public health implication of bovine tuberculosis (BTB) in Alage. Single intradermal comparative tuberculin (SIDCT) test, bacterial isolation and polymerase chain reaction (PCR) based typing were performed on both sputum samples of pulmonary tuberculosis patients and milk of reactor cows. The data was analyzed using SPSS version 11.5 for windows. Percentages were used to calculate the prevalence while chi-square test, univariable and multivariable logistic regressions were used to analyze the putative risk factors of BTB. Out of 355 tuberculin-tested animals, 101 animals were positive and 36 animals were doubtful reactors indicating a prevalence of 28.45% and 38.59% when the doubtful reactors are included. Out of 58 milk samples collected from SIDCT test-positive cows, two (3.54%) *M. bovis* were isolated by culturing on Löwenstein Jensen media and gave signal for the region of diversity (RD)4 based PCR typing. Similarly, 2 (50%) of 4 mycobacterial isolates from sputum samples of patients with pulmonary tuberculosis were *M. tuberculosis*. The reactor rates observed for animals under different parity levels were 3.9% for nullipara, 10.14% for unipara and 14.3% for pluripara. The reactor rates for cattle in different physiological states such as lactating pregnant, lactating non-pregnant, non-lactating pregnant and non-lactating non-pregnant were 0.28%, 16.05%, 6.47% and 5.63%, respectively. Cattle under different age-groups of <3 years old, 3-5 years old and ≥ 6 years old gave reactor rates of 1.41%, 12.67% and 14.36%, respectively. Classification of reactors by body condition scores (BCS) indicated that 9.86% were very thin while 13.24% and 5.35% were thin and moderate, respectively. Chi-square test for associations between parity, physiological status, age-groups and BCS with tuberculin reactivity was significant ($P < 0.05$). The stepwise logistic regression analysis using independent variables unipara, non-lactating non-pregnant animals and age-group <3 years old as reference category indicated that parity [pluripara ($P=0.049$, OR=3.54, 95% CI=1.01-12.45)], physiological status [(lactating pregnant ($P=0.045$, OR=0.093, 95% CI=0.009-0.953), lactating non-pregnant ($P=0.000$, OR=0.101, 95% CI=0.03-0.34) and non-lactating pregnant ($P=0.000$, OR=0.07, 95% CI=0.02-0.24)] and with age-groups [(3-5 years ($P=0.000$, OR=29.54, 95% CI=6.03-144.65) and ≥ 6 years ($P=0.000$, OR=56.96, 95% CI=9.54-340.12)] significantly affected tuberculin reactivity. This study

showed that the prevalence of SIDCT test positives and risk of acquiring the disease increased with age and parity level while lactation and pregnancy reduced tuberculin reactivity. Thus, more sensitive diagnostic techniques and control strategies should be considered on these risk groups. The high prevalence of BTB and the isolation of *M. bovis* from raw milk have public health risk which needs immediate attention from the concerned bodies and effective management strategies against the disease should be implemented.

bovine TB

skin test

Key words: Age, BTB, *M. bovis*, Prevalence, Public health, Risk factors, SIDCT-test

Algae dairy farm

1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by mycobacteria that are of major health risk to humans and animals. It is widely distributed throughout the world affecting all age groups of humans and animals. Human TB of animal origin, particularly *Mycobacterium bovis*, is increasingly becoming important in developing countries (Cosivi *et al.*, 1998). *Mycobacterium bovis* is one of the etiological agents of mammalian TB that is antigenically grouped in *M. tuberculosis* complex (MTBC) (Barwinnek and Taylor, 1996).

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a well-known zoonotic disease, which affects cattle worldwide. The Office International des Epizooties (OIE) classifies bovine tuberculosis in List B of diseases. It is of great socio-economic, public health importance and of significant in international trade of animals and animal products (Cousins *et al.*, 2003). The BTB is re-emerging in many countries as a devastating disease due to the positive potentiating by the human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS) (Ayele *et al.*, 2004).

There were 8.8 million new cases of TB in 2003 (140 cases per 100,000 people), of which 16% were in sub-Saharan Africa (WHO, 2005). Globally, TB causes more adult death than any other single infectious disease. Approximately, 95% of cases and 98% of deaths occur in the developing world (Lawson *et al.*, 2006). Ethiopia is one of the top sixteen countries in the world, and one of the top three in Africa, with regards to the number of TB patients. Over a third of the population has been exposed to TB. The annual risk of TB infection is about 2.2%. An estimated 377,030 Ethiopians (0.62% of the population) have active TB of all kinds, with more than 120,000 new cases in the year 2003/04, nearly a third of which were smear-positive. It remains difficult to explain the extraordinary proportion of cases that are reported as extra-pulmonary tuberculosis (EPTB) (>34% in 2003 with regional variations ranging between 29-54%). The vast majority of EPTB were reported as lymph node TB. In 2003, the numbers of deaths of all cases of tuberculosis including HIV positive patients were 56,456 (WHO, 2005).

Similarly, the prevalence of BTB in Ethiopia is also high ranging from 3.4% in smallholder production systems to 50% in peri-urban (intensive) dairy production systems (Ameni and Roger, 1998; Kiros, 1998; Bogale *et al.*, 1999; Ameni *et al.*, 2001). In countries where BTB is still common and pasteurization of milk is not practiced, an estimated 10-15% of human TB is caused by *M. bovis* (Ashford *et al.*, 2001). Human infection due to *M. bovis* is thought to be mainly through drinking of raw milk. However, cases of pulmonary TB have also been reported particularly in patients from rural areas that live in close contact with cattle (O'Reilly and Daborn, 1995). The high prevalence of TB in cattle, the close contact of cattle and humans in rural areas, the habit of raw milk consumption in the community and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other mycobacteria between cattle and their owners (Ayele *et al.*, 2004).

BTB can be diagnosed by tuberculin test using purified protein derivative (PPD), milk bacteriology and meat inspection (Pritchard, 1988; Cosivi *et al.*, 1995). Human infection caused by *M. tuberculosis* and *M. bovis* are clinically and radiologically indistinguishable. Therefore definitive diagnosis can be achieved through isolating and typing of the etiological agent (Acha and Szyfres, 2001). PCR is a biochemistry and molecular biology technique for enzymatically replicating deoxyribonucleic acid (DNA) without using a living organism. It is a method that efficiently increases the number of DNA molecules in logarithmic and controlled fashion (Biet *et al.*, 2005). PCR have shown more promise for mycobacterial detection in clinical samples to detect sequences specific for *M. tuberculosis* and *M. bovis* (Wards *et al.*, 1995). The procedure is very sensitive when used on tuberculosis culture and the test is much faster than culture and detects non-viable organisms (Acha and Szyfres, 2001).

The control of *M. bovis* in developing countries including Ethiopia is hampered by the high cost of a sustainable testing programme, the lack of veterinary expertise and communication networks (Ayele *et al.*, 2004). In 1996, a prevalence rate of 27.2% was reported in Alage intensive dairy cattle farm (Ameni (1996) cited by Ameni (2003)) using single intradermal tuberculin (SIDT) test. However, no further study was conducted there after. In the presence of several risk factors that promote *M. bovis* transmission from cattle-to-cattle and cattle-to-

* A paragraph of statement of justification to undertake this study

* Deletion typing PCR → Few sentences

humans, it is desirable to conduct in-depth investigation into the epidemiology and public health implication of BTB so as to take the necessary control measures.

Thus, the present study was conceived with the following objectives:

- To investigate the prevalence of BTB at the Alage Dairy Farm
- To identify the risk factors of BTB at the farm
- To assess the public health implication of *M. bovis*

2. LITERATURE REVIEW



2.1. Etiology

2.1.1. Classification and taxonomy

Mycobacteria belong to the genus *Mycobacterium* of the family Mycobacteriaceae (Seifert, 1996). The genus *Mycobacterium* includes obligate parasites, saprophytes and intermediate forms differing in nutritional requirements (Tauro *et al.*, 1996). *Mycobacterium* species that are pathogenic to animals and man include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. avium* complex, *M. microti*, *M. marinum*, *M. leprae*, *M. lepraemurium*, *M. avium subsp. partuberculosis*, *M. senegalense* and *M. farcinogenes* (Quinn *et al.*, 2002). The causative organism of bovine tuberculosis is *M. bovis*, a member of the *M. tuberculosis* complex which includes *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* (Wedlock *et al.*, 2002).

