PREVALENCE OF SWINE TUBERCULOSIS IN CENTRAL ETHIOPIA AND MOLECULAR CHARACTERIZATION OF ITS CAUSATIVE AGENTS

By

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DEBRE ZEIT, ETHIOPIA

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"Thou art my God, and I will praise thee: thou art my God, I will exalt thee.
O give thanks unto the Lord; for he is good: for his mercy endureth for ever."
(Psalm 118:28-29)

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immuno-Deficiency Syndrom
ALIPB	Aklilu Lemma Institute of Pathobiology
ATPase	Adenosinetriphosphatase
BCG	Bacillus Calmette-Guerin
bp	Base Pair
BTB	Bovine Tuberculosis
C	Cytosine
DNA	Deoxyribonucleic Acid
DR	Direct Repeat Region
G	Guanine
GTPase	Guaninetriphosphatase
HCl	Hydrochloric Acid
HIV	Human Immuno Deficiency Virus
INF-γ	Interferon gamma
IS	Insertion Sequence
LJ	Lowenstein-Jensen
MAC	*Mycobacterium avium* Complex
MAH	*Mycobacterium avium* subsp. *hominissuis*
$MgCl_2$	Magnesium Chloride
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Numbers Tandem Repeats
ml	milliliter
µl	Microliter
MTC	*Mycobacterium tuberculosis* Complex
NaOH	Sodium Hydroxide
OIE	World Organization for Animal Health (Office International des Epizooties)
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
RD	Region of Defferentiation

RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minutes
rRNA	Ribosomal Ribonucleic Acid
Subsp.	Subspecies
TB	Tuberculosis
VNTR	Variable Number Tandem Repeats
WHO	World Health Organization

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ABSTRACT

A cross-sectional study was carried on 841 pigs slaughtered at Addis Ababa and Alema Swine Farm abattoirs in central Ethiopia to estimate the prevalence of swine tuberculosis (TB) and to investigate the potential risk factors associated with the prevalence as well as to isolate and characterize the causative agents. To achieve these objectives, detailed post-mortem examination, mycobacteriological culturing, molecular typing using multiplex polymerase chain reaction (PCR), regions of difference (RD)-4, and RD9 deletion typing, and spoligotyping were used. The prevalence of TB in the study pigs was 5.8% (49/841) on the basis of gross post-mortem lesion. The prevalence was significantly higher in older pigs than in younger pigs (10.8% versus 3.4%, $\chi^2 = 18.84$; $P<0.001$). The origin of pigs was also found to significantly affect the prevalence ($\chi^2 = 27.67$; $P< 0.001$). Pigs originated from Addis Ababa and its surrounding had prevalence of 12.8% and were more than 3 times likely to get the infection (OR=3.3, 95% CI=1.7:6.4, $P<0.001$) than pigs from Bishoftu which had a prevalence of 3.2%. In contrast, sex, floor type and water source did not significantly affect the prevalence ($P>0.05$). The multivariate analysis indicated that age and origin of pigs were the only statistically significant explanatory factor for variation in the recorded prevalence. Culture positivity was confirmed in 30.6% (15/49) of the pigs with TB suspected lesions. Of the 10 acid-fast positive isolates subjected to PCR 4 showed band for *Mycobacterium tuberculosis* complex on gel electrophoresis. Speciation of three isolates indicated that two of them were *Mycobacterium tuberculosis* (*M. tuberculosis*), and one was *M. bovis* and further strain characterization of the two *M. tuberculosis* showed that one was SIT 53 and the other was a new strain which had not been reported to the international spoligotype database before. The findings of human type *Mycobacteria* probably suggest interspecies transmission pattern. SIT 53 is known to be common in human population in the country further strengthening the suggested transmission pattern given the endemic nature of TB in Ethiopia. Hence launching feasible control methods for swine TB and avoiding physical contact between infected cattle or humans and swine would be required.

Key words: *Ethiopia, Molecular typing, postmortem lesion, prevalence, risk factors, swine tuberculosis*

1. INTRODUCTION

Tuberculosis (TB) is an infectious, granulomatous disease caused by acid-fast bacilli of the genus *Mycobacterium.* Although commonly defined as a chronic, debilitating disease, TB occasionally assumes an acute, rapidly progressive course. The disease affects practically all species of vertebrates (Thoen, 2005). The generic name *Mycobacterium was* introduced by Lehmann and Neumann (1896) to include the tubercle and leprosy bacilli. The tubercle bacilli are *Mycobacterium tuberculosis* (*M. tuberculosis*), the agent of the disease in primates, *M. bovis* in other mammals, and *M. avium* in birds. Host specificity is relative (Hartmans *et al.,* 2006).

The consequences of TB on society are immense. Worldwide, one person out of three is infected with *M. tuberculosis* – two billion people in total. Together with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and malaria, TB is recognized as one of the most important threats to human health (Dye *et al,* 1999; Smith 2004; Young 2008). Although great advances have been made in the control of TB, the number of deaths from this disease is still enormous (WHO, 2011). The neglect of TB is a scandal that has gone on for decades and is only now beginning to be addressed (Enarson, 2003). TB is the only disease ever declared a global emergency by the world health organization (WHO) (Ritacco *et al.,* 2007).

The impact of the disease varies both by continent and economic status of individual countries. However, in all regions where it is present, it has an adverse influence on agricultural economics (Pollock *et al.,* 2005), constitutes both a human health hazard and an animal welfare problem, with economic implications particularly in terms of trade restrictions, productivity losses, and massive annual expenditure on eradication programs (Schiller, 2009). An estimated 10 to 15% of human TB cases are caused by *M. bovis* in countries where the disease is common and milk pasteurization is not practiced (Ashford *et al.,* 2001). In Africa this is favoured by the higher risk of the majority of human (82%) and cattle (85%) populations being exposed to TB (Cosivi *et al.,* 1995).

In Ethiopian bovine TB is an important infectious disease which has been documented in many parts of the country. The prevalence of the disease ranges between 3.4% and 50% in

smallholder and intensive dairy production systems, respectively (Ameni *et al.*, 2001; Ameni *et al.*, 2007). Other studies showed the endemicity of BTB in many regions of the country with prevalence ranging from 1.5% in Addis Ababa, 78.7% in Bishoftu dairy farms (Asseged *et al.*, 2000; Ameni *et al.*, 2003; Asseged *et al.*, 2004), 24.7% in Adama and 4.2% in Yabello slaughterhouses (Demelash *et al.*, 2009).

There is increasing evidence that *M. bovis* infections may be much more significant than generally considered (Shitaye *et al.*, 2007). The direct correlation between *M. bovis* infection in cattle and the disease in the human population has been well documented in developed countries, whereas scanty information is available from developing countries (Cosivi *et al.*, 1995). In particular, there is no report on the status of TB in swine in Ethiopia so far except a very preliminary report from a retrospective meat inspection data analysis of slaughter cattle, sheep, goats and pigs by Shitaye *et al.* (2006), who indicated that the prevalence of bovine TB in pigs was 0.009% of 10,820 pigs slaughtered in Addis Ababa abattoir in the years 1996–2005. TB continues to cause significant economic losses to swine producers throughout the world. Although TB due to *M. bovis* has been nearly eradicated in many developed countries, lesions continue to be reported in the cervical and mesenteric lymph nodes of swine during meat inspection (Thoen, 2006).

The presence of TB in pigs in virtually all countries in which pigs are farmed has long been reported (Nuttall, 1986). In pigs, the disease levels reflect those in the local cattle population from which the infection derives either by: the ingestion of dairy products, feeding of offal from abattoirs or feeding on uncooked garbage, or by grazing over the same pasture as cattle (Radostits *et al.*, 2006), where mycobacteria may survive on pasture for two months or more (Thoen, 2005), and through wildlife-livestock interface (Quinn and Collins 2006). Nuttall (1986) suggested that there is a correlation between the occurrence of TB in pigs and the opportunity which the pigs have for direct or indirect contact with tuberculous humans (Pavlik, 2006), cattle or birds.

Ten to twenty percent of the local pigs are likely to be infected when the disease is common among dairy cattle in an area. A high prevalence has been observed in pigs bedded on shavings infected with *M. bovis* (Radostits *et al.*, 2006). Pigs are susceptible to all the three types of tubercle bacilli: human, bovine, and avian, respectively, *M. tuberculosis*, *M bovis*, and *M. avium* complex (MAC). Although *M. avium* subsp. avium is most frequently isolated

(Thoen, 2005), an interesting finding by Mohamed and co-workers showed multiplicity of mycobacterial species and particularly a high incidence of *M. tuberculosis*, *M. bovis*, and *M. avium* complex from slaughtered pigs in Egypt (Mohamed *et al.,* 2009).

In infected pigs granulomatous lesions are most often found in the submandibular, cervical, bronchial, mediastinal, hepatic, and mesenteric lymph nodes, but lesions may also be found elsewhere (Radostits *et al.,* 2006). Pigs with disease due to *M. tuberculosis* may have similar regionalized lesions. Susceptibility of pigs particularly to *M. bovis* usually occurs from shared grazing or ingestion of dairy products. This can cause a rapidly progressive, disseminated disease with caseation and liquefaction of lesions (Thoen, 2005).

There is no epidemiological information of mycobacterial infection of pigs and the impact on human health and other domestic animals in Ethiopia, mainly because it is erroneously assumed that pigs do not play a role in the epidemiology of TB. But several studies in different countries have shown the importance of pigs in the epidemiology of TB and non-tuberculous mycobacterial infections which have both public and veterinary health importance besides their economic impacts (Bono *et al.,* 1995; Falkinham III, 1996; Field *et al.,* 2004). The economical losses due to swine mycobacteriosis is attributed not only to condemnation of pig meat, head, and visceral organs at abattoir inspection points due to tuberculous lesions (Windsor *et al.,* 1984) but also involve losses among cattle due to bovine TB caused by dissemination of *M. bovis* in the environment by infected pigs (Machackova *et al.,* 2003; Phillips *et al.,* 2003). In addition, the dissemination of nontuberculous *Mycobacterium* species can sensitize cattle, with the subsequent nonspecific reaction to tuberculin skin test and losses from destruction of false-positive reactors (Pollock *et al,* 2000; Matlova *et al.,* 2005; Pavlik *et al.,* 2005).

On the other hand, the increasing reports of several types of mycobacterial infections among HIV–infected and immunocompromised patients (LoBue *et al,* 2001; Srikantiah *et al.,* 2005; Chaisson and Martinson, 2008) has drawn attention to animals as the source of these *Mycobacterium* species to humans. Previous studies have shown that pigs are among the possible sources of mycobacterial infections to humans and animals (Bono *et al,* 1995; Komijn *et al,* 1999; Phillips *et al,* 2003). Previously no study had been carried out in Ethiopia, however, about the epidemiological role of pigs and the causative agents for TB. Therefore,

detection, isolation and characterization of the causative agents and investigating the potential risk factors of swine TB are important steps in the control of TB.

General objective of the study:

- To investigate the epidemiology of swine TB in central Ethiopia

Specific objectives:

- To estimate the prevalence of swine TB
- To investigate potential risk factors associated with the occurrence of the disease
- To isolate, identify and characterize mycobacterial isolates of pigs using bacteriological and molecular techniques

2. LITERATURE REVIEW

2.1. Historical background of Tuberculosis

Tuberculosis (TB) – known in the past as the "White Plague" – is an ancient and often neglected disease (Palmero, 2007). The disease has a long history. It was present before the beginning of recorded history and has left its mark on human creativity, music, art, and literature; and has influenced the advance of biomedical sciences and healthcare. TB was documented in Egypt, India, and China as early as 5,000, 3,300, and 2,300 years ago, respectively (Daniel, 2006).

The earliest evidence of TB in man and animals is provided by bone finds – mainly fragments of vertebrae – showing the gibbous typical of tuberculous Pott's disease. The oldest examples of spinal TB, in the form of fossil bones, date back to about 8000 BC (Ayvazian, 1993). A bone from the Neolithic period (5000 BC), found in the region of Heidelberg, likewise shows evidence of tuberculous changes. Findings in certain Egyptian mummies clearly indicate that spinal caries existed around 2400 BC. The depictions of a gibbous in statuettes of ancient Egyptian and pre-Columbian origin represent clear evidence of spinal TB. There is the unique bacteriological finding of acid-fast bacilli in smears taken from a psoas abscess in the astonishingly well-preserved mummy of an Inca child from around 700 BC, clearly documenting a case of TB of the lumbar spine (Dubos and Dubos, 1952; Dubos, 1982).

Identification of genetic material from *M. tuberculosis* in ancient tissues has provided a powerful tool for the investigation of the incidence and spread of human TB in historic periods. It also offers potential new insights into the molecular evolution and global distribution of these microbes. Research on ancient DNA poses extreme technical difficulties because of the minute amounts of DNA remains, their oxidation/hydrolysis, and the extremely high risk of contamination with modern DNA (Cooper and Poinar, 2000).

The TB epidemic in Europe, later known as the "Great White Plague", probably started at the beginning of the 17th century and continued for the next 200 years. Death from TB was considered inevitable and, by 1650, TB was the leading cause of mortality. The high population density and poor sanitary conditions that characterized the enlarging cities of

Europe and North America at the time, provided the necessary environment, not met before in world history, for the spread of this airborne pathogen. The epidemic spread slowly overseas by exploration and colonization. TB existed in America before Columbus' arrival but was rare among the natives. The major outbreaks of TB among the native people of North America began in 1880, after they were settled in reservations or forced to live in barracks in prison camps. Death rates increased rapidly, and by 1886, reached 9,000 per 100,000 people (Bates and Stead, 1993).

TB was also rare among Africans who lived in small remote villages. When exposed to the disease by contact with Europeans, these populations experienced a high mortality rate. Africans taken as slaves were free from TB on arrival to the Americas. Then, cases of sub-acute fatal TB developed among them. After their liberation from slavery and movement into the cities, TB morbidity and mortality rose quickly, reaching 700 per 100,000 in 1912 (Bates and Stead, 1993). There is also evidence of the presence of the disease in pre-historic Asia, but it was only toward the end of the 19th century that peaks in incidence were observed in India and China (Sledzik and Bellantoni, 1994).

TB currently holds the seventh place in the global ranking of causes of death. Unless intensive efforts are made, it is likely to maintain that position through to 2020, despite a substantial projected decline in disease burden from other infectious diseases (Dye *et al,* 1999; Smith 2004; Young 2008).

2.2. The genus *Mycobacterium*

Mycobacteria belong to the Order Actinomycetales, Family Mycobacteriaceae, and genus *Mycobacterium* (Olsen *et al,* 2010). The genus *Mycobacterium* encompasses a number of medically important species that exact an alarming toll in human and animal morbidity and mortality. In 1993, the WHO declared a global emergency owing to the fact that TB was epidemic in many areas of the world. Other mycobacterial diseases continue to plague the world's populations as well. *Mycobacterium leprae*, the cause of leprosy, persists in developing countries, and other mycobacteria (ordinarily non-pathogens) have now become threats to individuals infected with the human immunodeficiency virus (HIV) (Saviola and Bishai, 2006). The genus consists of the members of the *M. tuberculosis* complex and greater

than 80 species of atypical mycobacteria, including pathogenic, opportunistic, and non-pathogenic species (Soini and Musser, 2001).

