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EPIDEMIOLOGY AND ZOONOTIC IMPORTANCE OF BOVINE TUBERCULOSIS IN SELECTED SITES OF EASTERN SHOA, ETHIOPIA

by

Tadele Kiros

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LIST OF ABBREVIATIONS

AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Disease Syndrome
ALERT	All African Leprosy Rehabilitation and Training
BCG	Bacillus Calmette-Guerin
BTB	Bovine Tuberculosis
CIDT	Comparative Intradermal Tuberculin Test
CMI	Cell Mediated Immunity
ELISA	Enzyme Linked Immuno sorbent Assay
ESAP	Ethiopian Society of Animal Production
EVA	The Ethiopian Veterinary Association
FAO	Food and Agricultural Organization
FRG	Federal Republic of Germany
γ-INF	Gamma Interferon
HIV	Human Immunodeficiency Virus
HTB	Human Tuberculosis
IAR	Institute of Agricultural Research
ILCA	International Livestock Center for Africa
ILRI	International Livestock Research Institute
IL	Interleukin
MAO	Ministry of Agriculture
MDRMB	Multidrug Resistant *M. bovis*
MHC	Major Histocompatibility Complex
MOH	Ministry of Health
NALC	N-Acetyl-L-cysteine
OIE	Office International des Epizooties
PA	Peasant Association
PPD	Purified Protein Derivative
SID	Single Intradermal tuberculin test
ST-CF	Short Term Culture Filtrate
TNF	Tumor Necrosis Factor
WHO	World Health Organization

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ABSTRACT

A survey was conducted in Debre Zeit and Addis Ababa, from January to October, 1997 in cattle and human tuberculosis patients to study the epidemiology of bovine tuberculosis and to assess the role of *M. bovis* in human TB cases respectively. The methods applied were comparative intradermal tuberculin test, postmortem diagnosis, cultural examination, biochemical tests and questionnaire survey on human TB patients and dairy farm workers.

A total of 788 animals were subjected to the comparative intradermal tuberculin test (CIDT) resulting in 23.9% positive and 5.8% doubtful reactors. There was a significant difference in prevalence between farms/sites ranging from 4.2% to 90.8% including the doubtful reactors (χ^2 test, $p<0.001$). Test for agreement between CIDT and postmortem as well as *γ-INF* assay and culture revealed moderate agreement in both cases with *Kappa coefficient* of 0.53 and 0.48 respectively.

Analysis for risk factors revealed that cattle under poor management were more likely to have high proportions of reactors (OR=14.9) than those under good management. The association between breed and prevalence was also statistically significant (χ^2 test, $p<0.001$). Exotic cattle were still more likely to be affected by tuberculosis even after controlling for the effect of management ($OR_{M\text{-}H}$=2.7, 95% CI=1.6-4.6). Animals with good body condition were 1.5 times more likely to react to tuberculin than those with poor condition, indicating an association. However, this association was not significant when the effect of breed was controlled ($OR_{M\text{-}H}$=0.8). There was no statistically significant association between age and prevalence (p=0.46).

A total of 265 sputum, peritoneal fluid, cattle milk and tissue samples were cultured resulting in 102 (38.5%) positive isolates on a primary culture. On subculture 81 of these isolates were positive for acid fast bacilli and subjected to niacin test out of which 36 (44.4%) were positive indicating *M. tuberculosis* and 45 (55.5%) were negative indicating *M. bovis* or atypical mycobacterium. Niacin negative organisms were isolated from milk (17/157), sputum (14/85) and tissue (14/22) samples. Of the 19 isolates from milk, 2 were niacin positive indicating the isolation of *M. tuberculosis* from raw milk which is the first report in the country.

Results of a questionnaire survey conducted on 138 human TB patients indicated that 38.4% were with extrapulmonary tuberculosis. The proportion of patients with EP TB was significantly high in younger age group (<15 years), in farmers, in patients with close contact to cattle, in those who frequently drink raw milk and in people from the rural areas ($p<0.001$ in all cases); however, there was no significant association between type of TB and sex (p=0.24).