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2.1.2. Morphology

Mycobacteria are of different lengths that ranges between 0.2-0.6 by 1.0-10.0 μm . Characteristically they appear in specimens of infected material in small clusters joined by one pole of the rod, thus forming an acute angle (Seifert, 1996). Most of the members are slightly curved or rods sometimes with branching filamentous and mycelia type growths may occur but usually get fragmented into rods or coccoid cells. Acid fast, non motile and do not form endospores, conidia or capsules. The cells are characterized by a high lipid content consisting of long, branched chains of mycolic acids. All are aerobic, although growth may occur in different depths of media (Tauro *et al.*, 1996).

2.1.3. Structure and composition

The cell wall of mycobacteria contains N-glycomuramic acid in place of N-acetylmuramic acid and has a high lipid content (60%) (Sharma and Adlakha, 1996). Lipids account for acid fastness, pathogenic and immunogenic properties. The various structural components of

mycobacteria, include surface mycosides (mostly glycolipids and peptidoglycolipids), mycolic acids and associated esters, wax-D, cord factor (dimycolil trehalose), sulfolipids (or sulfatides), phosphatidyl inositol mannoside, mycobactins, carotenoid pigments (chromogens) and tuberculin (peptides liberated into culture media during growth) (Biberstein and Hirsh, 1999). The genome sequence of *M. bovis* is at least 99.95% identical to that of *M. tuberculosis*, but deletion of genetic information has led to a reduced genome size (bases) (Garnier *et al.*, 2003).

2.1.4. Staining and cultural characteristic

Although mycobacteria are cytochemically Gram-positive, the high lipid and mycolic acid content of cell wall prevent the uptake of the dye in the Gram-stain (Quinn *et al.*, 2002). Their most noted staining property is their acid fastness. Once stained; they resist discoloration with 3% HCl in ethanol (Biberstein and Hirsh, 1999). The reason for this is because the organisms contain a thick cell wall composed of wax-D and lipid with high content of mycolic fatty acids (Ochei and Kolhatkal, 2005). The heated stain is able to penetrate the thick cell wall which binds the carbon fuchsin. Because they resist discoloration with mineral acid, the organisms stain red and are referred to as acid fast or Zeihl-Neelsen positive (Quinn *et al.*, 2002). The degree of acid fastness varies from species to species. For instance, *M. leprae* is much less acid fast than *M. tuberculosis*. Thus, weaker acid solution is used for the discoloration of such species like *M. leprae*. Routinely, acid-alcohol is used for discoloration of tubercle bacilli since it gives clear background. Hence, some investigators use the term acid-alcohol fast although the alcohol is not essential in the discoloration technique (Ochei and Kolhatkal, 2005).

Mycobacteria grow on protein enriched media except *M. leprae*, *M. lepraemurium* and the *Mycobacterium* causing bovine skin tuberculosis which do not grow on any artificial culture media. The cultural differentiation of mycobacteria is based on: growth rate, temperature of growth, production of pigments in light and darkness (Biberstein and Hirsh, 1999; Ochei and Kolhatkal, 2005) and colonial characteristics (Quinn *et al.*, 2002). The surface mycosides (glycolipids and peptidoglycolipids) determine the colonial characteristics and serologic specificities (Biberstein and Hirsh, 1999). The cultural characteristics and growth requirements of mycobacterial species are summarized in (Table 1).

Table 1: Summary of the cultural characteristics and growth requirements of mycobacteria

Cultural characteristics and requirements	<i>M. tuberculosis</i>	<i>M. bovis</i>	<i>M. avium</i> complex
Growth rate	Slow (3-8weeks)	Slow (3-8weeks)	Slow (2-6weeks)
Atmospheric requirements	Aerobic	Aerobic	Aerobic
Colonial features	Rough, buff, difficult to break apart	Cream coloured raised with central roughness, break apart easily	Sticky, off white, break apart easily
Essential growth supplement	None	None	None
Effect of added glycerol	Enhanced growth (Eugonic)	Growth inhibited (Dysgonic)	Enhanced growth (Eugonic)

Source: Quinn *et al.* (2002)

2.2. Epidemiology

2.2.1. Host range

Mycobacterium bovis has a broad host range in wildlife, captive wildlife, domestic livestock, non-human primates and humans as principal cause of TB (Biet *et al.*, 2005). Examples of these hosts include cattle, humans, monkeys/gorillas, goats, cats, dogs, pigs, buffalo, badgers, possums, deer and bison. Many of these susceptible hosts, including man, are spillover hosts in which infection is not self-maintaining (O'Reilly and Daborn, 1995). In Africa wild ruminants and carnivores are the natural reservoirs of *M. bovis*. As for the human, the highest risk groups

are individuals with concomitant HIV/AIDS infection (Ayele *et al.*, 2004). The rate of transmission of *M. bovis* is significantly influenced by the high density of the susceptible population and its prevalence (Ashford *et al.*, 2001)

2.2.2. Source of infection and mode of transmission

Cattle-to-cattle

The principal source *M. bovis* is the infected animals. It is usually excreted in the respiratory discharge, feces, milk, urine and semen intermittently throughout all stages of the disease and in particular during its advanced stages (Biberstein and Hirsh, 1999).

Tubercle bacilli are transmitted via the respiratory and alimentary routes. Percutaneous and transplacental routes of infection are unusual. Intrauterine infection of calves occurs when bovine tuberculosis is highly prevalent (Biberstein and Hirsh, 1999). Inhalation of *M. bovis* is the principal route to bovine infection and is facilitated by close, prolonged contact between infected and healthy animals. Intensive livestock farming promotes close contact between animals and in extensive livestock farming, close contact rates between animals occurs at watering points, at night gathering places, vaccination and artificial insemination centers, dipping tanks, auction stations, market places, during transportation, during concentration under trees or other shaded areas and salt supplementing sites favoring the spread of *M. bovis* in animals. Ingestion of *M. bovis* directly from infected animals or from contaminated pasture, water or utensils may also be very common (Ayele *et al.*, 2004).

Cattle-to-human

Although BTB primarily affects the lungs of cattle and only less than 2 % of animals suffer from infected udders (Collins, 2000), *M. bovis* shed directly from infected mammary tissues into the milk appears to be the only significant food-borne exposure to human (O'Reilly and Daborn, 1995; Cousins, 2001;). Gastrointestinal infection by ingestion of *M. bovis* is made possible by the fatty coating of the bacterium which protects it from digestion. The ileocaecal region is the

main route of infection for ingested organisms, where the bacterium migrates to mucosal glands and is carried to the Peyer's patches by phagocytes (Biet *et al.*, 2005). Poorly heat-treated meat and vegetable contaminated by *M. bovis* (as it can survive in the soil) as well as contaminated aerosol droplet from infected cattle are potential sources of *M. bovis* infection (Ayele *et al.*, 2004). Proud (2006) reviewed a 21-year evidenced research, (1890-1911), and concluded that tuberculous milk was the cause of significant loss of human life. This review resulted in effective action to make milk safe. In addition, proud meat is generally well cooked, and since the tubercle bacillus is destroyed by exposure to comparatively low temperature, this is an unlikely serious source of infection. The bacilli may be found but rarely in the flesh. For such reasons, in Edinburgh, 70% of carcasses from cattle with tuberculous lesions could be passed as fit for human consumption and only 20% were subjected to partial rejection while 10% were totally rejected (RUA-Domenech *et al.*, (2006). ? Is this name is correct?

Human-to-cattle

The detection of mixed infection with mycobacterial pathogens in humans and cattle (Prasada *et al.*, 2005) denotes the potential transmission of these pathogens from cattle-to-humans (zoonosis) and from humans-to-cattle (reverse zoonosis). Agricultural workers may acquire the disease by inhaling cough spray from infected cattle; they develop typical pulmonary tuberculosis (TB). Such patients may infect cattle and have a great role in human-to-cattle transmission of *M. bovis* (Cosivi *et al.*, 1995).

Human-to-human

The transmission of *M. bovis* between human-to-human is usually considered as a rare event, but in the context of high HIV prevalence and the presence of many risk factors, the epidemiology of the disease has greatly changed and currently human-to-human transmission is more common (Wedlock *et al.*, (2002). HIV associated *M. bovis* has resulted in hospital outbreaks, with a clear evidence of human-to-human transmission (Bouvet *et al.*, 1993 cited by Cosivi *et al.*, (1995).