Mycobacteria are aerobic, non-spore-forming, non-motile, rod shaped, acid-fast bacilli. Individual species differ in size; the rods of *M. bovis* and *M. avium* subsp. *avium* are slender and up to 4μm in length, whereas those of *M. avium* subsp. *paratuberculosis* are broad and are usually less than 2 μm long. Although mycobacteria are cytochemically Gram positive, the high lipid and mycolic acid content of their cell walls prevents uptake of the dyes employed in the Gram stain. The cell wall lipids bind carbol fuchsin which is not removed by the acid-alcohol decolourizer used in the Ziehl-Neelsen (ZN) staining method. Bacilli, which stain red by this method, are called acid-fast or ZN-positive. The mycobacteria include diverse species ranging from environmental saprophytes and opportunistic invaders to obligate pathogens (Quinn *et al.*, 2002).

The genus *Mycobacterium* comprises a number of Gram-positive aerobic bacteria and is the only member of the family Mycobacteriaceae within the order Actinomycetales. The genus shares an unusually high genomic DNA G+C content (62–70%) and the production of mycolic acids with closely related genera, *Nocardia* and *Corynebacterium*, within Actinomycetales. Phylogenetic trees are available which depict genetic relatedness based on homology of the 16S ribosomal gene sequence. Mycobacteria that have highly homologous rRNA sequences are closely related and are on neighbouring branches of the tree. The mycobacterial phylogenetic tree can be further subdivided into fast and slow growing bacteria. The fast growers form colonies on selective media in less than 7 days and the slow growers, in greater than 7 days (Saviola and Bishai, 2006). The genus *Mycobacterium* includes the *M. tuberculosis* and *M. avium* complexes, other pathogenic *Mycobacteria*, and numerous species of saprophytic microorganisms present in soil and water. Although some pathogenic *Mycobacteria* exhibit a particular host preference, they can occasionally infect other species. Mycobacterial diseases in domestic animals are usually chronic and progressive (Quinn *et al,* 2002).

2.3. *Mycobacterium tuberculosis* complex (MTC)

Together with other highly related bacteria, *M. tuberculosis* forms a complex, the *M. tuberculosis* complex (MTC), a single species as defined by DNA/DNA hybridization studies. The MTC comprises seven members which include *M. Tuberculosis,* the primary causative agent of human TB; *M. bovis* which cause progressive disease in most warm-blooded vertebrates, including humans; *M. africanum,* the main causative agent of TB in West Africa; *M. microti*, which is a pathogen of voles and rarely infects humans; *M. canetti*, a rare MTC strain that produces smooth and glossy colonies, with all known cases far isolated from individuals having been to the horn of Africa; *M. pinnipedii*, also known as the seal bacillus; and *M. caprae*, primarily isolated from goats (Fig. 1. A) (Thoen, 2005; Saviola and Bishai, 2006; Asiimwe, 2008; Olsen, *et al.,* 2010).

Brosch *et al.* (2002) described a new evolutionary scenario for MTC members which concluded that all animal adopted *M. tuberculosis* complex strains differ from human adopted *M. tuberculosis* strains by the absence of a specific chromosomal region (RD9). These results contradict the often presented hypothesis that *M. tuberculosis* evolved from *M. bovis*. It suggests that it is more likely that the common ancestor of the tubercle bacilli was *M. tuberculosis* or *M. canettii* alike and may already have been a human pathogen. The RD9 deleted lineage, which is almost phenotypically homogenous, excludes *M. tuberculosis* as well as *M. canettii*, but includes *M. africanum*, *M. microti*, *M. pinnipedii, M. caprae* and *M. bovis* (Brosch *et al.,* 2002).

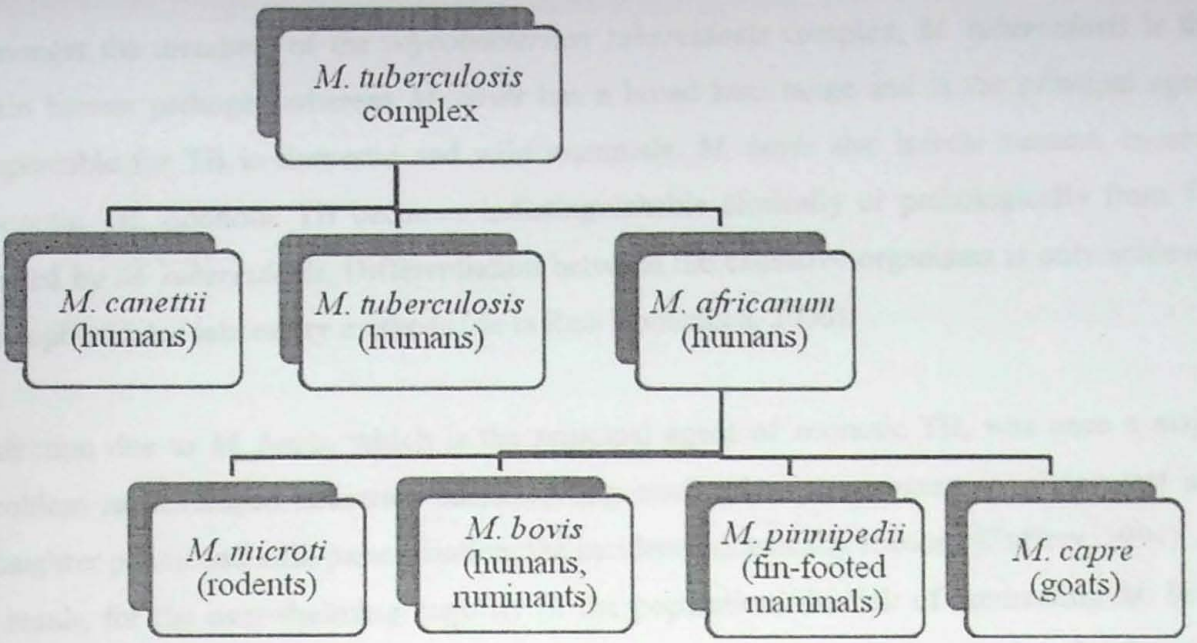
A recent study suggested using phylogenetically informative spacers, in combination with previously identified single nucleotide mutations and chromosomal deletions to identify different clades in the RD9 deleted lineage each with a separate host preference (Smith *et al.,* 2005). It is therefore suggested that the MTC is rather described as a series of host-adapted ecotypes than attributed a broad host range of distinct members of the MTC, like it was the case for a long time for *M. bovis* (O'Reilly and Daborn, 1995).

2.4. *Mycobacterium avium* complex (MAC)

The *Mycobacterium avium* complex includes *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. intracellulare* (fig. 1. B). All these species are common in the environment, as they are isolated from soil, water, food and domestic animals. Categorized as slow growing mycobacteria, these species grow optimally at 37°C and achieve growth in approximately 4–6 weeks (Havlir and Ellner, 2000). Colonies are non-pigmented and are both opaque and domed or flat and transparent (Toosi and Ellner, 1998). *Mycobacterium avium* causes a TB-like infection in chickens, pigeons and other birds, whereas *M. paratuberculosis* causes Johne's disease in ruminants, which is a contagious enteritis resulting in progressive wasting and eventual death (Thoen, 2005). *Mycobacterium paratuberculosis* has not been shown to cause human disease (Saviola and Bishai, 2006). *Mycobacterium avium* subspecies *avium* is the only species of consequence in birds, but it has a wide host range and is also pathogenic for pigs, cattle, sheep, deer, mink, dogs, cats, and some cold-blooded animals (Thoen, 2005).

In humans, *M. avium* and *M. intracellulare* can cause pulmonary disease, regional lymphadenitis or disseminated disease. Those individuals who are coinfected with HIV are prone to disseminated disease, whereas those who are not coinfected are likely to have pulmonary disease (Saviola and Bishai, 2006). *Mycobacterium avium* subsp. *hominissuis* (MAH) infection in swine may cause granulomatous lesions in lymph nodes that must undergo differential diagnosis with those caused by *M. tuberculosis* complex members. Moreover, MAH outbreaks can lead to severe economic losses due to condemnation of carcasses. A number of potential sources of infection for animals can usually be identified in contaminated farms. Molecular profiles obtained from MAH strains suggested a likely epidemiological link between clinical and environmental isolates cultured from sawdust and cooling systems from one breeding farm (Alvarez, 2010).

(A)



(B)

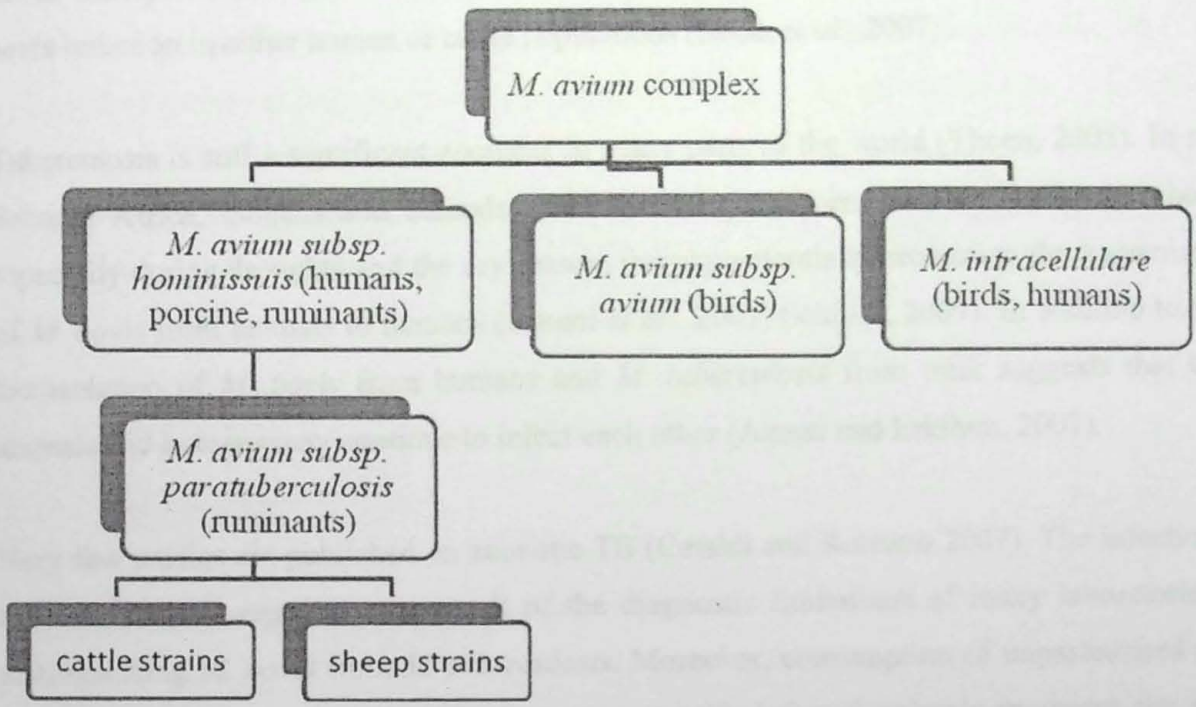


Figure 1: Proposed evolution of the *M. tuberculosis* and *M. avium* complexes.

The tree diagrams for the proposed evolution of the *M. tuberculosis* (A) and *M. avium* (B) complexes are shown (Olsen *et al.*, 2010).

2.5. Zoonotic importance of tuberculosis

Amongst the members of the *Mycobacterium tuberculosis* complex, *M. tuberculosis* is the main human pathogen whereas *M. bovis* has a broad host range and is the principal agent responsible for TB in domestic and wild mammals. *M. bovis* also infects humans, causing zoonotic TB. Zoonotic TB becomes indistinguishable clinically or pathologically from TB caused by *M. tuberculosis*. Differentiation between the causative organisms is only achieved by sophisticated laboratory methods (de la Rua-Domenech, 2006).

Infection due to *M. bovis*, which is the principal agent of zoonotic TB, was once a major problem in developed countries but following eradication programmes involving test and slaughter policy and milk pasteurization, the incidence drastically reduced (Caffery, 1994). As a result, for the overwhelming majority of the population, the risk of contracting *M. bovis* infection from animals appears to be extremely low in industrialized countries. On the other hand, in most developing countries, human TB is widely known to be caused by *M. tuberculosis*; however, an unknown proportion of cases are due to *M. bovis*. In Africa, bovine TB is widespread throughout the continent, but very little is known about risk factors for *M. bovis* infection in either human or cattle populations (Sarah *et al.*, 2007).

Tuberculosis is still a significant zoonosis in many parts of the world (Thoen, 2005). 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; Schiller, 2009). In addition to this, the isolation of *M. bovis* from humans and *M. tuberculosis* from milk suggests that both animals and humans may continue to infect each other (Ameni and Erkihun, 2007).

Very few studies are published on zoonotic TB (Cataldi and Romano 2007). The infection in humans is under-reported as a result of the diagnostic limitations of many laboratories in distinguishing *M. bovis* from *M. tuberculosis*. Moreover, consumption of unpasteurised milk and poorly heat-treated meat and close contact with infected animals represent the main sources of infection for humans (Ayele *et al.*, 2004). In Ethiopia bovine TB is endemic, although the zoonotic importance of the disease is not well known like other developing countries (Ameni *et al.*, 2006; Ameni *et al.*, 2007). Few study in different parts of the countries confirmed *M. bovis* infection in human. For example, a study conducted on cattle

owned by TB patients had higher prevalence than cattle owned by non tuberculous owners (Regassa *et al.*, 2008).

2.6. Swine tuberculosis

TB continues to cause significant economic losses to swine producers throughout the world. Although TB due to *M. bovis* has been nearly eradicated in many developed countries, lesions continue to be reported in the cervical and mesenteric lymph nodes of swine during meat inspection. Available information indicates that 30–50% of the carcasses of slaughter swine from some large confined herds have granulomatous lesions from which *M. avium* complex serovars were isolated (Payeur and Songer, 1999). The mammalian type of TB is still frequently reported from several developing countries (Sulieman and. Hamid, 2002; Mohamed *et al.*, 2009). The processing of tuberculous swine carcasses is costly and results in significant economic losses (Payeur and Songer, 1999). Regulations of the Meat and Poultry Inspection Program of the USDA require that unaffected portions of swine carcasses with tuberculous lesions in more than one primary site, such as cervical and mesenteric lymph nodes, be cooked at 76.7°C for 30 minutes before being approved for human food. The value of a cooked carcass is only about 20–25% of the value of a carcass not cooked. In processing plants where facilities are not available for cooking, the carcass is condemned and there is no salvage value. The public health significance of *M. avium* complex infections has been recognized. Of special interest are reports on the isolation of some of the same serovars of *M. avium* complex from patients with acquired immune deficiency syndrome and from swine (Chin *et al.*, 1994; Komijn *et al.*, 1999).