A five year hospital data from Debre Zeit and ALERT hospitals revealed that there was significantly higher proportion of male patients than females ($p<0.001$) and more than 70% of the patients were in the active age group (15-45 years). In ALERT hospital 19.7% of the TB patients were also concurrently test positive for HIV.

In conclusion, incidence of bovine tuberculosis is increasing gradually in the livestock population particularly in the intensified dairy farms, this increment together with the habit of

the community to consume raw milk and meat may necessitate to develop an appropriate and feasible control measure for bovine tuberculosis in cattle and prevention of its zoonotic importance in Ethiopia.

1. INTRODUCTION AND OBJECTIVES

The World human population is growing at a rate much faster than food production which may reach 7.2 billion at the end of 2010; this increase will be mainly in developing countries which are unable to assure adequate food for their people.

Developing countries have nearly 2/3 of the world livestock population, but produce less than a third of world's meat and a fifth of its milk (ILRI/FAO, 1995). Ethiopia, one of the developing countries in sub-Saharan Africa, stands first in livestock population in Africa and tenth in the world. Although statistical data for livestock in Ethiopia have never been consistent the latest estimates indicate that there are 27 million cattle, 24 million sheep, 18 million goats, 7 million equine, 1 million camel, 52 million poultry (ILCA, 1992). Despite the country's huge livestock population, the meat and milk production is very low estimated to be 246,000 tons and 960,000 tons respectively with *per capita* consumption of 17.1 kg milk and 5.6 kg meat per year in 1983-1985 (ESAP, 1995).

To feed their people developing countries need to intensify their livestock production system; but intensification has been hindered by diseases such as mastitis, respiratory diseases, tuberculosis, etc. Bovine tuberculosis is one of the important diseases of intensification not only due to its effect on animal production but also due to its public health significance (O'Reilly and Dabron, 1995). Nevertheless, governments and veterinarians in sub-Saharan Africa have focused on rinderpest and trypanosomosis as these two diseases are considered to be the most rampant animal diseases in the region (Alaku and Moruppa, 1993). However, as man and animals in this region are sharing the same micro environment, zoonotic diseases such as tuberculosis should also be the area of focus (WHO, 1993).

Tuberculosis had at times presented one of the deadly threats to human beings. Between 1750 and 1900, almost every one from the temperate region was infected with TB; in certain regions every fourth death was due to TB (Kleeberg, 1984). Because of the effective control measures applied, the disease had practically disappeared from most of the developed countries, remaining an increasingly serious problem of the developing world. It is still the greatest single cause of human morbidity and mortality in many developing countries resulting in 3 million deaths and 10 million new cases every year the world over (Kochi, 1991) of which 98% of the deaths and 95% of the new cases are in the developing countries (Harries and Maher, 1996).

Africa with 1.4 million new cases annually is estimated to have the highest incidence of 272 cases per 100,000 population and an over all mortality of 660,000 per year (WHO, 1992). In Ethiopia the annual risk of infection ranges from 1.5%-3% and TB stands third (6.2%) among diseases that cause hospitalization and is the leading cause of death (14.3%) in hospitalized patients (MOH, 1992). These days, due to the rapid increase of TB cases world over, TB has been declared a 'global emergency' gaining world-wide emphasis together with HIV. According to WHO (1993) morbidity and mortality due to TB during the decade (1990-1999) is estimated to be 88.2 million of which 8 million will be attributed to HIV; among these 30 million will die, 2.9 million being with HIV. Among all other risk factors HIV is the most powerful factor known to increase risk for TB; compared to an individual who is not infected with HIV the infected one has 10 times increased risk of developing tuberculosis (Dabron and Grange, 1993).