2.2.3. Risk factors

Cattle:

The prevalence of BTB within a country varies from area to area. The highest occurrence is generally observed where intensive dairy cattle production is most common, notably in the milk sheds of larger cities (White, 2004). Herds with the following risk factors had a significantly greater prevalence of intradermal test positives: >50 cattle in the herd; herds housed inside at night and herds in contact with wildlife. Furthermore, villages that experience annual flooding have a higher prevalence rate of infection (Cleaveland *et al.*, 2006).

Factors that play major roles in predisposing animals to infection include types of production systems. Dairy cattle are at high risk because husbandry methods allow close contacts between animals during milking and when housed together. Suckling calves become infected by ingesting contaminated milk (Quinn *et al.*, 2002). Prevalence of intradermal test positives increased significantly with cattle age (Cleaveland *et al.*, 2006). This is because susceptibility to *M. bovis* infection increases with the age of cattle. Nevertheless, immature animals often develop more severe lesions than older ones (Biberstein and Hirsh, 1999).

Genetic variation in the susceptibility of cattle to *M. bovis* infection exists (Foster *et al.*, 2002). Breed susceptibilities differ: zebu cattle (*Bos indicus*) are more resistant than European breeds (*Bos taurus*). Natural exposure to *M. bovis* or environmental mycobacteria may assist in the development of specific immunity. Immunological suppression in the periparturient period can produce anergic reactors, which may act as a constant source of infection for cattle-to-cattle transmission. Circumstantial evidence suggests that inadequate intake of mineral, vitamin and protein increases the susceptibility of cattle. In addition, weather patterns have also been implicated in the susceptibility of herds to *M. bovis* infection (Foster *et al.*, 2002).

Human:

Factors that contribute to acquisition of infection by humans are demography (age and number of individuals per m² in a dwelling); eating habits (consumption of raw milk and raw milk products and consumption of raw meat); occupation (abattoir workers, veterinarians, laboratory technicians, animal care takers in the zoo, workers in animal reservations and national parks) and living status of families (sanitation, income, education, family ownership of cattle, previous livestock ownership and history of working with animals) (Ayele *et al.*, 2004).

M. bovis in human patients has been associated with families in which a confirmed diagnosis of tuberculosis had previously been made and with households far from the neighbors (Cleaveland *et al.*, 2006). Close physical contact between humans and potentially infected animals is present in some communities, especially in developing regions. For example, in many African countries cattle are an integral part of human social life. They represent wealth and are at the center of many cultural events (Idigbe *et al.*, 1986).

2.2.4. Occurrence

Although BTB is widespread throughout Africa, very little is known about risk factors for *M. bovis* infection in either human or animal populations. According to Cosiyi *et al.* (1998), 60% of the African, 47% of Asian and 38% of the Latin American and the Caribbean countries reported sporadic to endemic occurrence of BTB. Of 36 Asian nations, 16 reported a sporadic/low occurrence of BTB, and one (Bahrain) described the disease as endemic. Ten did not report BTB and the remaining nine did not have data. Of 34 Latin American and Caribbean countries, 12 reported BTB sporadic occurrence, seven reported it as endemic, and one (Dominican Republic) described the occurrence as high. Twelve countries did not report BTB. No data were available for the remaining two countries (Thoen and Steele, 1995).

Of 55 African countries, 25 reported sporadic occurrence of BTB, six reported endemic occurrence. Two, Malawi and Mali were described high occurrence, four did not report the disease, and the remaining 18 countries did not have data (Thoen and Steele, 1995). Among East

African countries, Somalia, Djibouti and Rwanda (Cosivi *et al.*, 1998), Ethiopia (Kiros, 1998; Ameni *et al.*, 2001) have reported BTB as having variable occurrences. In Ethiopia a number of studies have shown that bovine tuberculosis is common among cattle of the local and exotic breeds. Table 2 shows the prevalence of bovine tuberculosis in cattle based on tuberculin testing in the country.

Table 2: The prevalence of bovine tuberculosis in cattle in different parts of Ethiopia

Area	Test method employed	Number of cattle		Prevalence of BTB (%)	Source
		Tested (n)	Reactors (both positive and doubtful)		
Eastern Shoa	SIDCT	788	234	29.7	Kiros, 1998 ✓
Addis Ababa	SIDCT	1241	128	10.31	Bogale, et al., 2001 ✓
North west Shoa	SIDCT	1041	259	24.9	Regassa, 2005 ✓
Alage dairy farm	SIDT	202	96	27.2	Ameni, 1996 ✓
Wollaita Soddo	SIDT	416	149	35.8	Regassa, 1999 ✓
Fitche town	SIDT	735	31	4.2	Belay, 2000 ✓
Asela town	SIDCT	514	18	3.5	Redi, 2003 ✓
Addis Ababa	SIDCT	1071	197	18.4	Kebede, 2005 ✓
Adama	SIDCT	524	59	11.3	Aklilu, 2005 ✓
Bodji District	SIDT	460	19	4.1	Laval and Ameni, 2004 ✓
Bodji District	SIDCT	320	12	3.8	Laval and Ameni, 2004 ✓
Dire-Dawa	SIDCT	301	93	3.1	Adugna, 2005 ✓

SIDCT: Single intradermal comparative tuberculin test

SIDT: Single intradermal tuberculin test

Check the difference with Ameni, 2005?
OK

2.3. Public health importance

Human tuberculosis of animal origin (zoonotic TB) is an important public health concern in developing countries. TB infection in humans is principally caused by *M. tuberculosis*. However, human TB of animal origin caused by *M. bovis* is becoming increasingly prevalent (Bedard *et al.*, 1993) due to the lack of both control and diagnostic measures and pasteurization of milk, coupled with a high prevalence and incidence of HIV/AIDS in humans. TB is a major opportunistic infection in HIV-infected people. According to the WHO estimate, 70% (6 million) of the people co-infected with TB and HIV live in sub-Saharan Africa (Cosivi *et al.*, 1998). *M. bovis* can infect humans, primarily by ingestion of raw (unpasteurized) milk or dairy products and through aerosols and breaks in the skin. Infections in humans may result in asymptomatic infections, pulmonary tuberculosis, or disseminated infections. The symptoms of pulmonary infection include fever, cough, chest pain, and hemoptysis. Untreated infections may be fatal (OIE, 2005). Every year, there are 8-10 million incidence cases and 2-3 million case-fatalities due to TB (Davis and Grange, 2001). However, the exact percentage of TB due to *M. bovis* is unknown. The global prevalence of human TB due to *M. bovis* is estimated at 3.1% cases. Of these, 2.1% are pulmonary and 9.4% extra pulmonary infections (Cosivi *et al.*, 1998).

The risk of contracting TB is 20-50 times greater in Africa than in Europe (Bedard *et al.*, 1993). In all countries of sub-Saharan Africa, an estimated 90% of the total milk produced is consumed raw or soured. Nevertheless, the importance of *M. bovis* in human TB cases is not mentioned in any of the national reports submitted to OIE and World Health Organization (WHO) by African countries. This indicates that cases of *M. bovis* infection in humans are underreported as a result of diagnostic limitations and inadequate sampling in research of this disease (Cosivi *et al.*, 1998). Strikingly, in Africa, where 50% of cattle happen to be in countries without any sanitary measures for BTB, there is substantial lack of knowledge of the epidemiological patterns and transmission dynamics of this important zoonosis (WHO, 1994).

Results from several studies conducted in different African countries have clearly established the importance of BTB as a major public health problem. For instance, in Malawi, a survey of human sputum cultures from human TB patients showed that 42.8% of positivity for *M. bovis*

(Wedlock *et al.*, 2002). In Burkina Faso, the Ministry of Health (MOH) reported 1,334 human cases of zoonotic TB and isolation of mycobacteria in 26% of 60 retailed milk samples collected from market centers (Coulibaly and Yameogo, 2000). In an investigation by two Egyptian health centers, the proportions of sputum-positive TB patients infected with *M. bovis*, recorded during three observations, were 0.4%, 6.4%, and 5.4% (Nafeh *et al.*, 1992). Furthermore, isolation of *M. bovis* from sputum samples from patients with pulmonary TB has also been reported from Nigeria. Of 102 *M. tuberculosis* complex isolates, 4 (3.9%) were *M. bovis* (Idigbe *et al.*, 1986). Another study in Nigeria reported that one out of 10 mycobacteria isolated from sputum-positive cultures was *M. bovis*. In Zaire, *M. bovis* was isolated from gastric secretions in two of five patients with pulmonary TB (Mposhy *et al.* (1983) cited by Cosivi *et al.*, 1998). In Tanzania, seven of 19 lymph node biopsies from suspected extra pulmonary TB patients were infected with *M. tuberculosis* and four with *M. bovis* (Daborn *et al.*, 1997). In Ethiopia *M. bovis* has been recovered from tissues and milk samples obtained from animals and, sputum and fine needle aspiration samples collected from humans (Kiros, 1998; Bogale, *et al.*, 2001; Regassa, 2005).