2.6.1. Epidemiology

Historically, the primary agents of mycobacterial disease in swine are the same as those causing TB in other animals. The disease is transmitted through animal to animal contact. Early in the 20th century when TB in cattle and human was more prevalent, mycobacteriosis in swine was either *M. bovis* or *M. tuberculosis* in countries which have now controlled human and bovine TB. Early 20th, however, *M. avium*, the cause of TB in birds, began to occur more frequently in swine. The most common serotypes of *M. avium* in pigs are 1, 2, 3, 4, 6, and 8. Outbreaks of disease due to these serotypes appear to have originated in soil,

contaminated litter, usually sawdust, wood shavings or straw and contact from infected fowl (Payeur and Songer, 1999).

Swine are susceptible to infection with serovars of *M. avium* complex, *M. tuberculosis*, and *M. bovis*. The occurrence of TB in swine, therefore, is in part related to the opportunity for direct or indirect contact with tuberculous cattle, humans, and fowl or the organism in the environment (Radostits *et al.*, 2006; Thoen, 2006). Tuberculous chickens may continue to be a primary source of infection for swine, although other environmental sources are also significant (Fischer *et al.*, 2001; Matlova *et al.*, 2003). Garbage feeding is a possible, but infrequent, means of spreading swine mycobacteriosis. Improper handling of chicken wastes fed to swine also may allow transmission of the disease. Soil and water are other possible reservoirs of infection for pigs. Pathogenic mycobacteria may survive for more than 4 years in soil and litter contaminated by chickens with TB (Payeur and Songer, 1999). Several *M. avium* complex serovars have been isolated from swine in the United States, Australia, Brazil, Denmark, France, Germany, Hungary, Japan, South Africa, and the Czech Republic. These reports indicate the worldwide distribution of TB in swine due to *M. avium* complex. The similarity of *M. avium* and so-called *M. intracellulare* has led to the proposal that the latter be considered serovars of *M. avium* complex (Thoen, 2006).

Studies have shown that sawdust or wood shavings used for bedding may be a source of mycobacteriosis in swine. *M. avium* is often found in samples of sawdust and wood shavings where it survives for long periods. The mycobacteria may multiply under proper conditions of moisture and temperature which could explain the seasonal occurrence of the disease in some herds. Seasonal changes may produce less favourable conditions for survival of organisms in wood shavings and thus cause the infection rate to decrease (Payeur and Songer, 1999).

The presence of infected sites in the intestinal wall with subsequent pig-to-pig transmission probably is due to shedding of mycobacteria in the faeces. Mycobacterial infections of lungs, mammary glands, and uterus also may occur with the potential for transmission of organisms from these sites. Thus, the addition of infected breeding stock could introduce the disease into a herd, and transmission from infected sows to their litters may maintain the disease within a herd (Payeur and Songer, 1999).

2.6.2. Pathogenesis

Pathogenic mycobacteria all produce granulomatous lesions in tissues of humans and a wide range of domestic and wild animals. Although the tubercle bacillus was discovered more than 120 years ago, definitive information on its pathogenesis is not yet available, although understanding is developing at a remarkable rate (Olsen *et al,* 2010). Pigs usually become infected with *M. avium* by ingesting the organism. After ingestion, the organism penetrates the wall of the pharynx near the tonsils or the wall of the small intestine and becomes localized in the submandibular and mesenteric lymph nodes respectively. Small areas of infection develop in these lymph nodes, and the organisms rarely escape these initial sites (Payeur and Songer, 1999). The development of disease in swine depends on the ability of the tubercle bacillus to multiply within tissues of the host and to induce a host response. Although acid-fast bacilli initially encounter granulocytes and humoral components, activated mononuclear macrophages are considered to be more important in protection of the host against mycobacteria (Thoen, 2006).

As successful pathogens, mycobacteria have developed strategies to turn sets of genes on and off in response to the distinct environments encountered during different stages of infection. First, the microorganisms gain entry into host macrophages, where they survive and replicate intracellularly. Subsequently, mycobacteria reside within a membrane-bound vacuole and prevent the maturation of this compartment. This arrest involves a series of alterations in the phagosome, such as changes in the pattern of acquisition of Rab proteins and GTPases, exclusion of the proton ATPase from the phagosome, and retention of a phagosome coat protein that would normally be released from this compartment prior to phagolysosome fusion (Olsen *et al,*2010).

The capacity of *M. avium* to produce progressive disease may be related to certain complex lipids present in the cell wall, such as the glycopeptidolipids localized in the exterior portion of the cell envelope (Rastogi and Barrow, 1994). However, it appears that the effect of these components alone or together on phagolysosome fusion cannot account for virulence. Available information suggests that a combination of toxic lipids and factors released by virulent tubercle bacilli may cause disruption of the phagosome, interfere with phagolysosome formation, alter the release of hydrolytic enzymes from the attached lysosomes, and/or inactivate the lysosomal enzymes released into the cytoplasmic vacuole

(Thoen and Bloom, 1995). Certain serovars of *M. avium* are susceptible to bactericidal mechanisms of macrophages; however, the importance of reactive nitrogen intermediates and oxygen radicals in macrophages of swine exposed to virulent tubercle bacilli remains to be elucidated (Olsen *et al,* 2010).

Although the mechanisms by which mycobacteria produce disease in swine have not been clearly defined, experimental studies in piglets revealed that nonspecific esterase activity was elevated in mononuclear macrophages of lymph nodes 7 days following inoculation of *M. avium* complex serovar 8 (Momotani *et al.,* 1980). Granulomas of varying stages were observed in mesenteric and submaxillary lymph nodes and intestinal mucosa at 14 days postinoculation (Payeur and Songer, 1999). In other investigations, sensitized lymphocytes and detectable mycobacterial antibodies have been reported to occur at 14–28 days post exposure to *M. avium* or *M. bovis* (Olsen *et al,* 2010; Thoen, 2006).

2.6.3. Clinical signs

Tuberculous lesions in cervical lymph nodes usually cause no clinical abnormality unless they rupture to the exterior. Although generalized disease are not common cases, if occur, present a syndrome similar to that seen in cattle. Tuberculous involvement of the meninges and joints rather is more common (Radostits *et al.,* 2006). Lesions caused by *M. avium* subsp. *avium* are most often seen in lymph nodes associated with the gastrointestinal tract (Thoen, 2005). The health and condition of the infected pigs usually are not affected, and it is often impossible to establish a clinical diagnosis of mycobacteriosis in these animals. *M. avium* has been isolated from the lymph nodes of swine that were negative to skin tests, presented no lesions in any tissue, and had no signs of illness (Payeur and Songer, 1999).

2.6.4. Necropsy findings

The primary mycobacterial lesion is the granuloma, which is found in affected tissues and the draining lymph nodes. It consists of a core of infected macrophages and macrophage-derived giant cells surrounded by T cells, B cells, neutrophils, and fibrotic tissue. The structure of the granuloma contains the mycobacteria and prevents spread to other areas, but granulomas also provide a niche where the bacteria can hide from the immune system. Although the bacteria

can lie quiescent for many years, the granuloma appears to be a dynamic structure where a balanced cytokine milieu seems important to control the infection (Olsen *et al.*, 2010).

Granulomas are localized in the tonsils, and the submandibular, 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. Because of the regressive nature of the disease in pigs, these lesions are often negative on culture (Radostits *et al.*, 2006).

The lesions vary in appearance from small, yellowish white, caseous foci a few millimetres in diameter to diffuse enlargement of the entire node. The disease may be localized in one group of nodes or may involve the liver, spleen, lungs, kidneys, and many lymph nodes along the digestive tract. Generalized lesions from infection with *M. avium* tend to be diffuse. The cut surface is usually smooth, and there is no great tendency toward encapsulation by fibrosis. There may be foci of caseation, but calcification is not pronounced. Lesions resulting from infection with mammalian tubercle bacilli, however, are likely to be discrete, caseous, and well circumscribed by fibrosis. Calcification is prominent (Olsen *et al.*, 2010; Thoen, 2006).

In most cases the mycobacteria are contained within the granuloma and the infection is under control, often referred to in humans as "latent TB". Whether latency is a feature of domestic livestock infected with *M. bovis* is still open for debate. Clearly there are animals that are skin-test and/or IFN-γ positive but in which no lesions can be detected, and this might reflect latency. Such animals can obviously represent a possible source of re-infection, whether they represent true latency with reactivation or a slowly developing chronic infection (Olsen *et al.*, 2010). Granulomatous lesions in the lymph nodes of infected pigs are small abscesses that are detected by repeatedly slicing the lymph nodes with a knife (Payeur and Songer, 1999).

Gross differentiation between tuberculous adenitis caused by avian tubercle bacilli and that caused by mammalian tubercle bacilli is difficult, but in general, some features are characteristic of each. In an infection of avian origin the lymph nodes may be enlarged and firm with no discrete purulent foci, or there may be one or more soft caseous areas with indistinct borders. Calcification is seldom demonstrable. The cut surface of the lesion has a neoplastic appearance with a few caseous foci. Although there may be diffuse fibrosis, there

is little tendency to encapsulation. Relatively large areas of caseation may be present and occasionally will involve the entire lymph node (Thoen, 2006).

The lesions due to tubercle bacilli of the avian type are generally not easily enucleated. In contrast, when the infection is due to *M. bovis* or *M. tuberculosis* the lesions tend to be well encapsulated and are relatively easy to separate from the surrounding tissue. In addition, calcification is usually prominent in lesions. The individual foci appear to be discrete and caseous. These distinctions are by no means absolute, and there are many variations in the gross appearance of tuberculous lesions in lymph nodes of swine. Moreover consistent histopathological differentiation between lesions caused by mammalian and avian tubercle bacilli is not possible. Also chronic granulomatous lesions may be difficult to differentiate grossly because of parasitic nodules and neoplasm (Thoen, 2006).

2.6.5. Diagnosis

A clinical diagnosis of TB in swine is presumptive at best. Generally, the tuberculous lesions are limited to small foci in a few lymph nodes of the digestive tract. It is difficult to conceive that such non-progressive morbid changes may elicit signs detectable by physical examination (Payeur and Songer, 1999). In extensive tuberculous infection, signs may be suggestive of an infectious disease, but the symptoms and changes are not sufficiently characteristic to establish a diagnosis of TB. Although necropsy and histopathological appearance of TB in swine are sufficiently characteristic to permit a tentative diagnosis of TB, they are not specific. The great similarity between localized tuberculous lesions and those associated with *R. equi* and other bacteria, parasitic nodules and neoplasms confuses and makes gross differentiation of chronic granulomatous lesions difficult (Thoen, 2006).

Culture and microscopic examination

Acid Fast Bacili (AFB) smear microscopy plays an important role in the early diagnosis of mycobacterial infections because most mycobacteria grow slowly and culture results become available only after weeks of incubation. In addition, AFB smear microscopy is often the only available diagnostic method in developing countries. Smear staining is based on the high lipid content of the cell wall of mycobacteria which makes them resistant to decolourization by acid-alcohol after the primary staining. To determine that a clinical specimen contains AFB,

the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background. The slide is observed under the microscope for the detection of AFB. Several methods can be used for determining the acid-fast nature of an organism. Two methods, Ziehl-Neelsen and Kinyoun, utilize basic fuchsin in ethanol for primary staining. In both cases, AFB appears red after decolourization with acid alcohol. Ziehl-Neelsen is a hot acid-fast stain because the slide has to be heated during incubation with fuchsine. In contrast, Kinyoun staining is a cold acid-fast staining procedure and therefore does not require heating. Kinyoun's cold carbol fuchsine method is inferior to the Ziehl-Neelsen staining (Somoskovi *et al.*, 2001; Van Deun *et al.*, 2005).

Culture techniques have been estimated to detect as many as 10–1,000 viable mycobacteria per mL of specimen. A positive culture for *M. tuberculosis* confirms the diagnosis of active disease. For culturing of mycobacteria, two types of clinical specimens are considered: contaminated specimens and specimens collected aseptically from normally sterile sites. Sterile specimens can be inoculated directly onto the culture medium. Specimens from non-sterile bodily sites are considered contaminated and therefore require processing before culturing in order to eliminate the associated flora. If not properly eliminated, this flora will overgrow the culture medium long before mycobacteria have the chance to develop visible colonies (de Waard and Robledo, 2007).

Several methods have been used to minimize culture contamination when specimens from non-sterile body sites are processed. Most of these methods include the digestion of mucus or organic debris and treatment to eliminate micro-organisms from the normal flora. Both steps are done to maximize the probability of isolating mycobacteria in culture. No single decontamination method is applicable to all circumstances, laboratories and clinical specimens; therefore, a laboratory should use the best suited method that keeps the contamination rate between 3% and 5%. A contamination rate lower than 3% may indicate that the procedure used is too harsh and may be killing the mycobacteria (Della Latta, 2004). The decontamination methods most commonly used are:-

- **Sodium hydroxide**. This method uses sodium hydroxide at concentrations ranging between 2 % and 4 % to digest and, at the same time, decontaminate the specimen.

Each laboratory should determine the lowest concentration for optimal digestion and decontamination (Della Latta, 2004).

- **N-acetylcysteine-sodium hydroxide, sodium chloride and sodium hydroxide**. This method, one of the most used worldwide, uses N-acetylcysteine for mucus digestion and sodium hydroxide as the decontaminant (Della Latta, 2004).
- **Oxalic acid**. This method is recommended for decontamination of clinical specimens that may have Pseudomonas aeruginosa as a contaminant (Della Latta, 2004).
- **Cethyl pyridinium and sodium chloride**. This method is useful to preserve specimens from contaminant flora overgrowth while in transit to the laboratory and also fulfils the decontamination step required prior to culture (Della Latta, 2004).

Different culture media are in use for the isolation of mycobacteria. The most common are based on egg and also contain high concentrations of malachite green to overcome contamination with other bacteria. In general, after the centrifugation step, sediments are inoculated onto two Löwenstein-Jensen slants. *Mycobacterium bovis* and other species of the *M. tuberculosis* complex (*M. microti* and *M. africanum*) are unable to use glycerol as a carbon source due to the lack of a functioning pyruvate kinase. Thus, these organisms will often fail to grow on Löwenstein-Jensen medium, which contains glycerol as the only available carbon source (Keating, *et al.*, 2005). Stonebrink medium has the same composition as Löwenstein-Jensen, with the exception that glycerol is replaced by 0.5 % sodium pyruvate. Many diagnostic laboratories that employ egg based medium for the isolation of mycobacteria, omit the use of Stonebrink medium. This probably leads to an underestimation of the actual weight of *M. bovis* as a human TB agent, especially in developing countries (de Waard and Robledo, 2007).

Tuberculin skin test

The tuberculin test for the diagnosis of TB in swine appears to be a useful procedure on a herd basis. Of the various techniques described for this test in swine, the operator should select the method that proves by experience to be most suitable. Separate simultaneous tests with *M. avium* and *M. bovis* tuberculin must be made (Thoen, 2006). A number of investigators have found that some tuberculous swine may fail to react to the intradermal tuberculin test. Therefore, tests should be repeated in a herd in which animals with positive reactions have

been found and excluded (Radostits *et al.*, 2006). The intradermal test, usually on the ear (Radostits *et al.*, 2006), or vulva may be employed. Because swine are susceptible to infection with *M. tuberculosis* complex and *M. avium* complex, avian and mammalian tuberculin should be used. At present, intradermal injection of PPD tuberculin in the dorsal surface of the ear is the recommended procedure for applying the tuberculin test in swine. The injection site should be observed at 48 hours (Thoen, 2006).

The amount of tuberculin used and the site of injection have varied depending on the investigator. The recommended method for a tuberculin skin test in swine is an intradermal injection of 0.1 ml avian PPD in the dorsal surface of the ear. The response to injection is read and recorded 48 hours later. Positive reactions usually include swelling and redness, and they may vary in size and intensity (Payeur and Songer, 1999). Haemorrhage and ulceration may occur at the injection site. The reliability of the tuberculin test when used on individual pigs has been questioned. The tuberculin test can be used successfully as a herd test although false positive and negative reactions occur (Radostits *et al.*, 2006).

The characteristic pathological features of TB in swine and the presence of acid-fast microorganisms in such lesions provide important indications on which to base a diagnosis of TB. However, an unequivocal diagnosis can be made on the basis of bacteriological procedures designed for the isolation, identification by biochemical and seroagglutination tests, and/or by molecular techniques (Kaneene and Thoen, 2004)

2.6.6. Control and prevention

The eradication of TB in swine, as well as in other species, is dependent on the availability of an economical and specific means of detecting infected animals. Additional information is needed to determine adequate measures for cleaning and disinfecting the premises where *M. avium* complex persists in the soil, in buildings, or on equipment. Also, we need to know how long these organisms will remain viable in the environment. Investigations also should be made to determine the sources and modes of transmission of the different serovars of *M. avium* complex (Thoen, 2006).

There is no vaccine available for the control of swine mycobacteriosis. Preventing the disease in non-infected herds is more effective than trying to eliminate the disease from infected

herds. It is important not to mix swine and poultry production activities on the same farm. Feeding uncooked garbage, unpasteurized milk, or other materials that might contain viable mycobacteria to pigs must be avoided. Breeding stock should be purchased from mycobacteriosis-free herds. Efforts should be made to prevent all contact between pigs and wild birds. The potential for transmission of mycobacteriosis from infected wild birds to pigs is probably slight but must be considered. Pigs should not be housed in old poultry buildings unless they have first been thoroughly cleaned and disinfected. The use of wood shavings for bedding, especially in farrowing buildings, should be eliminated. Wood shavings should be kept dry at the saw mill and on the farm and protected from contamination by wild birds (Payeur and Songer, 1999).

2.7. Detection and identification methods in *Mycobacteria*

2.7.1. Conventional methods

Identification of Mycobacterium species is normally based on the traditional methods of Ziehl-Neelsen acid-fast staining and growth characteristics such as pigment production, growth rate, and gross and microscopic colony morphologies of cultures of the isolated causative organism (Pourahmad, 2007). To the species level, mycobacterial isolates have traditionally been identified based on their reactions in a series of phenotypic and biochemical tests. However, the biochemical reactions of isolates of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained. Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using molecular methods for species identification (Soini and Musser, 2001).

2.7.2. Molecular techniques

Nucleic acid-based diagnostics gradually are replacing or complementing culture based biochemical, and immunological assays in routine microbiology laboratories. Similar to conventional tests, the first-generation deoxyribonucleic acid assays determined only a single analyte. Recent improvements in detection technologies have paved the way for the development of multiparameter assays using macroarrays or microarrays, while the

introduction of closed-tube real-time polymerase chain reaction systems has resulted in the development of rapid microbial diagnostics with a reduced contamination risk. The use of these new molecular technologies is not restricted to detection and identification of microbial pathogens but also can be used for genotyping, allowing one to determine antibiotic resistance or to perform microbial fingerprinting (Jannes and De Vos, 2006).

The basis of all molecular genotyping techniques relies on the acquisition of genetic mutations over time within specified regions of the bacterial genome, thus creating unique profiles between isolates. Although two bacterial strains of the same species have virtually identical genomes, differences will inevitably occur at a few loci. These differences may be small, such as the alteration of a single nucleotide that may either create or remove a specific restriction enzyme site, or larger changes may occur leading to the replication or deletion of DNA fragments hundreds of base pairs in size. Therefore, epidemiologically related strains will show identical or very similar genetic profiles, whereas unrelated clinical isolates will have divergent patterns (Harris, 2006).

The specificity and discriminatory power (the ability to differentiate among closely related strains) of any molecular epidemiology technique are dependent on the frequency at which the targeted genetic element is present in the bacterial genome. The higher this frequency, or copy number, the greater the certainty that two identical results from one method represent epidemiologically related strains. When typing methods only detect low copy numbers of a genetic element, no conclusion about clonal identity among bacterial strains can be drawn, and other methods may be needed to verify relationships. Thus, the choice of methods for molecular epidemiology work must be selected with respect to the end goal, be it epidemiological identification of strains involved in a disease outbreak or a more global perspective of biogeographic distribution of isolates from a phylogenetic perspective (Harris, 2006).

Many new molecular techniques have been developed over recent years to aid the differentiation of isolates belonging to the Mycobacteria. Among these techniques, the most widely used are spoligotyping (Kamerbeek *et al.,* 1997), restriction fragment length polymorphism (RFLP), and variable number of tandem repeat (VNTR) typing (Frothingham and Meeker-O'Connell, 1998; Filliol *et al.,* 2000). RFLP with the IS6110 probe has proved to be very useful for the discrimination of *M. tuberculosis* isolates, which generally harbour a

high copy number of this insertion sequence (IS) (Goyal *et al.,* 1999). However, for *M. tuberculosis* isolates with a low copy number of IS6110 and for the majority of *M. bovis* isolates from cattle which present only one copy of this IS (Kamerbeek *et al.,* 1997), spoligotyping has been shown to be more discriminating than IS61110 RFLP. DR-RFLP, which detects polymorphism within the same region of the chromosome as spoligotyping, is equally discriminating. For all the RFLP techniques, DNA extraction is required. In addition, the technique itself is time-consuming and technically demanding and problems of reproducibility have been reported (Durr *et al.,* 2000).

In contrast, spoligotyping is a rapid, simple, and reproducible technique which can be performed on cell lysates and even on clinical specimens (Goyal *et al.,* 1999). It is based on the polymorphism of a region called DR. The DR region is unique to bacteria belonging to the *M. tuberculosis* complex and is constantly present in this group of mycobacteria. In the current protocols, spoligotyping involves the simultaneous detection of the presence or absence of 43 unique short DNA sequences (35 to 41 bp) called spacers. Spoligotyping is considered a very useful technique, at least at the level of a first screening (Kamerbeek *et al.,* 1997), and the results are produced in an intrinsically qualitative form, as the response for each spacer is either present or absent (Durr *et al.,* 2000).

In general, the finding of genetic differences between two strains of *M. bovis* implies that they presumably originate from different sources, which is a fundamental question to be answered during a disease outbreak. Confidence in this assumption can be increased by using more than one typing system to detect genetic differences, as various genetic markers target changes or mutations within different parts of the mycobacterial genome (Harris, 2006).

Genomic deletion (Regions of Difference) analysis

Comparative genomics have uncovered several variable genomic regions in the members of the MTC. In two studies, differential hybridization arrays identified 14 regions of difference (RD1-14), ranging in size from 2 to 12.7 kb, that were absent from bacillus Calmette-Guerin Pasteur relative to *M. tuberculosis* H37Rv (Gordon *et al.,* 1999). Using strains of the MTC, Brosch *et al.* (2002) studied the distribution of 20 variable regions resulting from insertion-deletion events in the genomes of the tubercle bacilli. They showed that the majority of these polymorphisms did not occur independently in the different strains of the MTC but, rather,

resulted from ancient, irreversible genetic events in common progenitor strains, and the findings contradicted the hypothesis that *M. tuberculosis* evolved from *M. bovis*. Regions of difference are now used to differentiate rapidly and unambiguously between members of the species within the MTC (Huard *et al.*, 2006).

Deletion analysis in conjunction with molecular typing and analysis of specific mutations was shown to be a very powerful approach for the study of the evolution of the tubercle bacilli and for the identification of evolutionary markers. In a more practical perspective, these regions, primarily RD9 and TbD1 but also RD1, RD2, RD4, RD7, RD8, RD10, RD12, and RD13 represent powerful diagnostic tools for the rapid and unambiguous identification of members of the *M. tuberculosis* complex. Genetic approaches for differentiation could replace the often-confusing traditional division of the *M. tuberculosis* complex into rigidly defined subspecies. Moreover, functional analyses will show whether the TbD1 deletion confers some selective advantage to modern *M. tuberculosis*, or whether other circumstances contributed to the pandemic of the TbD1-deleted *M. tuberculosis* strains (Brosch *et al.*, 2002; Huard *et al.*, 2006).

Spacer Oligonucleotide typing (Spoligotyping)

Spoligotyping is based on amplification of the DR region and subsequent differential hybridization of the amplified products with membrane bound oligonucleotides complementary to the variable spacer regions localized between the DRs (Kamerbeek *et al.*, 1997). Strains that are similar or different can be distinguished by their spoligotype patterns, characterized by the number and identity of spacers (Van Soolingen *et al.*, 1995). The presence of the spacer sequences varies in different strains and is visualized by a spot on a fixed site of the hybridization membrane. The differentiating power of spoligotyping is less than IS6110 typing when high copy number strains are being analyzed, but it is superior for the evaluation of low copy number strains. It distinguishes *M. tuberculosis* and *M. bovis* and can be used with culture negative specimens. A simultaneous detection and strain differentiation based on this method has been developed (Kamerbeek *et al.*, 1997). The method is simple, rapid and robust but lacks discrimination (Kanduma *et al.*, 2003).

The DR region is composed of multiple well conserved direct repeats of 36bp which are separated by distinct, non-repetitive spacer sequences of similar size (Van Soolingen *et al.*,

1991). In the standard spoligotyping scheme, a PCR with primers complementary to the DR-sequence is used to amplify all spacer sequences of a given strain. One of the two primers is labelled with a biotin marker. The PCR products are denatured and hybridized to a standard set of 43 oligonucleotides covalently linked to a membrane. These oligonucleotides correspond to 37 spacers from *M. tuberculosis* H37Rv and 6 additional spacers from *M. bovis* BCG P3. If any of these spacers are also present in an investigated strain, they will be amplified during the PCR and hybridized to the spacers on the membrane. The successful hybridization can be visualized by incubation with Streptavidin peroxidase (which binds to the biotin molecule), subsequent addition of a chemiluminescent Streptavidin peroxidase substrate and exposure to a light sensitive film (De Boer *et al.*, 2000). Compared to other molecular typing techniques, spoligotyping is not very discriminatory although its resolving power could be increased by inclusion of additional spacers in extended spoligotyping protocol (Anh *et al.*, 2000).

Spoligotyping also allows the differentiation of MTC members; e.g., *M. bovis* strains characteristically lack spacers 3, 9, 16 and 39-43. Moreover, strain groups and families can be readily identified (Pfyffer *et al.*, 2001) and extensive databases (www.Mbovis.org and SpolDB4 (Niemann, 1997)) have facilitated the comparison of strains isolated in different countries. It is assumed that the DR locus has a unidirectional evolution (spacers can only be lost and not reacquired) and spoligotyping can therefore be particularly useful for phylogenetic analyses (Bifani, 1999).

Restriction fragment length polymorphism (RFLP)

Differentiation of strains of *M. tuberculosis* complex using nucleic acid-based technology is based on strain specific differences and frequencies of certain DNA sequences in chromosomal DNA. This is usually demonstrated by digestion of the genomic DNA with specific restriction enzymes and analysis of the generated patterns after separation of the DNA fragments on agarose gel: restriction fragment length polymorphism (RFLP) (Collins and Lisle, 1984; Patel *et al.*, 1996). This kind of analysis is technically possible and no hybridization step with defined probes is needed. However, interpretation of the results is difficult because the large number of fragments generates a complex pattern and only a small number of different RFLP types are observed (Kanduma *et al.*, 2003).

The insertion sequence IS6110 is a transposable (mobile) element belonging to the IS3 family of insertion elements, and it is found only in members of the *M. tuberculosis* complex (Cave *et al.,* 1991). However, the total number of IS6110 copies found in the genome differs widely between the various *M. tuberculosis* complex species. For example, the *M. tuberculosis* genome may harbor up to 19 copies of IS6110, whereas *M. bovis* only contains one to five copies (van Soolingen *et al.,* 1991).

The foundation of any RFLP technique relies on digestion of genomic DNA followed by probing with a specific marker using Southern blotting techniques. Thus, the combination of the restriction enzyme used and the DNA sequence of the probe allows for polymorphisms in the genomic DNA to be visualized. For IS6110-RFLP, the variation in the copy number of IS6110 is a major source of genetic polymorphisms between bacterial isolates. This variation has significantly contributed to IS6110-RFLP becoming the standard reference typing technique for *M. tuberculosis* (Cave *et al.,* 1991). Unfortunately, IS6110-RFLP is less useful for differentiating *M. bovis* isolates because strains of *M. bovis* only harbor one to five copies of IS6110. Furthermore, this element is routinely present in the same 1.9-kb Pvu II fragment of many *M. bovis* strains (van Soolingen *et al.,* 1991), limiting the differential capacity of this technique. However, utilization of different DNA probes against the same IS6110 target increases the resolution of this method. Reprobing the same strains with a fragment of the inverted repeat region of the insertion element produced two bands per IS6110 copy, allowing a single-band cluster of isolates to be differentiated into two distinct groups (Harris, 2006).

Variable-number tandem repeat typing (VNTR)

A more recent PCR-based typing method for the *M. tuberculosis* complex was first described by Frothingham and Meeker-O'Connell (Frothingham and Meeker-O'Connell, 1998). This method is based on the variable number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR). In mycobacteria, isolates are typed by determining the number of copies of specific repeat units at various MIRU-VNTR loci scattered throughout the genome. These short-sequence repeat units are 52–77 nucleotides in length, thus classifying these loci as minisatellite regions. In MIRU-VNTR genotyping, the number of repeat units within a specific locus is determined by PCR amplification of the entire locus, followed by gel electrophoresis, where the number of repeats is determined by the size of the PCR amplicon. The MIRU minisatellite regions have been classified into three major types:

type 1, which consists of 77 nucleotide repeat units, type 2, which has a 24-bp gap in the 3' portion of the type 1 sequence, and type 3, which has a 15-bp gap in the 5' portion of the type 1 sequence. Mixed type 2 and type 3 MIRUs have also been discovered, which contain gaps in both the 3' and 5' regions (Supply, 2000).