2.4. Diagnosis

reference

Diagnosis of BTB in animal, human and food of animal origin can be achieved by history of contact, physical, chemical, immunological and/or molecular methods (Quinn *et al.*, 2002; OIE, 2003).

2.4.1. Delayed hypersensitivity test

Infection with *M. tuberculosis* or *M. bovis* induces delayed hypersensitivity (allergy) and immunity (Ochei and Kolhatkal, 2005). The term allergy implies a state of super sensitiveness to the products of infecting organisms on the part of an animal suffering from specific disease, for example, sensitivity to tuberculin in TB. Since allergy is highly specific it is possible to achieve a high standard of efficiency in differential diagnosis by the application of suitable allergic tests. However, in the case of related infections and variants of any given infection, the specificity of allergic reaction is reduced. So it is not possible to produce a tuberculin of potency sufficient to cause a reaction in all animals infected with BTB which will not cause a reaction in animals

sensitized with the other acid fast organisms like *M. avium*. Because of this it is necessary to use the comparative test with avian and bovine tuberculin (Biberstein and Hirsh, 1999).

Tuberculin is the name given to extracts of *M. tuberculosis*, *M. bovis* and *M. avium* used in skin test in order to identify those with tuberculosis. PPD tuberculin is prepared by growing organisms in synthetic medium, killing them with steam and filtering. The PPD tuberculin is precipitated from this filtrate with trichloroacetic acid, washed and finally resuspended in buffer ready for use. Its major antigenic component is probably a heat shock protein, HSP 65 (Proud, 2006).

The tuberculin reaction is an immunologically specific inflammatory reaction mediated by T-cells. The SIDCT test is useful when a high prevalence of avian tuberculosis or Johne's disease is anticipated. PPD from *M. bovis* is more specific in cattle than *M. tuberculosis*, giving less cross-reaction with *M. avium* and is used together with avian PPD (Ashford *et al.*, 2001). The SIDCT test and other skin tests are internationally accepted standard for the detection of *M. bovis*-infected cattle and are considered the best tests available for the diagnosis of TB in live animal (RUA-Domenech *et al.*, 2006). Other types of tuberculin tests in use include the SIDT test, the short thermal test and the stormont test (Biberstein and Hirsh, 1999) (Table 2).

Table 3: Types of tuberculin tests used in cattle

Test	Usage	Advantages	Disadvantages
SIDT	Routine testing	Simple	Prone to false positivity
SIDCT	When avian TB or Johne's disease is prevalent	More specific than SIDT	More complex than SIDT
Short thermal test	Used in post-partum animals and in heavily infected animals	High efficiency	Time consuming
Stormont test	Used in post-partum animals and in advanced cases	Very sensitive and accurate	Three visits required and may sensitize an animal

Source: Biberstein and Hirsh (1999)

2.4.2. Bacteriological culture

Bacteriological isolation of *M. bovis* from the lesion or secretions is the only way to make a definitive diagnosis. It is found desirable to use two different culture media for the primary isolation of *M. bovis*: agar media for rapid growth and egg media for control of contamination. Additional control of contamination can be achieved without adversely affecting the viability by treating the specimen before culture with 0.075% hexadecylpyridinium chloride. To determine the significance of cattle that give a positive reaction in diagnostic tests but do not have visible lesions, a bacteriological examination is necessary (Corer, 1994). The cultural characteristics and growth requirements of some mycobacterial species is shown in Table 1.

2.4.3. Molecular techniques

The nucleic acid amplification procedure, PCR technique is much faster than culture and reduces the time for diagnosis from several months to 2 days. It also detects *M. bovis* when rapidly growing *Mycobacterium* species are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms are non-viable. This method could be used as a rapid screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of BTB (Liebana *et al.*, 1996). DNA probes complementary to specific sequence of ribosomal ribonucleic acid (rRNA) and DNA restriction endonuclease analysis or DNA fingerprinting are also available (Quinn *et al.*, 2002). Rapid radiometric mycobacterial detection system and gas and liquid chromatography have also been developed (MoARD, 2005).

Individual strains of *M. bovis* can be distinguished according to their genetic fingerprint. The principal typing method is known as spoligotyping. It is based on DNA polymorphism present at one particular chromosomal locus, the direct repeat (DR) region, which is unique to MTBC. This method distinguishes between strains by identifying the presence or absence of known spacer oligonucleotides. Strains are grown, heat killed and subjected to PCR using primers based on the DR region allowing amplification of the spacers in between. Forty-three unique spacer oligonucleotides are hybridized permanently to a nylon membrane. The PCR products from the killed strains are hybridized to the membrane at right angle to the spacers. After hybridization,

the membrane is developed by an enhanced chemoluminescence system. An image, showing the presence or absence of spacers and giving impression of the individual bars representing each sample, is developed by autoradiography (Jahans and Worth, 2006). Acid-fast organisms which are not recognized as *M. bovis* by spoligotyping or not show growth characteristics typical of *M. bovis* are tested by multiplex PCR. The amplification process is moderated by several oligonucleotide primers that, typically, are 20-30 nucleotides long. This is where the multiplex PCR differs from the conventional PCR which generally uses only one set of primers. The primers are single-stranded DNA that has sequences complementary to the flank regions of the target DNA. Following PCR the amplification product can be detected using gel electrophoresis. Visible bands are measured against reference DNA bands added at either end of the gel pre-electrophoresis. When the gel is run the reference DNA produces a ladder of bands at intervals of 100 base pairs (bp). All members of the *Mycobacterium* genus produce a band at 10-30 base pairs (bp). However, The MTBC show a second band at 372bp, *M. avium* a second band at 180 bp and *M. intracellulare* a second band at 850 bp (Blissitt, 2006).

2.5. Control

2.5.1 Test and slaughter

Conventional control methods based on test and slaughter policies have, in a number of countries, led to a successful eradication of bovine tuberculosis. This method involves diagnosis of tuberculosis in live animals through whole herd testing and removal of infected animals. A test and slaughter program must be integrated with a series of other control measures to successfully control the disease. There should be control of movement of animals from infected farms in order to stop disease spread (Wedlock *et al.*, 2002).

2.6.2 Vaccination

Vaccination of domestic animals is a possible option for countries in which there are wild reservoirs, whereas in developing countries, an effective vaccine for vaccination of domestic animals or wildlife may be the affordable solution for control (Wedlock *et al.*, 2002). A number



of tuberculosis vaccines including attenuated *M. bovis* strains using Bacillus Calmette-Guèrin (BCG), killed mycobacteria, protein and DNA vaccines have been developed for cattle (Buddle *et al.*, 2002).

3. MATERIALS AND METHODS

3.1 Study area and population



3.1.1. Study area

The Alage Agricultural Technical Vocational and Educational Training (TVET) College is situated at 217 km south west of Addis Ababa in the vicinity of Abijata and Shalla lakes, west of Bulbula town 32km away from Addis Ababa-Shashemene highway. Its absolute location is at a longitude of 38° 30' East and a latitude of 7° 30' North. It rests on 105 'gasha' or 4200 hectares of land with an altitude of 1600m above sea level. The area is characterized by mild subtropical weather with minimum and maximum temperature ranging from 11 to 29°C. The area experiences bimodal rainfall distribution with annual average of 700 to 900 mm. The three defined seasons based on rainfall distribution are: short rainy season (March to April), long rainy season (June to September) and long dry season (October to January). The dominant soil type is black clay soil (vertisoil) with a PH of 7.9. The area is suitable for livestock production. The animal populations in the different farms of the college are about 403 dairy cattle and 203 beef type local breed (Borans), 200 pigs, 60 sheep and goats, 3000 poultry and 15 camels. There is a protected natural forest in the college which contains many wildlife species including monkeys, apes, bushbucks, warthogs and others.

The human population in the area is estimated to be 12,362 (7,219 males and 5,143 females). The annual students' population ranges from 3,098 to 7126. Alage Seble Clinic is involved in the diagnosis of TB which largely depends on sputum smear examination and clinical signs. The Alage Dairy Farm serves milk shed for in and around Alage. Figure 1 shows the study area.

3.1.2. Animals

Of the total dairy cattle population (403) in Alage Dairy Farm all cattle older than 6 months of age (355) were included in the study.

* More information
on the dairy cows and
Farm

$$n = \frac{Z\alpha^2 P(1-P)}{d^2}$$



Where, n = samples size estimate for diary cattle

$Z\alpha = 1.96$ (at $\alpha = 0.05$ or 95% confidence interval (CI))

P = expected prevalence (27.2%) of BTB (Ameni, 1996 cited by (Ameni *et al.*, 2003))

d = expected level of precision (0.05)

$$n = \frac{(1.96)^2 0.272 (1-0.272)}{(0.05)^2} = 308$$

3.3. Study design and Sampling technique

A Cross sectional study design was used to conduct SIDCT test on cattle in Alage dairy farm so as to study the prevalence of BTB and subsequently milk sample from lactating reactor cows (n=58) was taken for mycobacterial isolation. All cattle above six months old (n=355) were included in the study. Similarly, a health institution-based cross-section study design was used to assess the occurrence of *M. bovis* in the sputum specimens of recently diagnosed PTB patients (n=8) in Alage Seble Clinic during October 2007 to March 2008.

3.4. Single intradermal comparative tuberculin (SIDCT) test

All animals in the dairy farm were identified by ear tag. The individual animal ear tag number and its sexual category were recorded by personal observation. Afterwards, breed (Holstein Friesians and zebu (Brahmans)), parity level, age, pregnancy (yes/No) and lactation (Yes/No) were recorded from the individual animal record cards which were kept for each animal in the dairy farm. In addition, body condition score (1-5) of each animals was determined (scored) according to Nicholson and Butterworth (1986) and recorded (Annex 1). Based on their BCS cattle in the dairy farm were classified into three groups: very thin (1, 1.25 and 1.5), thin (1.75, 2.0, 2.25 and 2.5) and moderate (2.75, 3.0, 3.25 and 3.5).

Two sites 12 cm apart on the mid neck of an animal were shaved and the skin fold thickness were measured (in millimeters) with digital calipers before the injection of tuberculin. Aliquot of

0.1 ml of 20,000 IU/ml bovine PPD (Veterinary Laboratory Agency (VLA) and 0.1ml of 25,000 IU/ml avian PPD (VLA) were injected in to the dermis at these sites (Annex 5). Interpretation of the result was made based on difference in skin indurations at bovine PPD (B) and avian PPD (A) injection sites. A difference (B-A) of 4 mm or above was considered as positive while a difference of less than or equal to 2 mm was considered as negative. Readings of difference between 2 and 4 mm were considered doubtful or inconclusive OIE (2004).

3.5. Mycobacterial Culture

3.5.1. Sputum culturing

Sputum specimens were collected by clinicians in Seble Clinic as they collect for the diagnosis of PTB by sputum smear examination. The specimens for mycobacterial isolation were collected by using sterile, leak proof, disposable plastic material (cup) labeled with the patient's code number, type of specimen and date of collection. Subsequently, they were kept at 4°C until transported to the laboratory.

The sputum specimens were cultured for isolation of microbacteria in the Laboratory of Microbiology at the Institute of Pathobiology, Addis Ababa University. All the culturing process was done in biological safety cabinet (BSC) II in accordance with WHO (1998). The specimens were decontaminated using 2% NaOH (1:3 ratio), agitated in a vortex mixer for 15 minutes at room temperature and then centrifuged at 3000 revolution per minutes (rpm) for 15 minutes at 4°C. 70% denatured alcohol with equal weight to the specimen was used to balance in the centrifuge during centrifugation (Ochei and Kolhatkal, 2005). The supernatant was decanted and the sediment was suspended in 2 ml of sterile physiological saline solution. One to two drops of 0.05% phenol red was added to indicate the PH change and then neutralized with concentrated HCl until the color of the suspension changed from deep purple to yellow. After neutralization, the sediment from each specimen was inoculated on to two L-J media (one with pyruvete) and the other with glycerol. The culture was incubated at 37°C and was observed for bacterial growth for 8 weeks. The growth of white, moist, flat and non-pigmented friable colonies on the

pyruvate-enriched medium were considered as the primary cultures of *M. bovis* WHO (1998). Annex 2 shows the preparation of L-J media.

3.5.2. Milk culturing

About 30 ml of the last few streams of milk was aseptically drawn into sterile universal bottle from the 4 quarters of each of 58 cows which were positive for the SIDCT test. The samples were transported at 4°C and stored at -20°C until they were processed for mycobacterial culture according to Kazwala *et al.* (1998).

About 30 ml of milk was added in-to sterile centrifuge tube and centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was decanted and the sediment was suspended with 2 ml of sterile physiological saline, decontaminated with equal volume of 4% NaOH, centrifuged again, and neutralized using concentrated HCl using 0.05% phenol red as an indicator. After neutralization, the sediment from each sample was inoculated on the two L-J media. The culture was incubated at 37°C and was observed for bacterial growth for 8 weeks.

3.6. Molecular technique

3.6.1. Polymerase chain reaction

Initial identification of mycobacterial species was based on the rate of growth, pigment production and colony morphology. Species belonging to the MTBC show a slow growth rate. Growth of *M. bovis* is enhanced by pyruvate. For further characterization of the species using molecular techniques, extraction of mycobacterial genomic DNA was conducted as described by Zumarraga *et al.* (1999), with minor modifications. One colony from each isolate was suspended in 100 µl of sterile distilled water and heat killed to 80°C for 20 minute. The heat treated suspensions were kept preserved in deep freeze (-20°C) until tested. Annex 4 shows DNA extraction (PCR directly from culture).

DNA amplifications was done in 20 μ l reaction volumes consisting: Two μ l of genomic DNA used as a template, 10 μ l hot taq (MgCl₂, dNTP; Taq polymerase and PCR buffer) for each sample, 0.3 μ l internal primer per sample, 0.3 μ l forward (RD4 flanking forward 5'AATGGTTTGGTCATGACGCCTTC) and 0.3 μ l reverse (reverse 5'CCGTAGCGTTACTGAGAAATTGC) primer per each sample and 7 μ l of distilled water per sample. The reaction mixture was then heated in thermal cycle as follows: 95⁰C for 15 minutes for enzyme activation; 94⁰C for 1 minute for denaturation; 60⁰C for 2 minute for annealing; 72⁰C for 3 minute for extension; and finally 72⁰C for 10 minutes for post extension, involving 35 cycles all in all. The amplified DNA were visualized after gel electrophoresis at 100 volt (V) for 40 minute in 2% agarose gel stained with ethidium bromide and viewed in UV-transilluminator (Zumarraga *et al.* 1999). Annex 5 shows polymerase chain rection.

3.7. Data Management and analysis

The difference between the change in skin thickness at the bovine injection site and the thickness change at the avian injection site, from the initial skin reading to the skin reading at 72 hour, was used as the outcome.

The data was encoded and entered into Micro Soft Excel spread sheets for descriptive and statistical analysis. The percentage or the prevalence of tuberculin reactors was calculated by dividing the number of reactors by the total number of animals tested. (Suspicious or inconclusive reactors were classified as negative for the purpose of establishing prevalence). Chi-square test for association (between age-group, breed, BCS, sex, physiological status, parity level and a positive tuberculin reaction), univariable logistic regression for determining the strength of association (by odds ratio (OR)) were conducted by Statistical Package for Social Science Students (SPSS)-version 11.5 for Windows.

Since individual animals were classified as being tuberculin positive or negative (dichotomous variable), a stepwise logistic regression procedure by SPSS-version 11.5 for Windows was used to control confounding factors and determine the effects of age, BCS, physiological status and

4. RESULT

4.1. Descriptive statistics and Chi-square test for association of risk factors of BTB

The result of SIDCT test in Alage intensive dairy cattle farm showed that 61.41% (218/355) were negative while 10.14% (36/355) and 28.45% (101/355) were doubtful and positive reactors respectively. Accordingly, the prevalence of BTB was found to be 28.45% (101/355) considering the inconclusive (doubtful) reactors as negative while the prevalence was 38.59% (137/355) given the doubtful were considered positive. The reactors rate for Holstein Friesians was 27.89% (99/344) while among local zebu it was 0.56% (2/11). Of the 351 female animals, 28.45% (101/351) were positive while no positive reactor was found among the 4 male animals. The reactor rates observed for animals under different parity levels showed 3.94% (14/123) for nullipara, 10.14% (36/148) for unipara and 14.37% (51/80) for pluripara.