Bacteria belonging to the *M. tuberculosis* complex contain 41 different MIRUs randomly located throughout their genomes (Supply, 2000; Roring, 2002). Within the genome, these MIRUs generally contain an open reading frame that overlaps the termination and initiation codons of their flanking genes. However, some are also found entirely within predicted coding regions (Supply, 2000; Roring, 2002; Skuce, 2002). The purpose of these minisatellite regions in the bacterial chromosome is uncertain, but some evidence indicates that similar to the PE–PGRS genes, they may code repeating amino acid motifs, thus providing another source of antigenic variation (Skuce, 2002). Alternatively, these MIRU-VNTR regions may function in transcriptional or translational regulation (Frothingham and Meeker-O'Connell, 1998; Roring *et al.*, 2004).

Several early studies identified small sets of these MIRU loci that had sufficient allelic variation to differentiate nonrelated bacterial strains, demonstrating the utility of these genetic markers for molecular epidemiology studies. In a landmark study by Frothingham and Meeker-O'Connell, a systematic analysis of the *M. tuberculosis* type strain H37Rv identified 11 tandem repeat loci (Frothingham and Meeker-O'Connell, 1998). These 11 loci were PCR amplified and could be reproducibly found in all strains of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and *M. africanum* tested. Only five of these loci (designated ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E) were suitable for epidemiological studies, as the remainder did not demonstrate sufficient allelic diversity to allow discrimination between nonrelated strains (Frothingham and Meeker-O'Connell, 1998). Subsequent analyses of both the *M. tuberculosis* and *M. bovis* genomes have identified other novel randomly dispersed loci containing MIRUs suitable for strain discrimination as well (Supply, 2000; Sola, 2003).

Interestingly, one locus from the *M. tuberculosis* genome, MIRU 21, is absent from all *M. bovis* strains tested to date (Supply, 2000). One of the drawbacks of these studies is the lack of uniformity in nomenclature between the various MIRU loci. Thus, careful analysis of the literature is necessary to accurately interpret data regarding specific minisatellite regions and their potential utility in epidemiology studies. To this end, a standardized system of

nomenclature has been proposed that uses the first four digits of the exact position of the MIRU with reference to the *M. tuberculosis* H37Rv chromosome (Roring, 2002). It appears that the degree of discrimination provided by VNTR typing is dependent on both the individual VNTR loci used and on the test panel of bacterial isolates chosen for analysis. As a consequence, certain MIRU-VNTR markers may be useful for larger population-based studies, whereas others may be more appropriate for outbreak analyses (Harris, 2006).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Addis Ababa and Bishoftu (Figure 2). Addis Ababa is the capital and largest city of Ethiopia, located on a well-watered plateau surrounded by hills and mountains, in the geographic centre of the country having an altitude of about 2500m above sea level. The average annual temperature and rainfall are 21 °C and 1800 mm, respectively. Bishoftu is located 46 km south-east of Addis Ababa, 8° 44' N and 39° 02' E, at an altitude of 1880m above sea level. The area receives mean annual rain fall of 865 mm in a bimodal distribution with mean minimum and maximum temperatures of 15 °C and 28 °C, respectively.

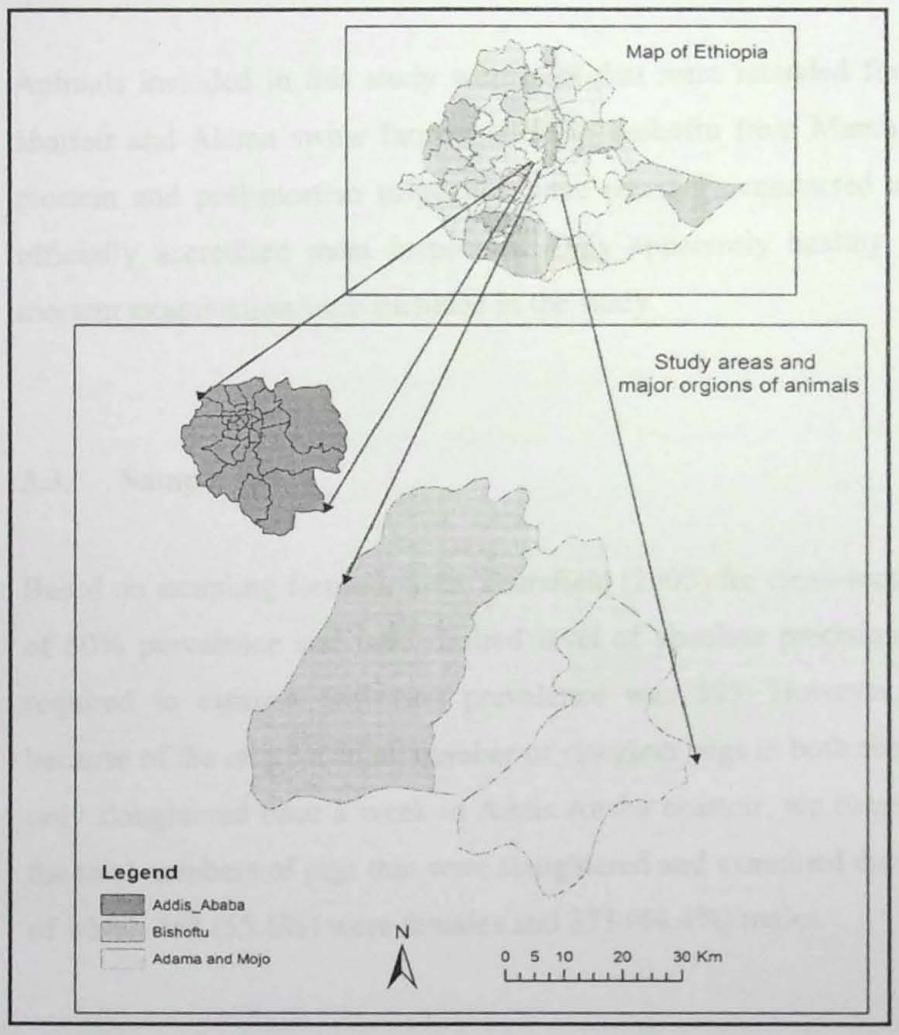


Figure 2: Map of the study areas and major origin of study animals

Although the pig industry is growing in recent years and many piggeries are being established in many parts of the country public slaughtering is carried out only in the slaughterhouse ofAddis Ababa Abattoirs Enterprises. Alema Swine farm has its own slaughterhouse and pork processing plant. In addition to these are several backyard slaughters in many of the small scale swine production systems which are favoured by increasing demand for pork by foreigners particularly from Asia. The two slaughterhouses were selected because they slaughtered larger number of pigs and for their convenience. Pigs that were destined for slaughter at the Addis Ababa Abattoir were originated principally from Addis Ababa and surrounding Special Zones of Oromia and from Bishoftu, Adama and Mojo (Figure 2). While Alema Swine Farm slaughtered pigs mainly from its own farm stock and rarely buys pigs from other surrounding small scale producers when the demand is higher.

3.2. Study population

Animals included in this study were pigs that were intended for slaughter at Addis Ababa abattoir and Alema swine farm abattoir in Bishoftu from March to September 2011. Ante-mortem and post-mortem inspection were routinely conducted at Addis Ababa abattoir by officially accredited meat inspectors. Only apparently healthy pigs that passed the ante-mortem examination were included in the study.

3.3. Sample size

Based on sampling formula from Thursfield (2005) for cross-sectional study and assumptions of 50% prevalence and 0.05 desired level of absolute precision, the minimum sample size required to estimate infection prevalence was 385. However, to increase precision and because of the relative small number of slaughter pigs in both abattoirs and because pigs were only slaughtered once a week in Addis Ababa abattoir, we considered taking a census. Thus the total numbers of pigs that were slaughtered and examined during the study period was 841 of which 468 (55.6%) were females and 373 (44.4%) males.

3.4. Study design

A cross-sectional study was conducted on 841 pigs slaughtered at Addis Ababa Abattoir, Addis Ababa, and Alema Swine Farm Abattoir, Bishoftu, from March to September 2011. Risk factors considered being associated with the occurrence of TB in swine such as age, origin, feed and water source, and other husbandry practices were investigated by collecting data from the owners or attendants at the abattoirs. Inspection was made once a week at Addis Ababa abattoir because pigs were slaughtered in weekly basis. The slaughtering in Alema Swine Farm was demand driven and made on daily basis. Contact was made with slaughter personnel in Alema Swine Farm before the study began. All pigs slaughtered on each day were inspected and samples from suggestive of TB lesion were collected with assistances from the meat inspectors when the number was higher to handle by self alone.

3.5. Post-mortem examination

Unlike bovine species, swine are not tested routinely with tuberculin. For this reason source of information on the prevalence and geographic distribution of tuberculosis in this species can only be collected at abattoirs during meat inspection. Detailed post mortem examination was conducted following the procedure described by (Gracy, 1986). The researcher was trained on detailed post-mortem examination before the actual work. This was achieved thorough visual inspection, palpation and incision of the carcasses and internal organs of known TB positive animals in closes supervision by the research's advisor. Samples of lungs, liver, kidneys, and thoracic, head, mesenteric, and hepatic lymph nodes were subjected to a detailed post-mortem examination in the abattoirs. Each suspected lymph node and organ was incised into smaller slices that facilitate detection of lesions and inspected for the presence of TB lesions. Tissues, organs and lymph nodes suspected of these lesions were collected into sterile universal bottles filled with 5ml of 0.9% saline solution for bacteriological culture. Then they were transported in an ice box with ice packs to keep the cold chain until they reached to the Aklilu Lemma Institute of Pathobiology (ALIPB) TB laboratory. In the laboratory samples were either processed in the following few days while kept at 4°C till the time of processing or kept refrigerated at −20°C for a maximum of 10 days for later processing.

3.6. Culturing and identification of *Mycobacteria*

Samples from suspicious TB lesions were further processed for isolation of mycobacteria in accordance with World Organization for Animal Health (OIE) protocols (OIE, 2010). In the laboratory, the tissue samples were dissected using sterile blades and manually homogenized using a pestle and mortar. This was followed by decontamination by shaking the homogenate in an equal volume of 4% NaOH and concentration by centrifugation at 3,000rpm for 15 min. After discarding the supernatant the sediment was neutralized with 1% (0.1N) HCl using phenol red as an indicator. Neutralization was achieved when the colour of the solution turned from purple to yellow. Then about 0.1ml of the suspension was spread on to each slant of two Lowenstein–Jensen media one enriched with glycerol and the other with pyruvate.

The slants were incubated aerobically at 37°C for six to eight weeks and examined on a weekly basis for growth of mycobacterial colonies. Cultures were considered positive if visible growth is detected within eight weeks of incubation. Microscopic examination of colonies using the Ziehl–Neelsen staining method was performed to select AFB positive isolates.

3.7. Molecular characterization of mycobacterial isolates

3.7.1. Mycobacterial genus typing

All AFB positive isolates were tested by multiplex PCR according to the technique described by Wilton and Cousins (1992), which detects and differentiate *M. tuberculosis* complex from *M. avium* complex, *M. intracellularae* and other mycobacterial species. Heat killed AFB positive samples were used as source of DNA template.

In brief, the PCR amplification reaction was made in thermocycler (Applied biosystem; PTC-100™) with 20 µl reaction mixture used for PCR reaction. This total volume consisted of 5 µl of genomic DNA as a template, 8 µl HotstarTaqMasterMix (Qiagen, United Kingdom), 0.3 µl of each of the six different primers used for multiplex PCR and 5.2 µl Qiagen water. The primers for amplification were MYCGEN-F, 5'AGA GTT TGA TCC TGG CTC AG 3' (35ng/µl); MYCGEN-R, 5' TGC ACA CAG GCC ACA AGG GA 3' (35ng/µl); MYCAV-R,

5' ACC AGA AGA CAT GCG TCT TG 3' (35ng/µl); MYCINT-F, 5' CCT TTA GGC GCA TGA TGT CTT TA 3' (75ng/µl); TB1- F, 5' GAA CAA TCC GGA GTT GAC AA 3' (20ng/µl); TB-1- R, 5' AGC ACG CTG TCA ATC ATG TA 3' (20ng/µl). *M. tuberculosis* strains (H37Rv) and *M. avium* were used as positive control while Qiagen water was used as negative control. The reaction mixture was then heated at 95^0C for 10 minutes. This was followed by 35 cycles of reaction consisting of 95^0C for 1 minute for de-naturation; 61^0C for 0.5 minute for annealing; 72^0C for 2 minutes for extension. Finally, the reaction mixture was maintained at 72^0C for 10 minutes.

The PCR product was electrophoresed in 1.5 % agarose gel in 10x TAE running buffer. Ethidium bromide at ratio of 1:10, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis. All members of the genus *Mycobacterium* produce a band of 1030bp, *M. avium* or subspecies such as *M. avium* subsp. *paratuberculosis* produces a band of 180bp, *M. intracellularae* a band of 850bp while members of *M. tuberculosis* complex produce a band of 372 bp.

3.7.2. RD4 Deletion typing

The RD4 deletion typing was carried out on isolates that showed band for *M. tuberculosis* complex by multiplex PCR. For this deletion typing, the procedure used by Cadmus *et al.* (2006) was followed. Primers that were used include RD4intF 5'-ACA CGC TGG CGA AGT ATA GC-3', RD4flankF 5'-CTC GTC GAA GGC CAC TAA AG-3' and RD4flankR 5'-AAG GCG AAC AGA TTC AGC AT-3' to check for the presence of RD4 locus. The HotStarTaqMasterMix system from Qiagen was used for PCR, with the primers described above. *M. tuberculosis* H37Rv and *M. bovis* were used as positive control while Qiagen water was used as negative control.

The reaction mixture was 10µl of HotStarTaq Master Mix, 0.3 µl each of the three primers (RD4flankR, RD4flankF and RD4int), 2 µl DNA template and 7.1 µl H_2O Qiagen to a final volume of 20 µl. The reaction mixture was heated in Thermal Cycler (Applied biosystem; PTC- 100^{TM}) at 95 °C for 15 minutes. Then, the reaction underwent 35 cycles consisting of de-naturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute. Finally the reaction mixture was maintained at 72 °C for 10 minutes.

The PCR product was electrophoresed in 1.5% agarose gel in 10x TAE running buffer, Ethidium bromide at ratio of 1:10. 100bp DNA ladder and orange 6x loading dye were used in electrophoresis. The gel was visualized in Multi–image™ light cabinet using Alpha innotech version 1.2.0.1 (Alpha Innotech Corporation). The presence of RD4 (*M. tuberculosis*, *M. africanum*) gives a product size of 335bp (RD4int+RD4flankR) and its absence (*M. bovis*) gives a product size of 446bp (RD4flankR+RD4flankF).