The reactor rates for cattle in different physiological state such as lactating pregnant, lactating non-pregnant, non-lactating pregnant and non-lactating non-pregnant were 0.28% (1/28), 16.05% (57/151), 6.47% (23/100) and 5.63% (20/72), respectively. Of cattle under different age-groups, the reactor rates of <3 years old, 3-5 years old and ≥ 6 years old were 1.41% (5/91), 12.67% (45/177) and 14.36% (51/87), respectively. Furthermore, classification of reactors based on body condition scores indicated that 9.86% (35/95) were very thin while 13.24% (47/160) and 5.35% (19/100) were thin and moderate, respectively.

As in Table 4, the Chi-square for the association indicated that parity ($X^2=69.155$, $P<0.001$), physiological status of the animals ($X^2=17.993$, $P<0.05$), age groups ($X^2=63.31$, $P<0.001$) and BCS ($X^2=7.714$, $P<0.05$) were significant while sex and breed were not significant at $P<0.05$.

Association

Table 4: Chi-square test showing the putative risk factors with tuberculin reactivity of cattle in Alage Dairy Farm

Variable	No. tested	No. positive (prevalence)	P- Value
Parity			
Nullipara	123	14 (3.94)	0.000
Unipara	148	36 (10.14)	
Pluripara	80	51 (14.37)	
Physiological status			
Lact. preg. ^a	28	1 (0.28)	0.001
Lact. non-preg. ^b	151	57 (16.05)	
Non-lact. preg. ^c	100	23 (6.47)	
Non-lact. non-preg. ^d	72	20 (5.63)	
BCS			
Very thin	95	35 (9.86)	0.021
Thin	160	47 (13.24)	
Moderate	100	19 (5.35)	
Age			
<3 years	91	5 (1.41)	0.000
3-5 years	177	45 (12.67)	
≥6 years	87	51 (14.36)	
Breed			
Holstein Friesian	344	99 (27.89)	0.351
Zebu (Brahmans)	11	2 (0.56)	
Sex			
Male	4	0 (0.00)	0.260
Female	351	101 (28.5)	

^aLact.-preg.: --Lactating pregnant

^c non-lact. preg.: --non-lactating pregnant

^b Lact. non-preg.: -- Lactating non-pregnant

^d non-lact. non-preg.: --non-lactating pregnant

4.2. Unavailable logistic regression analysis of risk factors to assess the strength of their association with BTB positivity

The univariable logistic regression analysis of the putative risk factors showed statistically significant difference on tuberculin reactivity between animals with moderate and very thin body condition score. Odds of tuberculin reactivity among animals with moderate BCS was 0.40 and 95% CI = 0.21-0.77; $P = 0.006$ as compared to animals with very thin BCS. Within parity the rate of reactors was significantly higher among pluripara (OR=14.1, 95%CI = 6.9-29.1; $P=0.000$) and unipara (OR=2.5, 95% CI = 1.3-5.07; $P= 0.005$) than nullipara, and also the rate was significantly higher among pluripara than unipara (Table 4). Furthermore, comparison of the physiological status of animals with tuberculin reactivity showed significantly lower reactor rates among lactating pregnant animals (OR=0.096, 95% CI=0.012-0.757; $P<0.05$) as compared to non-lactating non-pregnant ones; but there was no significant difference among animals of other physiological status.

Furthermore, the univariable logistic regression analysis has found that tuberculin reactivity was significantly different among the different age-groups. Thus, tuberculin reactivity was significantly higher in age-groups within 3-5 years (OR=5.86, 95% CI =2.24-15.36; $P = 0.000$) and in those animals ≥ 6 years old (OR=24.37, 95% CI = 8.99-66.07; $P = 0.001$) as compared to animals under three years of age.

Table 5: Univariable logistic regression analysis of the putative risk factors and tuberculin reactivity of cattle in Alage Dairy Farm

Variable	OR	P-Value	95% CI (OR)
Parity			
Unipara	2.5	0.005	1.33-5.07
Pluripara	14.1	0.000	6.92-29.12
Nullpara*			
Physiological status			
Lact ^a . preg ^b	0.096	0.026	0.01-0.76
Lact. non preg.	1.577	0.145	0.86-2.91
Non lact. Preg.	0.777	0.476	0.39-1.56
Non lact. non preg*			
BCS			
Thin	0.71	0.218	0.42-1.22
Moderate	0.40	0.006	0.21-0.77
Very thin*			
Age			
3-5 years	5.864	0.000	2.24-15.36
≥6 years	24.367	0.000	8.99-66.07
<3 years*			

*Reference category

4.3. Multivariable stepwise logistic regression analysis of risk factors and tuberculin reactivity

The difference for parity level, age-group and physiological status based infection rates observed in the initial logistic regression model were also found evident after the control of confounding factors in the stepwise logistic regression analysis. On the other hand, BCS was excluded from the model indicating that BCS was not significantly affecting animals' reaction to tuberculin.

Of the sub groups within age-groups, parity level, and physiological status of animals risk factors which appeared significant for the stepwise logistic regression model were age-groups [significantly high reactor rates in cattle of 3-5 years old (OR=29.54, 95% CI 6.03-144.65; P<0.001) and cattle of ≥ 6 years old (OR=56.96, 95% CI= 9.54-340.12; P<0.001) as compared to cattle of < 3 years old]; parity [tuberculin reactivity was significantly higher among pluripara (OR=3.54, 95% CI=1.01-12.45; P<0.05) as compared to nullpara]. In addition, among cattle of different physiological status tuberculin reactivity was significantly lower in lactating pregnant cows (OR=0.093, 95% CI=0.009-0.953; P<0.05), lactating non-pregnant (OR=0.101, 95% CI=0.03-0.34; P<0.001) and non-lactating pregnant cows (OR=0.07, 95% CI=.02-0.24; P<0.001) as compared to non-lactating non-pregnant cows. This indicates that party, physiological status and age were significantly affecting animals' reaction to tuberculin skin test. Table 5 below shows the result of stepwise logistic regression analysis of risk factors and tuberculin reactivity of cattle in Alage Dairy Farm.

Table 6: Multivariable stepwise logistic regression analysis of risk factors and tuberculin reactivity of cattle in Algae Dairy Farm

Variable	OR	P-Value	95% CI (OR)
Parity			
Unipara	0.909	0.857	0.321-2.572
Pluripara	3.540	0.049	1.01-12.451
Nullpara*			
Physiological status			
Lact ^a . preg ^b .	0.093	0.045	0.009-0.953
Lact. non preg.	0.101	0.000	0.030-0.341
Non lact. preg.	0.070	0.000	0.020-0.241
Non lact. non preg.*			
Age			
3-5 years	29.538	0.000	6.032-144.645
≥ 6 years	56.958	0.000	9.538-340.120
<3 years*			

*Reference category

4.4. Summary of the bacteriological culture and PCR results

The bacteriological findings showed that out of 58 milk samples collected from SIDCT test positive lactating cows, two (3.54%) *M. bovis* were isolated through the culture on L-J media and gave signal for the RD4 based PCR typing (Figure 2: Lane 4 and 5). ~~The primers used in the RD4 based PCR typing were 5'AATGGTTTGGTCATGACGCCTTC (RD4 flanking forward) and 5'CCGTAGCGTTACTGAGAAATTGC (RD4 flanking reverse).~~ Out of 8 sputum specimens collected from human pulmonary TB patients and cultured L-J media, 4, 50% (4/8) were culture positives. 50% (2/4) of mycobacterial isolates obtained from human PTB cases were identified as *M. tuberculosis* and gave signal for the RD4 based PCR typing which is specific for *M. tuberculosis* and *M. bovis* (Figure 2: Lane 9 and 10). In the present study, *M. bovis* was not isolated from the sputum of human pulmonary TB patients. Table 7 below shows mycobacterial species isolated from sputum of human PTB cases and milk of SIDCT test positive cows in Alage.

Table 7: Mycobacterial species isolated from sputum of human PTB cases and milk of SIDCT test positive cows in Alage.