3.7.3. RD9 deletion typing

The RD9 deletion typing was applied to isolates that showed the presence of RD4 by RD4 deletion typing. For this deletion typing, the procedure used by Cadmus *et al.* (2006) was followed. The primers used for RD9 deletion typing were RD9flankF, 5'-GTG TAG GTC AGC CCC ATC C-3', RD9intR, 5'-CTG GAC CTC GAT GAC CAC TC-3', and RD9 flankR, 5'-GCC CAA CAG CTC GAC ATC-3'. The mixture was heated in Thermal Cycler (Applied biosystem; PTC- 100™) using an initial hot start of 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 1 minute, 55 °C for 2 minute, and 72 °C for 1 minute; a final extension step of 72 °C for 10 minutes to complete the cycle. Each PCR reaction tube contained 7.1µl H_2O Qiagen, 10µl HotStarTaqMasterMix, 0.3µl of each of the three primers (1.5µM final concentration), 2 µl of DNA templates of samples or controls making the total volume 20µl. *M. tuberculosis* H37Rv and *M. bovis* were used as positive control while Qiagen water was used as negative control. PCR products were electrophoresed in 1.5% agarose gel in 10x TAE running buffer, Ethidium bromide at ratio of 1:10, 100bp DNA ladder and orange 6x loading dye were used in electrophoresis. The gel was visualized in Multi–image™ light cabinet using Alpha Innotech version 1.2.0.1 (Alpha Innotech Corporation). The presence of RD9 (i.e. *M. tuberculosis*) gives a product size of 339bp (+RDint+RD10flankR) and its absence (*M. africanum*, *M. bovis*) gives a product size of 472bp (RD10flankF+RD10flankR).

3.7.4. Spoligotyping

Spoligotyping is a PCR-based technique, which exploits the variability of the direct repeat (DR) region, developed to simultaneously detect and type *M. tuberculosis* complex. Spoligotyping was performed as previously described by Kamerbeek *et al.* (1997) and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Iisselstein, The Netherlands). The DR region was amplified by PCR using oligonucleotide

primers (DRa: 5' GGT TTT GGG TCT GAC GAC 3'and DRb: 5' CCG AGA GGG GAC GGA AAC 3') derived from the DR sequence. The DRa is biotinylated in the 5'-end. A total volume of 25 µl of the following reaction mixture was used for the PCR: 12.5 µl of HotStarTaqMasterMix (Qiagen: this solution provides a final concentration of 1.5 mM MgCl2 and 200µM of each deoxynucleotides triphosphates), 2µl of each primer (20 pmol each), 5µl suspension of heat-killed cells (approximately 10 to 50ng), and 3.5 µl distilled water.

The mixture was heated for 15 minutes at 96 °C and then subjected to 30 cycles of 1 minute at 96 °C, 1 minute at 55 °C, and 30 seconds at 72 °C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 minutes in 2x SSPE (1x SSPE is 0.18 M NaCl, 10 mM NaH_2PO4, and 1 mM EDTA [pH 7.7])-0.5% sodium dodecyl sulfate at 60 °C and then incubated in 1:4000 diluted streptavidin peroxidase (Boehringer) for 45 to 60 minutes at 42°C. The membrane was washed twice for 10 minutes in 2x SSPE-0.5% sodium dodecyl sulfate at 42°C and rinsed with 2x SSPE for 5 minutes at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method (Amersham) and by exposure to X-ray film (Hyperfilm ECL, Amersham) as specified by the manufacturer.

3.8. Data collection, management and analysis

Data describing the pathological conditions suggestive of swine TB observed on lymph nodes and other organs along with husbandry practice, sex, age, origin of animals etc. was classified, filtered and coded using Microsoft Excel® 2007. The data was then exported to SPSS Windows version 18.0 (SPSS INC. Chicago, IL) for appropriate statistical analysis. Data were analysed to estimate the prevalence of gross TB lesions. Descriptive statistics was used to estimate prevalence of TB across the different factors. Univariate analysis was used to evaluate the association of risk factors with the presence of TB using Chi-square test. Multivariate logistic regression was then used to analyse risk factors that were found significant in the univariate analysis (P=0.05) and those variables with p-value $\leq$ 0.2 were forced to be included. The multivariate logistic regression model analysis was built with backward stepwise (conditional) elimination procedure to include variables in the model. Effects were reported as statistically significant if P-value was less than 0.05. Model validity

was assessed using the Hosmer–Lemeshow goodness-of-fit test by default approach of categorising the data set into ten groups.

4. RESULTS

4.1. Husbandry characteristics

A total of 841 pigs were inspected of which 468 (55.6%) were females and 373 (44.4%) males. More than 62% (528/841) of the pigs were slaughtered in Addis Ababa Abattoir while the remaining 37.2 % (313/841) were slaughtered in Alema Farms abattoir. Majority (67%) of the pigs were less than one year of age.

Information regarding husbandry characteristics was collected from the abattoir and farm. Pig owners or attendants who brought pigs to Addis Ababa abattoir were interviewed. Personal observation and communication was made with the farm manager and personnel in Alema swine farms. Pigs in the farm are reared in an intensive production system where they are kept indoors in a well constructed housing, fed with mixed commercial feed, and with a skilled management input. According to the response by owners or attendants who brought pigs for slaughter to Addis Ababa abattoir, the origin of pigs were principally (60%) from Bishoftu followed by Addis Ababa and surrounding Special Oromia Zones (30%) . With regard to the production system the response also showed that 88% of the pig owners used commercial mixed feed while only 12% fed their animals with one or combination of hotel/household leftover or poultry offal and litter as a supplement to grazing on pasture and/or roaming for garbage.

However very small are the responses admitting practices of poor production system, personal observation revealed poor production practices are dominating the pig production systems in the study areas except a very few number of intensive production systems with good practices and bio-security measures. This poor practice holds particularly true to small scale extensive production systems and is characterized by feeding swill, poultry litter and offal mixed feed, open access to garbage, herding to communal grazing pasture and sheltering with other domestic animals.

4.2. Prevalence of swine tuberculosis

The prevalence of swine TB was 6% (49/841) on the basis of detail post-mortem examinations. Detection of gross lesions suggestive of tuberculous in any of the tissues was used to classify the tissue as having TB lesions (Figure 3). The prevalence was significantly higher in older pigs (more than one year of age) than in younger (one year or less) pigs (10.8% versus 3.4%, $\chi^2 = 18.85$; $P<0.001$, Table 1).

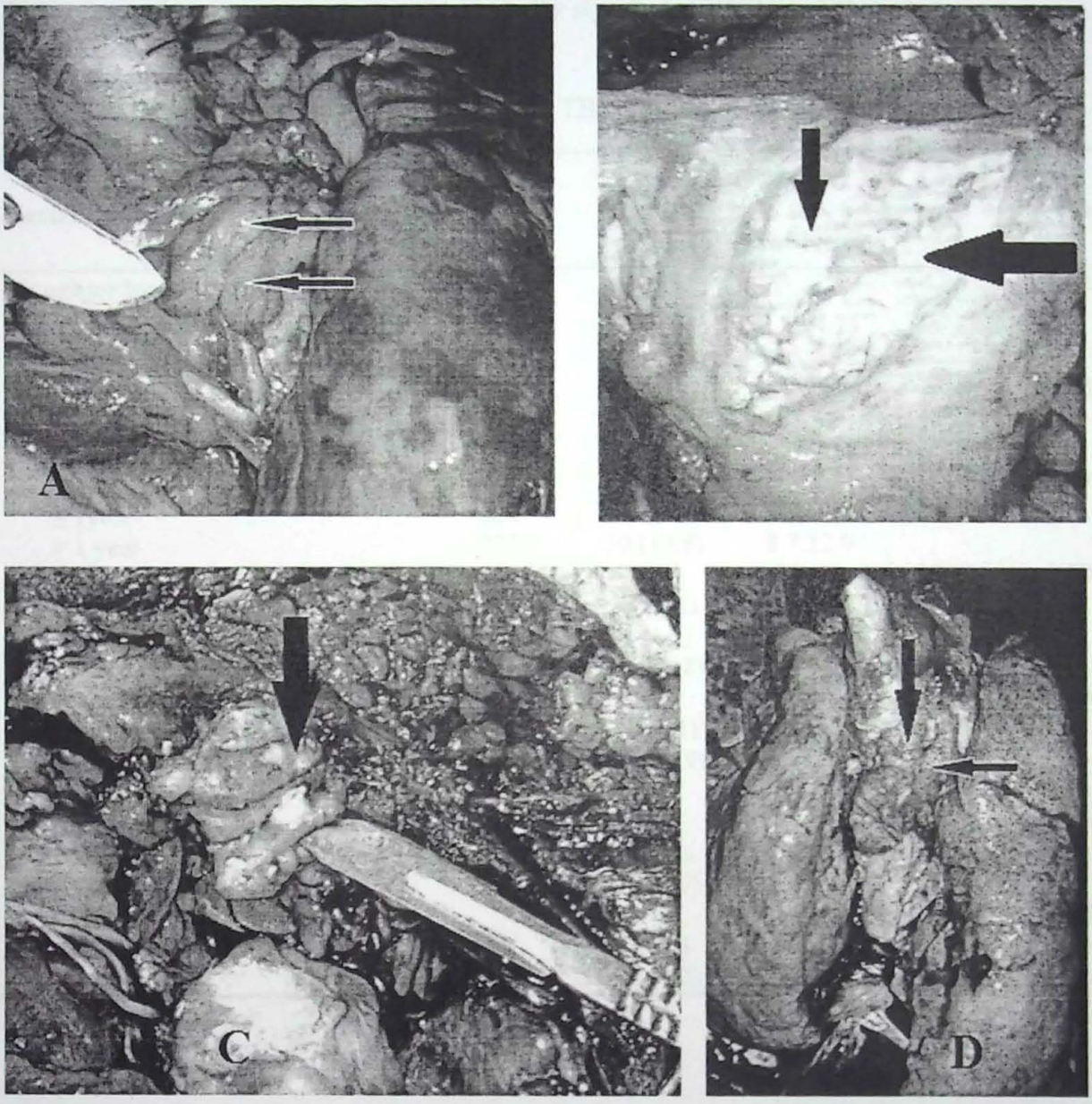


Figure 3: Tuberculous Lesion from various tissues of pigs at slaughter

Tuberculous lesions indicated by solid arrows in various lymph nodes of pigs at slaughter. Bronchial lymph node (A), submandibular lymph nodes (B, C) and enlarged medistinal lymph node (D).

Furthermore, the origin of pigs significantly (χ^2 = 27.67; P< 0.001, Table 1) affected the prevalence. Pigs originated from Addis Ababa and the surrounding nearby villages had the highest prevalence of 12.8% being tuberculous. Similarly, the prevalence was affected by feeding practice (P<0.05) and location of slaughterhouse (P<0.05). Pigs that were kept on free grazing and fed with one or more of swill, poultry offal or left to roam for garbage as a supplementary feed were found to harbour mycobacterial infections more frequently than those fed on commercial mixed feed. In contrast, the variation in the prevalence between sex, floor type and water source was not statistically significant (P>0.05, Table 1).

Table 1: Univariate association between gross TB lesion and risk factors

Variables	No Examined	No Positive (%)	95% CI for Positive %	χ^2	*P*-value
Slaughter location				4.86	0.028
Alema Farms	313	11 (3.5)	2.26:4.74		
Addis Ababa Abattoir	528	38 (7.2)	5.45:8.95		
Sex				0.26	0.608
Male	373	20 (5.4)	3.87:6.93		
Female	468	29 (6.2)	4.57:7.83		
Age				18.85	< 0.001
≤ 1year	564	19 (3.4)	2.18:4.62		
> 1 year	277	30 (10.8)	8.7:12.9		
Origin				27.67	< 0.001
Bishoftu	501	16 (3.2)	2.01:4.39		
Adama and Mojo	114	4 (3.5)	2.26:4.74		
Addis Ababa and surroundings	226	29 (12.8)	10.54:15.06		
Feed source				5.37	0.021
Commercial mixed feed	740	38 (5.1)	3.61:6.59		
Grazing with unconventional type of supplements	101	11 (10.9)	8.79:13.01		
Water source				0.44	0.833
River	21	1 (4.8)	3.36:6.24		
Tap water	820	48 (5.9)	4.31:7.49		
Floor type				0.33	0.565
Soil with/without litter	70	3(4.3)	2.93:5.67		
Concrete	771	46 (6.0)	4.39:7.61		

Considering the significance of associations in the univariate analysis (i.e., a cut-off P>0.2) sex, water source and floor type were not included in the multivariate analysis. Thus based on the results in Table 1, slaughter location, age, origin of pigs, and feed type were included into the logistic regression as the main explanatory variables for the prevalence of swine TB in slaughtered pigs.

The model fits the data as it is evaluated by the Hosmer–Lemeshow goodness-of-fit test (H-L χ^2=4.63; P=0.33). Age and origin of the animals were good indicators of swine TB prevalence in the study areas. The result is shown in Table 2. Older pigs were more than twice (OR=2.55, CI = 1.36:4.78; *P*=0.004) as likely to present as gross TB lesion than pigs less than one year of age. Likewise, origin of pigs significantly affected the prevalence of TB. Pigs originated from Addis Ababa and nearby villages were more than three times (OR=3.29, CI=1.69:6.41; *P*<0.001) likely to show tuberculous lesion during slaughter than pigs originated from Bishoftu.

Table 2: Multivariate association between gross TB lesion and risk factors

Variables	OR	95% CI for OR		*P*-value
		Lower	Upper	
Age				
≤ 1 year				0.003
> 1 year	2.55	1.34	4.78	0.004
Origin				
Bishoftu				0.001
Adama and Mojo	0.87	0.28	2.70	0.812
Addis Ababa and surroundings	3.29	1.69	6.41	<0.001

4.3. Mycobacteriology

Mycobacterial culture positivity on L-J media was observed in 30.6% (15/49) of the pigs examined for suggestive gross TB lesions. Acid fast staining confirmed 12 of these were AFB positive. The percentage of gross TB lesion observed was highest in submandibular lymph nodes (28%) while culture positivity was higher in both the lungs and submandibular lymph nodes (each of 40%) than in mesentric lymph nodes (20%). The result is summarized on Figurers 4 and 5.

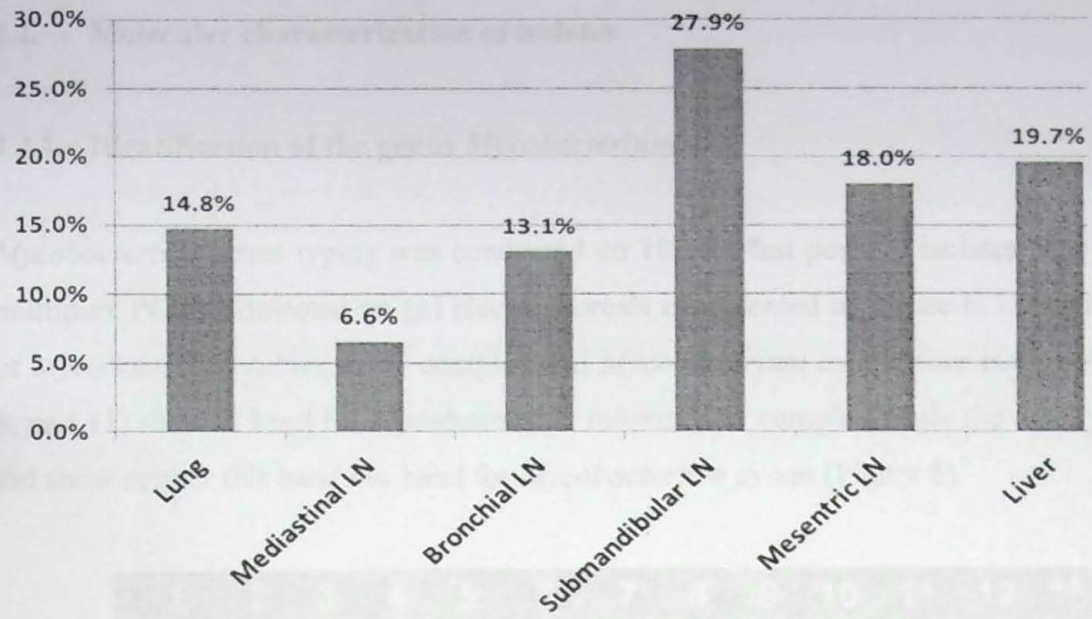


Figure 4: Proportion of tissues observed with gross TB lesion from pigs at slaughter.

LN=Lymph Node

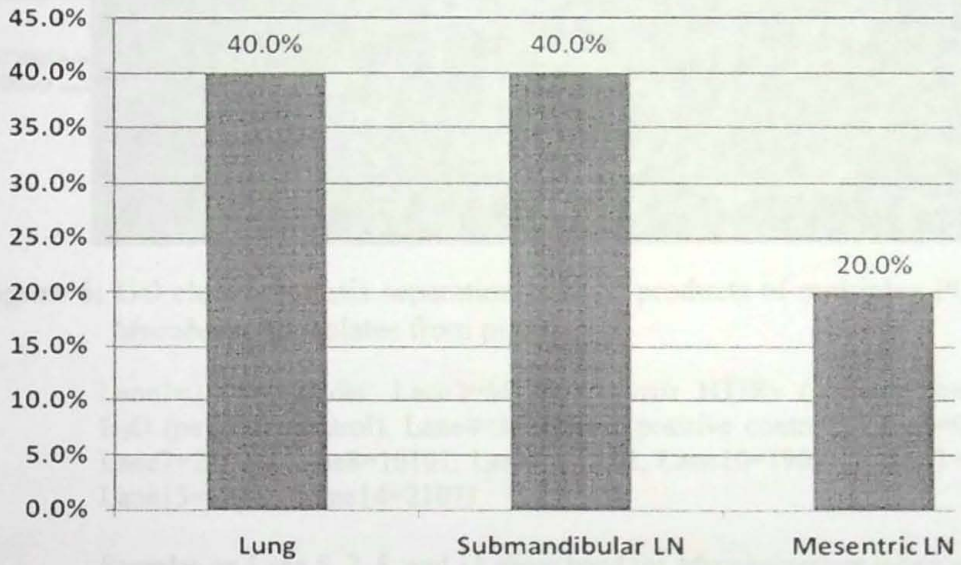


Figure 5: Proportion of tissues yielded mycobacterial growth.

LN=Lymph Node

4.4. Molecular characterization of isolates

4.4.1. Identification of the genus *Mycobacterium*

Mycobacterium genus typing was conducted on 10 acid-fast positive isolates. The product of multiplex PCR as detected on gel electrophoresis is presented in Figure 6. Using the primers of *Mycobacterium tuberculosis* complex and *Mycobacterium avium*, four isolates (Lane 5, 7, 8, and 11) showed band for *Mycobacterium tuberculosis* complex while the other six isolates did show neither this band nor band for *Mycobacterium avium* (Figure 6).

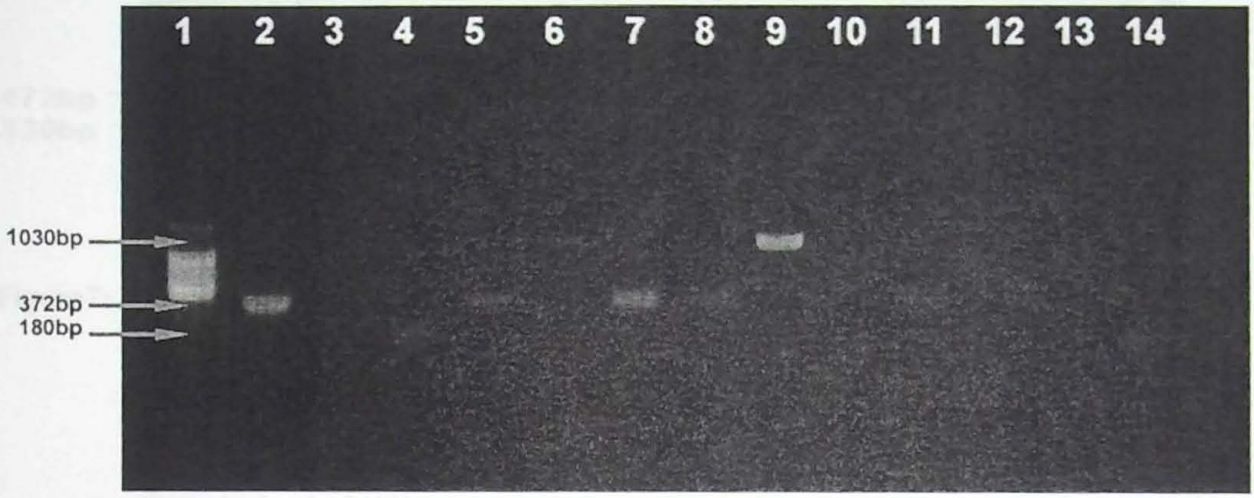


Figure 6: Gel electrophoresis separation of PCR products of multiplex PCR genus typing on *Mycobacteria* isolates from pigs.

Lane1=100bp Ladder, Lane2=*M. tuberculosis* H37Rv (positive control), Lane3=Qiagen H_2O (negative control), Lane4=*M. avium* (positive control), Lane5=0706, Lane6=23073c, Lane7=21074, Lane8=10101, Lane9=10102, Lane10=19091, Lane11=10105, Lane12=936, Lane13=08076, Lane14=21071

Samples on Lane 5, 7, 8, and 11 show band for *Mycobacterium tuberculosis* complex

4.4.2. Speciation of *Mycobacterium tuberculosis* complex isolates

Three *M. tuberculosis* complex isolates were then subjected to RD4 deletion typing and the result showed that two of the isolates were *M. tuberculosis* and the other was *M. bovis*. The result of RD4 deletion typing was further confirmed by RD9 deletion typing and the two isolates were further confirmed to be *M. tuberculosis* and the other one as *M. bovis*.

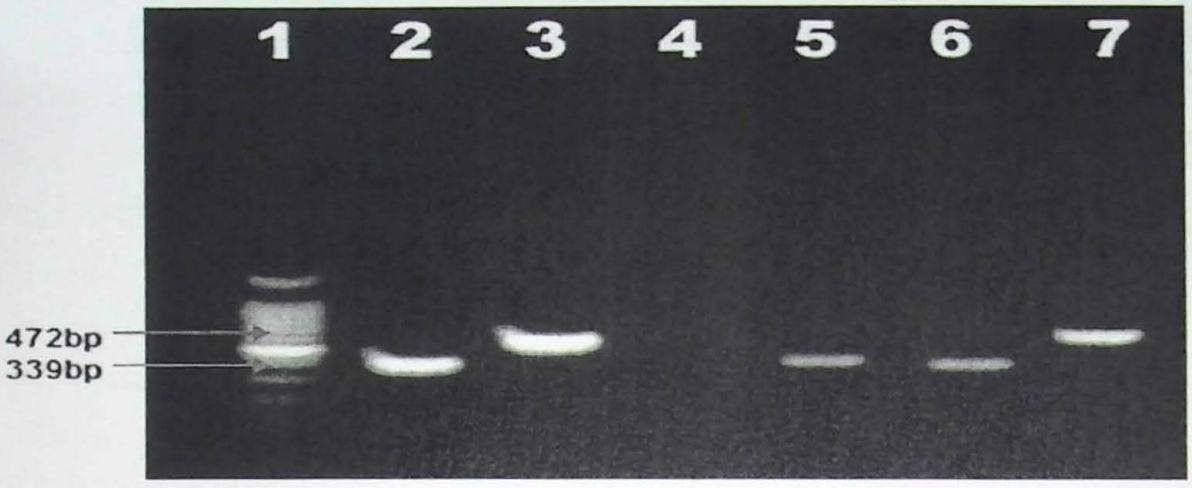


Figure7: Gel electrophoresis separation of PCR products from RD9 deletion typing of *Mycobacteria* isolates from pigs.

Lane 1 =100bp DNA ladder, Lane 2 = *M. tuberculosis* (positive control), Lane 3 = *M. bovis* (positive control), Lane 4 =Qiagen H_2O (negative control), Lane 5–7 were isolates from pigs. Sample on lane 5 and 6 showed a band of 339bp while sample on lane 7 showed a band of 472bp, confirming for *M. tuberculosis* and *M. bovis*, respectively.

4.4.3. Identification of strains of *Mycobacterium tuberculosis* complex

To further characterize the *M. tuberculosis* isolates to a strain level two of the isolates were subjected to spoligotyping. This revealed that the two isolates had distinct spoligopatterns. In comparisons of spoligopatterns with the international spoligotype database of the *M. tuberculosis* complex, SpolDB4 (Brudey *et al.*, 2006), the genotype family assignment showed one of the isolates belonged in the T family, was strain SIT 53 while the other had not been reported before to the database, hence was confirmed to be a new strain. The result is shown below in Figure 8. Sample on Lane E is strain SIT† 53 while sample on Lane D is the new strain.

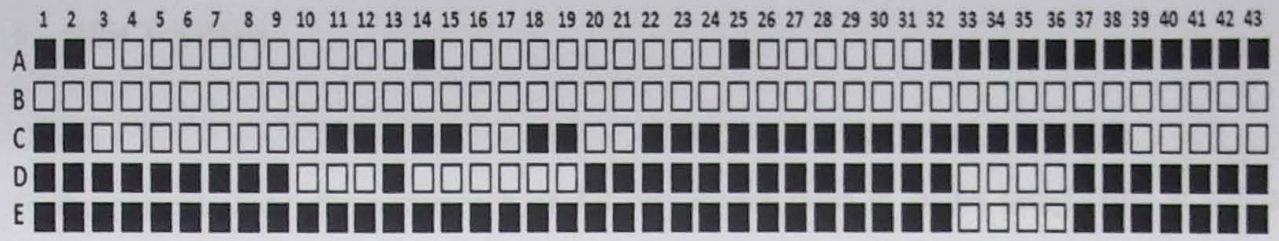


Figure 8: A schematic diagram representing the spoligotype patterns of the two isolates of *M. tuberculosis* from tuberculous lesion of swine

Lane **A**= *M. tuberculosis* H37Rv (positive control), Lane **B**= dH_2O (Negative control), Lane **C** =*M. bovis* BCG (Positive control), Lane **D**= taste sample 1 (New strain), Lane **E**= taste ample 2 (SIT† 53)
The black boxes indicate the presence and white blank areas the absence of the specific spacer at position 1 to 43 in the DR locus.

† SIT=Spoligo-International-Typing number according to the SPOLDB4/ SITVIT databases (Brudey *et al.*, 2006; http://www.pasteur-guadeloupe.fr:8081/SITVITDemo)

5. DISCUSSION

In the present study, the occurrence of TB was investigated in swine slaughtered at Addis Ababa and Bishoftu abattoirs. Post-mortem and bacteriological examinations as well as molecular typing were applied. The prevalence of TB on the basis of lesion was 6%, and is lower than prevalence reported from other African countries including Cameroon (Awa *et al.,* 1999), Egypt (Mohamed *et al.,* 2009) and Uganda (Muwonge *et al.,* 2010). On the other hand, the presently recorded prevalence is higher than those reported from developed countries including New Zealand (Nuttall, 1986), The Netherlands (Komijn *et al.,* 1999), Czech Republic (Pavlik *et al.,* 2005) and USA (Thoen, 2006). Such differences in the prevalence of swine TB in developing and developed countries could be associated with the eradication of TB in cattle in the developed world, where cattle served as sources of infection to swine (Caffery, 1994; Thoen, 2006).

A significant association was detected among the age class and the prevalence of TB lesion in pigs. Tuberculous lesions occurred more frequently in older pigs than younger pigs which could be partly due to chronic nature of the disease that require longer time to produce detectable lesion and due to longer period of exposure in older pigs. This is in consistent with reports from other countries and in other species of animals (Radostits *et al.,* 2006; Muwonge *et al.,* 2010; Mamo *et al.,* 2011).

Higher prevalence was recorded in swine from Addis Ababa and surrounding areas as compared to those slaughtered at Alema Swine Farm. Swine from Adama and Mojo had also a slightly higher occurrence of infection than those slaughtered at the farm. This variation in the prevalence of swine TB could be a reflection of the husbandry system in that swine reared in Addis Ababa and surrounding areas are kept under various poor husbandry practices including feeding swill, poultry litter, abattoir offal and garbage; free range feeding and sheltering with other domestic animals; and confinement in poor housing system where they have close contact with other animals and humans as compared to those reared in Alema Swine Farm. In Ethiopia, the traditional small scale system, which is the predominant swine production system particularly in Addis Ababa and its surrounding, is characterised by absence or minimal health care and supplementary feeding and proper housing. It has been also reported that swine production is aggregated in the central part of the country (Abdu and

Gashaw, 2010), which could also promote the transmission of mycobacterial infections among the different farms through the movement of swine from one farm to another particularly during establishment of new farms.

In our study, gross TB lesions were observed in the lungs, liver, head and mesenteries and associated lymph nodes. The lesions appeared more frequently in the head and mesenteric lymph nodes and in the liver. The presence of tuberculous lesions in the liver and the lymph nodes of the head and mesenteries may indicate infection by ingestion of contaminated feed, offal or infection from scavenging on contaminated garbage (Radostits *et al.,* 2006; Thoen, 2006), while lesions in the lung and associated lymph nodes suggest aerogenic transmission (Menzies and Neill, 2000). The observation of higher proportion (>65%) of lesion in the lymph nodes of the head and gastrointestinal organs suggests that ingestion to be the most common route of transmission in pigs in this study. Similar observations were reported by other investigator (Thoen, 2006).

Only about 31% of the lesions yielded mycobacterial growth on culture. Similar observations have been made in Czech Republic (Pavlik *et al.,* 2005), Egypt (Mohamed *et al.,* 2009) and Uganda (Muwenge, *et al.,* 2010). The failure to demonstrate tubercle bacilli could be due to inadequacy in methods for isolating tubercle bacilli, occurrence of healed processes that contain no viable tubercle bacilli, cause of the lesions by some microorganism other than tubercle bacilli, such as *Rhodococcus equi* or *R. sputi* (Thoen, 2006; Radostits *et al.,* 2006). It could also be due to subjective difference in identifying tuberculous lesions (Berg *et al.,* 2009). Nevertheless, this needs further investigation so as to enable maximum yield of viable organisms from culture for a correct interpretation of the results and thereby to appropriately explain the magnitude of the disease.