Type of Specimen or sample	No. examined	Growth on L-J medium (%)	Speciation by RD4 PCR typing	
			<i>M. bovis</i> (%)	<i>M. tuberculosis</i> (%)
Sputum	8	50% (4/8)	0%	50% (2/4)
Milk	58	5.17% (3/58)	2.66% (2/3)	0%

RD4 PCR typing results

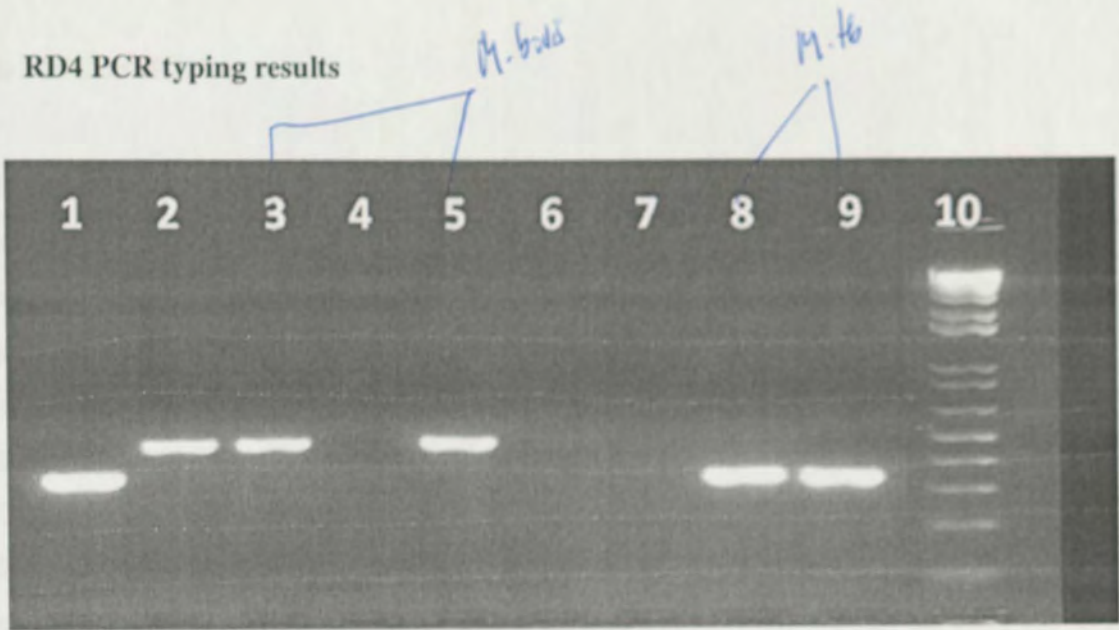


Figure 2: Picture showing the results of RD4 PCR typing

lane: 10=1kb ladder, 9=105H/G, 8=108H/G, 7=104H/G, 6= 101H/G, 5= 539M/P, 4=643M/G, 3=923M/ P, References: 2= *M. bovis* and 1=*M. tuberculosis*

5. DISCUSSION

The prevalence (28.45%) of BTB recorded by the present study is comparable with the previous result (27.2%) reported by Ameni (1996) cited by Ameni *et al.* (2003) in the same farm. This is because the earlier investigation was done by SIDT test which might have included more false positive animals. On the other hand, the prevalence of the present study ^{was} 38.3% when the inconclusive reactors ^{were} are considered as positives. This difference is due to the continued transmission of disease within the herd in the presence of both infected and susceptible animals. Given the scarce resource in dairy breeds in Ethiopia, there is a tendency to keep animals with a long production life without culling; reinforcing the chance that they participate in the BTB spread (Ameni *et al.*, 2003). Hence, even if one animal is infected in such a farm with a large herd size, there is a high chance of transmitting the disease to other members of the herd.

In contrast, the prevalence rate recorded in the present study is less than 87.14% in Debre Zeit State Farm, 78.85 % in Debre Zeit Military Camp, 79.46% in Chaffa Farm (Wollo) and 53.67% in Mullo State Farm (Ameni *et al.*, 2001). This could be associated with the good management system in the farm that permits separate watering and feeding troughs. In addition, animals move out of their barn regularly for exercise and sunbath (no total confinement). The most important reason could be due to the reduced infection rate in the young stock due to the distant separation and segregation from the infected herd.

2003 → not correct reference?

In the univariable logistic regression analysis, significant difference ($P < 0.05$) in tuberculin reactivity was observed between non-lactating non-pregnant and lactating pregnant cattle as indicated by lower tuberculin reactivity in the later group ($OR = 0.096$). This is inconsistent with the finding of (Bogale *et al.*, 2001). Furthermore, after elimination of confounders in the stepwise logistic regression analysis, significantly ($P < 0.05$) lower tuberculin reactivity rates were observed among lactating pregnant ($OR = 0.093$), lactating non pregnant ($OR = 0.101$) and non lactating pregnant ($OR = 0.070$) cattle as compared to non lactating non pregnant ones. This is because pregnant animals show lower tuberculin reactivity as a result of stress-induced immunosuppression (Biberstein and Hirsh, 1999). It is also reported that Immunological suppression in the periparturient period can produce anergic reactors or induce anergic condition

2. Not the correct reference:
(Ashford *et al.*, 2001). This is observed by the present study where only 1 (0.026%) of the lactating pregnant animals reacted positive. The higher prevalence of BTB in dairy than beef cattle may reflect greater productivity stress among dairy cows. Exemption from pregnancy and lactation may explain the lower disease prevalence in bulls than cows (Biberstein and Hirsh, 1999).

The reactor rate for cattle aged 3-5 years old was significantly ($P=0.000$) higher than cattle below three years of age and they were 29.54 times more likely to react to tuberculin test. Furthermore, reactor rate for older cattle ≥ 6 years of age was significantly ($P=0.000$) higher than those below three years old and they were 56.96 times more likely to react for the tuberculin test. This is in agreement with O'Reilly and Daborn (1995). According to O'Reilly and Daborn (1995), the reaction to tuberculin test in cattle increases uniformly by 7.5% for every year of life reaching 40% at 6-7 years old. As explained by other workers (Barwinnek and Taylor, 1996) as age increase the probability of acquiring TB infection increases. The higher prevalence in dairy than beef cattle may reflect closer confinement, longer life spans, and greater productivity stress among dairy cows. This may explain the lower disease prevalence in bulls than cows (Biberstein and Hirsh, 1999).

This study revealed lower reactor rates among male animals (0.0%) and Brahmans (0.56%) cattle as compared to the females (28.45%) and Holstein Friesians (27.89%), respectively. However, there was no significant ($P=0.260$) association between sex and tuberculin reactivity of the animals. Similarly, no significant ($P=0.351$) association was observed between sex and tuberculin reactivity. These could be due to the small number of observations of males and Brahmans compared to the females and Holstein Friesians cattle, respectively, involved in this study. On the other hand, Barwinnek and Taylor (1996) reported that genetically improved cattle might suffer more severely from malnutrition and poor housing systems in tropical countries, and consequently become more susceptible to infection.

BCS had no effect on tuberculin reactivity. This is in agreement with Bogale *et al.* (2001). However, this is in contrast to the established fact that poor nutrition predisposes to tubercular infection (Biberstein and Hirsh, 1999). It can be suggested that the risk associated susceptibility to malnutrition could be offset by a lower reactivity in malnourished cattle. Circumstantial

evidence suggests that inadequate intake of mineral, vitamin and protein increases the susceptibility of cattle, although weather patterns have been implicated in the susceptibility of herds to *M. bovis* infection (Proud, 2006). It is concluded that some reduction in the susceptibility of cattle to *M. bovis* infection can be achieved by modifications to the management system to minimize risk factors (Foster *et al.*, 2002).

In the present study, 2 (50%) of 4 mycobacterial isolates from sputum samples of patients with pulmonary TB were identified as *M. tuberculosis* whereas no *M. bovis* was detected by RD4 based PCR typing. This is in contrast with 4 (3.9%) of 102 *M. tuberculosis* complex isolates from sputum samples of patients with pulmonary TB from Nigeria were *M. bovis* (Idigbe *et al.*, 1986). Nevertheless, the importance of *M. bovis* in human TB cases is not mentioned in any of the national reports submitted to OIE and WHO by African countries. This indicates that cases of *M. bovis* infection in humans are underreported as a result of diagnostic limitations and inadequate sampling in research of this disease (Cosivi *et al.*, 1998). Hence, *M. bovis* was not isolated from humans due to lack of adequate samples (PTB cases) and diagnostic limitations for sampling EPTB in the study area.

On the other hand, 2 (3.45%) *M. bovis* was isolated from milk of 58 cows positive to SIDCT test. In other similar studies, *M. bovis* isolation of 13.3% (4/30) and 1.4% (7/486) were reported by Yehualashet (1995) cited by Kiros (1998) and 8.7% (4/46) by (Bogale *et al.*, 2001). This substantiates the fact that only about 1% of the tuberculous cows excrete the bacilli in their milk (Birberstein and Hirsh, 1999). The isolation of *M. bovis* from the milk of reactor cows signifies the actual and potential risks of BTB to humans. Although the number of *M. bovis* positive milk samples was low, pooling milk from the farm does pose a great public health danger to milk consumers. It has been reported that one cow can secrete enough viable bacilli to contaminate the milk of up to 100 cows when the milk is pooled, which may be the case in the governmental dairy collection system (Cosivi *et al.*, 1998).



6. CONCLUSIONS AND RECOMMENDATIONS

In the present study very high prevalence (28.45%) of BTB was recorded in Alage dairy cattle by SIDCT test. The doubtful reactors increased the prevalence to 38.59%. The risk factors of parity, physiological status and age were associated with tuberculin reactivity of the animals while, high rate (71.8%) of low BCS (thin and very thin) observed in the dairy cattle; the mixing of cattle during the parturition period lowered the resistance to the disease, increased parity level as well as the keeping of cattle for long production life seems to account for the acquisition of infection and its transmission. It is concluded that some reduction in the susceptibility of cattle to *M. bovis* infection can be achieved by modifications to the management system to minimize risk factors. This study also showed that risk of acquiring the disease increases with age and parity level while lactation and pregnancy reduces tuberculin reactivity due to anergy. Thus, more sensitive diagnostic techniques and control strategies should be considered on this risk group. Due to the small number of human pulmonary TB cases reported in the area, no *M. bovis* was isolated from sputum samples. Whereas it was isolated from 3.54% of raw milk samples from tuberculin reactor cows which imply the inevitable risk of exposure of the public to infection in the absence of milk pasteurization. Moreover, the high prevalence of BTB in the farm and the isolation of the agent from milk suggest the risk of BTB in food hygiene.

Therefore, based on the above conclusion the following recommendations are forwarded.

- The herd health management should be improved by better nutritional management, periodic tuberculin testing of the herd by using more sensitive diagnostic techniques especially for pregnant and lactating cows, complete segregation of positive animals and culling of advanced case, higher parity and older cows
- Public education about food safety measures and pasteurization of milk should be compulsory
- Further study on the occurrence of zoonotic TB should be conducted especially to investigate the importance of the beef type local breed (Borans) and wild life in the area particularly warthog and bushbuck in the epidemiology of BTB

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7. ANNEXES

Annex 1. Body condition score for dairy cattle

BCS 1. Thin, deep depression around the tail head and no fat over the rump and loin

BCS 2. Shows shallow cavity around the tail head and a small amount of fat covering the rump and loin area

BCS 3. Shows no cavity around the tail head and fatty tissue over the whole rump and loin area

BCS 4. Shows fold of fatty tissue over the tail and patches of fat over the rump with fairly heavy fat covering over the loin

BCS 5. Shows the tail head buried in fatty tissue and heavy fat covering over the rump and loin area

Source: Nicolson and Butterworth (1986)

Annex 2. Preparation of Lowenstein-Jensen media

Ingredients

1. Mineral salt solution

Potassium dihydrogen phosphate	2.4
Magnesium sulphate	0.24
Magnesium citrate	0.6 gm
Asparagin	3.6 gm
Glycerol	4 ml
Pyruvate	12 gm
Distilled water	600 ml

2. Malachite green solution 2%

Malachite green water	2 gm
Sterile distilled water	1.00 ml

3. Homogenized whole eggs

Fresh hen's eggs not more than 7 days old

For the preparation of complete media the following ingredients are essentially pooled in a large sterile flask and mixed well.

Mineral salt solution 600 ml

Malachite green solution 20 ml

Homogenized eggs (20-25 depending on size) 1000 ml

These are mixed well and will be added in each of the universal bottle for media preparations. Bottles are put in autoclave in slant position at 80-85°C for 45 minutes. The slant media is solidated and cooled after which it is kept in deep freezer.

Source: WHO (1998)

Annex 3. Polymerase chain reactions

1. Add 12.5 ml of hot start (Dntp + DNA polymerase +MgCl₂ + TE buffer) on each tube.
2. Add sterile distilled water 3.5 ml on each tube.
3. Add primer 10 pmol on each tube 7 ml.
4. Add heat killed mycobacteria sample 2 ml (1 mg/ml) in all tubes.
5. Put the microfuge tube epindorf tubes (PCR tubes) to the PCR thermal cycler and leave for 3 hours and 15 minutes until the PCR complete its process.
6. Prepare 1.5% agarose gel for electrophoresis until the PCR machine completes its work.
 - 15 ml of Tris EDTA buffer solution +0.75 g agarose power + 50 ml distilled water mixed and heated.
 - Add 8 ml of Ethidium broide dye
 - Add the mixture into a simple plexi-glass apparatus at room temperature for 30 minutes to form gel (solidified), when cooled.
 - Prepare a fenestration on the gel before it solidifies
7. After 3 hours and 15 minutes, when the thermal cycler complete it process the microfuge tube taken out, 8 µl loading dye (bromophenol blue) was add to all PCR product.
8. Place the solidified agarose gel to the horizontal agarose gel electrophoresis of simple plexi-glass apparatus which is filled with a solution of Tris-EDTA.
9. Inject the PCR product to each fenestrations of the agarose gel and connect to electrophoresis power supply (120 V) and leave it for 30 minutes and above.
10. Take out the gel and put it in to the photographing machine so that the ethidium bromide fluoresce and the image visualized by machine and appreciated on the computer compare the size of the sample base pair to the known positive control of *M. bovis* and *M. tuberculosis*.

Source: Zumarraga *et al.* (1999)

Annex 4. DNA extraction (PCR directly from culture)

1. Scrape a few colonies off L-J slant using disposable loop.
2. Add to screw cap microfuge tube containing 400 μ l distilled water and vortex well.
3. place in heating block at 100⁰C for 10minutes (to release DNA and inactivate bacteria)
4. Use 2 μ l aliquot for PCR reaction.

Source: Zumarraga *et al.* (1999)



Annex 5. Photographs



Single intradermal comparative tuberculin test on the mid-side of neck of cattle in Alage



8. CURRICULUM VITAE



A. Biographical Data:

Name: Dawit Shimelis Taye
Date of Birth: June 27, 1980
Place of Birth: Asseba-Teferi, Ethiopia
Sex: Male
Qualification: Doctor of Veterinary Medicine
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B. Educational Background

<u>Level</u>	<u>Place</u>	<u>Award</u>
Primary education	Arberekete and Asseba-Teferi	Certificate
Secondary education	Asseba-Teferi	Certificate
Higher education	Debre Zeit (AAU, FVM)	DVM Degree

C. Professional Experience

2005 – Present Senior Lecturer in Ardayta and Alage TVET Colleges as course instructor of

- Anatomy and physiology of farm animals
- Introduction to animal health
- Introduction to beef cattle production
- Introduction to veterinary parasitology
- Introduction to pathology
- Introduction to microbiology
- Non infectious diseases of cattle
- Technical report writing

	➤ Introduction to basic knowledge of drugs
2005 – 2006	Research and Extension committee in Animal Science Department in Ardayta College
2006 – 2008	Parasitology and Microbiology Course Coordinator
2008	Practical Coordinator of animal Health Department

D. Research Output/ Technical Paper

- ♣ Seminar on *the principles of participatory epidemiology*
- ♣ Seminar on *the epidemiology and public health importance of avian influenza*
- ♣ Research on *the epidemiology of Taenia saginata taeniosis and cysticercus bovis in North Gonder zone, Northwestern Ethiopia*

E. Training

- ♣ As trainer of senior meat inspectors in introduction to veterinary parasitology, microbiology, pathology and poultry anatomy

F. Reference Person

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9. SIGNED DECLARATION SHEET

This thesis is my original work, has not been presented for a degree in any university and that all sources of material used for the thesis have been duly acknowledge.

Name _____

Signature _____

Date of submission _____

This thesis has been submitted for examination with my approval as a university advisor

Dr. Moses Kyule (BVM, MSc, MPVM, PhD, Associate Professor) M. Kyule