Isolation of *M. tuberculosis* complex species from tuberculous lesions of swine suggests their transmission from humans or cattle to pigs, which could occur as a result of close contact between pigs and humans and cattle. Previous studies have also suggested interspecies transmission of mycobacterial species in Ethiopia (Asseged *et al.,* 2000; Ameni *et al.,* 2003; Ameni *et al.,* 2007; Ameni and Erkihun, 2007; Shitaye *et. al.,* 2007; Berg *et al.,* 2009; Demelash *et al.,* 2009; Ameni *et al.,* 2010). Similar findings were reported from other countries as well with high burden of TB in humans and animals and transmission between these two (Sulieman and Hamid, 2002; Mohamed *et al.,* 2009).

Nonetheless, most of the report from developed countries showed that the lesions of swine TB were due to members of the *Mycobacterium avium* complex (Komijn *et al.,* 1999; Payeur and Songer, 1999; Pate *et al.,* 2004; Thoen, 2006; Cvetnić *et al.,* 2009). This difference may be due to the control of mammalian TB achieved by developed countries. Early in the 20th century when TB in cattle and human was more prevalent, mycobacteriosis in swine was either *M. bovis* or *M. tuberculosis*. However, for instance in USA by 1925, the avian type TB began to occur more frequently in swine. Today isolation of mycobacteria other than *M. avium* from swine is uncommon in the USA. In rare cases it occurs in pigs which are kept on the same premises with *M. bovis* infected cattle or which have been in close contact with *M. tuberculosis* infected humans (Payeur and Songer, 1999). After the enforcement of strict regulations for test and slaughter the prevalence of bovine type of TB in swine gradually declined concurrently with the eradication of the disease in cattle in most western countries (Caffery, 1994; Komijn *et al.,* 1999; Thoen, 2006).

Two of the AFB positive isolates did not show signal for MTC up on genus typing using multiplex PCR in this study and hence assumed to be members of non-tuberculous mycobacteria (NTM). Isolation of NTM from swine was reported from other countries as well (Nuttall, 1986; Mohamed *et al.,* 2009). In Ethiopia, NTM have been isolated from cattle (Berg *et al.,* 2009) and camels (Mamo, *et al.,* 2011) with tuberculous lesions which indicate their involvement in broad range of animal species (Phillips and von Reyn, 2001) and their role as a cause of tuberculous lesions.

From the online international spoligotype database, The SITVIT Database, we confirmed that one of the *M. tuberculosis* isolates was SIT-53 strain. This strain was reported as extremely drug resistant TB isolate in a study carried out in Ethiopia (Agonafir *et al.,* 2010). Other studies have also shown that this strain is frequent in Ethiopia (Bruchfeld *et al.,* 2002; Legesse, 2011). The other isolate was confirmed to be a new strain and it had not been reported to the database previously. Several studies are keeping on discovering the emergence of new strains of *Mycobacteria* throughout the world. Since 2009 alone, for instance, several researchers discovered new strains of tubercle bacilli in Ethiopia (Berg *et al.,* 2009; Ameni *et al.,* 2010; Agonafir *et al.,* 2010; Mamo *et al.,* 2011; Legesse, 2011). The increasing finding of new mycobacterial strains would probably suggests a threat of facing an even increasing

population of multi drug resistant strains of *Mycobacteria* which would complicate the control of TB and continue to be a real threat to world health.

6. CONCLUSION AND RECOMMONDATIONS

In conclusion, this study, to the best of our knowledge, is the first survey in swine TB in Ethiopia. The study showed a fairly high prevalence of swine TB in central part of the country. The prevalence was found to be significantly affected by age and origin of pigs while several other factors did not appear to influence it. With the limitation of data quality regarding the husbandry practices of pig production system the interpretation of the study should, however, be considered within this context. The isolation of *M. tuberculosis* and *M. bovis* from pigs indicates the probability of potential risk of interspecies transmission given the endemic nature of TB in Ethiopia. Moreover, with the expected rapid expansion of swine production within a couple of decades, it is likely that the country will get challenge from a rise in the incidence of TB. Frequent findings of new strains of *Mycobacteria* in several studies would suggest an increasing threat of emerging multidrug resistant bacilli which complicate the control of TB in general and continue to be a real threat for world health.

Therefore, based on the above concluding remarks the following points are forwarded:

- ✓ Launching feasible control methods of swine TB would be required.
- ✓ Avoiding physical contact between infected cattle or humans and swine would minimize interspecies transmission of *Mycobacteria*.
- ✓ Comprehensive studies should be conducted so that a countrywide epidemiology of swine TB is established
- ✓ Strain typing of isolates is required when study on TB is carried out for a clear epidemiological understanding of the disease

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8. APPENDICES

Appendix I. Preparation of Löwenstein-Jensen egg-based medium

1. Preparation of mineral salt solution

Approximate Formula* Per 600 mL

Ingredients

- ✓ Potassium dihydrogen phosphate anhydrous (KH_2PO4) 2.4 g
- ✓ Magnesium sulphate (Mg SO_4. 7H2O) 0.24 g
- ✓ Magnesium citrate ($Mg_3(C_3H5O7(2))$ 0.6 g
- ✓ Asparagines 3.6 g

i. Weigh and add the above listed minerals salt ingredients in to a flask that labelled with P (Pyruvate) and G (Glycerol),

ii. Add 300ml distilled water to each flask,

iii. Add 12ml of Glycerol (*M. tuberculosis*) to the flask labelled with G,

iv. Add 10g of Pyruvate (*M. bovis*) to the flask labelled with P,

v. Dissolve the mineral salt ingredients to the distilled water by heating,

vi. Autoclave the minerals salt solution at 121ₒc for 30 minutes to sterilize,

vii. Cool at room temperature before use,

viii. Keep the solution indefinitely and may be kept in suitable amounts in refrigerators.

2. Preparation of 2% malachite green solution

Ingredient

- ✓ Malachite green dye 2.0g
- ✓ Sterile distilled water 100 ml
- ✓ Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours.

3. Preparation of homogenized whole eggs

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

- ✓ Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand, brush in warm water and plain alkaline soap.
- ✓ Let the eggs soak for 30 minutes in the soap solution.
- ✓ Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes.
- ✓ Before handling the clean dry eggs scrub the hands and wash them.

- ✓ Crack the eggs with a sterile knife into a sterile flask till 1000m obtained.
- ✓ Then beat with sterile egg whisk or by using magnetic stirrer until it fully homogenized

4. Preparation of the complete medium (Mixing of the different solution)

- ✓ Measure and add 500ml of homogenized eggs in to sterile flask that labelled with P and G,
- ✓ Add 300ml mineral salt solution to each flask(remember to add P labelled salt solution to P flask and labelled and G labelled mineral salt to G labelled),
- ✓ Add 10ml malachite green solution,
- ✓ Steer well the complete medium
- ✓ Distribute the complete egg medium in 6-8 ml volumes in sterile 14 or 28ml McCartney bottles or in 20 ml volumes in 20 x150 mm screw- capped test tubes and the tops tightly closed,
- ✓ Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the

Heavier ingredients

5. Coagulation of medium (Inspissation)

- ✓ Before loading heat the inspissations to 80oc to hasten the build up of the temperature,
- ✓ Place the bottles in a slanted position in the inspissator and coagulate the medium at 80- 85^{o}^{c} for 45 minutes.

6. Sterility checking

- ✓ After inspissations, the whole batch of media or a representative samples of culture bottles should be incubated at 35-37^{o}^{c} for 24 hours as a sterility check

7. Storage

- ✓ Date the media for storage,
- ✓ Store or keep the dated media in the refrigerator for several weeks with caps tightly closed to prevent the medium from drying out.

Source: WHO (1998)

Appendix II. Culturing of tissue samples

1. Homogenization of tissue samples

- ✓ Remove the surrounding flesh with sterilized blade/scissor
- ✓ Cut the tissue into pieces by using sterilized scissor
- ✓ Add these pieces of tissue into mortar that contain small amount of sterilized sand
- ✓ Then grind the tissue with pestle
- ✓ Add small amount of sterilized saline
- ✓ Filter the grinding tissue using sterilized sieve
- ✓ Take(harvest) the filter liquid, and add into centrifuge tube

2. Decontamination

- ✓ Add equal volume 4% NAOH to centrifuge tube
- ✓ Mix well for about 10 minutes
- ✓ Centrifuge the mixed liquid at 3000 rpm at 4oc for 15 minutes
- ✓ Decant the supernatant
- ✓ Mix the sediment

3. Neutralization

- ✓ Add drop of phenol red indicator to well mixed sediment and mix well until it becomes purple
- ✓ Add 1% HCL to the sediment and mix well until it becomes yellow

4. Inoculation

- ✓ Add 1-3 drop of neutralized sediments of the tissue into LJ media (one labeled with P and other with G)

5. Incubation

- ✓ Incubate at 37°C with 10% $C0_2$ air at slant position for a week,
- ✓ Then put at upright position for 8 weeks at the some temperature and air condition until complete growth is observed.
- ✓ Monitor every week for growth

Source: WHO (1998)

Appendix III. Ziehl-Neelsen stain reagents preparation

1. Carbol-Fuchsin Solution:

- ✓ Basic fuchsin 2.5 gm
- ✓ Distilled water 250.0 ml
- ✓ 100% alcohol 25.0 ml
- ✓ Phenol crystals, melted 12.5 ml
- ✓ Mix well, filter into brown bottle. Label bottle with date and initials, solution is stable for 1 year.

Caution: Carcinogen, toxin.

2. 1% Acid Alcohol:

- ✓ Hydrochloric acid 10.0 ml
- ✓ 70% Alcohol 990.0 ml
- ✓ Mix well, label with date and initials, stable for 1 year

Caution: Flammable, corrosive

3. Methylene Blue

3.1. Stock Solution:

- ✓ Methylene blue 0.7 gm
- ✓ Distilled water 50.0 ml
- ✓ Mix well, filter into bottle. Label with date and initials, stable for 1 year.

3.2. Working Solution:

- ✓ Methylene Blue Stock 5.0 ml
- ✓ Distilled water 45.0 ml
- ✓ Pour into coplin jar, stable for 2 months.

Caution: Avoid contact and inhalation

Source: WHO (1998)

Appendix IV. Ziehl-Neelson staining procedure for AFB

- ✓ Mix well the sample/sputum/ isolated colonies and make the smear,
- ✓ Fix the smear with heat,
- ✓ Flood the slide with the carbon-fishsin stain (primary stain),
- ✓ Gently heat the carbon-fuchin stain until it steam and maintain steaming for 5 minutes,
- ✓ Wash the slide thoroughly with tape water,
- ✓ Decolorize the slide with acid alcohol (3% HCL) for 5 minutes,
- ✓ Wash thoroughly the slide with tape water,
- ✓ Flood the slide with the methyl blue/ malachite green (counter stain) for 1 minute,
- ✓ Wash thoroughly the slide with tape water,
- ✓ Wipe the back of the slide with cotton or gauze and dry it,
- ✓ Examine the slide with 40x magnification power to check for quality of smear and Staining,
- ✓ Add one drop of oil immersion and examine the slide with 100x magnification power

Source: WHO (1998)

9. CURRICULUM VITAE

Personal information	
First name(s) / Surname(s)	**Sintayehu Mulugeta Arega**
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Gender	Male
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Work experience	
Dates	From October 2007 onwards
Occupation or position held	Assistant professor
Main activities and responsibilities	Teaching, advising and supervising veterinary students, Carrying out researches, Providing community services
Name and address of employer	University of Gondar, Faculty of Veterinary Medicine, P.O. Box 196, Gondar, Ethiopia
Type of business or sector	Educational Institution
Education and training	
Dates	From January 2010 to December 2011
Title of qualification awarded	MSc in Transboundary Animal Disease Management
Principal subjects/occupational skills covered	**General** Bridging module (Project assignment), Workshop module (Study design) Master thesis work, Workshop module (Data analysis) **Occupational** Epidemiology & biostatistics, Animal health information system & disease pattern, Risk analysis, surveillance & risk-based surveillance, Laboratory diagnosis applications, Emerging & re-emerging diseases & environmental interactions, Livestock & animal products trade & regulations, Delivery & evaluation of veterinary services, Prevention & control of transboundary animal diseases, Animal food value chains & food safety, Livestock production systems & disease ecology, Animal health economics, socio-economics, Community participation & project management, Wildlife, aquatic & ecosystem health management, and Marketing of animals & animal products **Field Work** Sample and data collection from field and abattoirs, Laboratory work (Mycobacteriology, PCR, Deletion typing, Spoligotyping)
Name and type of organisation providing education and training	Addis Ababa University, Faculty of Veterinary Medicine, P. O. Box 34, Debre Zeit, Ethiopia and Free University of Berlin, Faculty of Veterinary Medicine, Germany.
Dates	From October 2001 to August 2007
Title of qualification awarded	Doctor of Veterinary Medicine (DVM)

Principal subjects/occupational skills covered	**General** Veterinary Anatomy and Embryology, Biochemistry, Organic Chemistry , V. Pharmacology and Therapeutics, V. Physiology, V. Immunology, V. Parasitology, V. Pathology and V. Microbiology **Occupational** Biostatistics, V. Theriogenology, V. Surgery, V. Medicine, V. Public Health, Research Method and Experimental design and V. Epidemiology and Preventive Medicine **Externship Experiences** Farm experience, Clinical Field Experience, Abattoir Experience and Laboratory Experience.
Name and type of organisation providing education and training	Addis Ababa University, Faculty of Veterinary Medicine, P. O. Box 34, Debre Zeit, Ethiopia
Dates	From July 2004 to October 2004
Title of qualification awarded	Diploma in Computer Application Software
Principal subjects/occupational skills covered	Introduction to PC and Ms-Dos, Ms-Windows XP, Ms-Word, Ms-Excel, Ms-Access and Internet application
Name and type of organisation providing education and training	DELTA Computer Systems computer tainting centre, P. O. Box 81203, Addis Ababa, Ethiopia. E-Mail: deltaplc@telecom.net.et
Personal skills and competences	
Mother tongue(s)	**Amharic**
Other language(s)	**English, German**
Social skills and competences	Team work: Anti-AIDS Movement Club, Animal Welfare Club, Business Process Re-engineering Team. - Memberships: Anti-AIDS Movement Club, Animal Welfare Clubs and Ethiopian Veterinary Association.
Organisational skills and competences	- Worked in Graduation Committee. during the last year of my undergraduate study I was member of Faculty Recruitment Committee at UoG - I have successfully accomplished training on Pedagogical Skills at University of Gondar (UoG)
Computer skills and competences	- Good in Microsoft Office ™ tools (Word™, Excel ™ and PowerPoint ™) and Internet - Certified in Geographical Information System at level of ArcGIS-Desktop I
Artistic skills and competences	- Good experience in drawing and painting
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10. DECLARATION

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

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Place: Addis Ababa University, School of Veterinary Medicine

Date of submission: December, 2011

This thesis has been submitted for examination with our approval as university advisors:

Name of advisors: Signature

Dr. Gobena Ameni (ALIPB, AAU, Ethiopia) _____

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