M. tuberculosis is the most frequent cause of human morbidity and mortality, but some cases can be also due to *M. bovis*. The incidences of human TB caused by the bovine bacillus had

been of significant proportion before control measures were introduced in the developed countries. In 1930s and 1940s, *M. bovis* was responsible for more than 50% of the cervical lymphadenitis cases in children in Europe (Cosivi *et al.*, 1995). Currently primary human disease due to *M. bovis* is rare in developed countries where bovine tuberculosis has been eradicated by the test and slaughter policy, but the transmissible post primary (reactivation) form is still encountered in persons born before bovine TB was eradicated in these countries. These people can act as source of infection to cattle; in Germany 16 out of 49 herds were infected from old agriculturalists with pulmonary (70%) and urogenital (30%) tuberculosis due to *M. bovis* (Weber *et al.*, 1988).

Reactivation or infection with *M. bovis* occurs as a result of immunosupression due to age or diseases like HIV; two HIV related cases (one pulmonary and the other with cervical lymphadenopathy) of human tuberculosis due to *M. bovis* have been reported in England in 1991 (Dabron and Grange, 1993). Bouvet *et al.* (1993) have also reported a nosocomial outbreak of a multidrug resistant *M. bovis* (MDRMB) among five HIV infected patients in Paris Hospital.

Man acquires tuberculosis of bovine origin directly by the aerogenous route and indirectly by the consumption of milk and rarely meat of tuberculous cattle (Kleeberg, 1984). Milk products such as yoghurt, cream and cheese were also noted to have contained tubercle bacilli several days after being manufactured from un-pasteurized milk (Pritchared, 1988). As the main route of entry is the oral route, TB of bovine origin in man is mainly extrapulmonary resulting in bone and joint tuberculosis as well as infection of the cervical and mesenteric lymphnodes (Dabron and Grange 1993; Edelsten, 1996). In Ethiopia several cases of extra-pulmonary TB have been reported in various hospitals (MOH, 1992); but no attempt has been done to isolate the bacilli and hence, the exact cause of the extra-pulmonary TB cases is not yet identified.

TB can also be transmitted from man to cattle mainly through aerogenous route (Collins and Grange, 1983) and through the oral route as a result of contamination of feed by workers urinating in the cow shade (Huitema, 1969); but cow-to-cow and man-to-man transmission of tuberculosis is mainly via the aerogenic route and the primary foci are found in the lungs.

In Africa, BTB has received scant attention as a public health threat; many countries including Ethiopia, don't routinely undertake national tuberculin testing of cattle. The incidence of human TB due to *M. bovis* runs parallel to that of TB in cattle, and the introduction of modern farming methods without tuberculosis eradication campaigns can increase the level of infection in cattle and hence, in man. In addition to this, people in rural areas live in close contact with cattle which if infected with TB are an important source of infection to man. Exposure is also great where children herd cattle, people buy milk directly from farmers and milk is consumed raw (Kleeberg, 1984). HIV pandemic can also increase the incidence of human tuberculosis due to this bacilli which would inevitably result in increased transmission of *M. bovis* not only to other humans but also back to animals (Dabron and Grange, 1993).

With many of these preconditions suitably existing in Ethiopia, and due to the habit of people to consume raw milk, products from raw milk and raw meat as well as due to the close association of farmers with their cattle to the extent of sharing the same house, infection of man with *M. bovis* is expected to be high. Despite this, no systematic study has been done on the epidemiology and zoonotic importance of the disease. Therefore, knowledge of epidemiology and extent of zoonosis both in human and bovine population by the different species of mycobacteria would be of prime importance in targeting an effective control scheme in the country. Thus the objectives of this study are:

1. To study the epidemiology of bovine tuberculosis in local and exotic animals.
2. To assess the zoonotic transmission of mycobacterium from cattle to man and vice-versa.
3. To estimate the association between human TB cases and tuberculin reactor cattle.
4. To identify risk factors and quantify their degree of association with tuberculosis both in cattle and man.
5. To generate some baseline data that could be useful for the control of bovine TB in cattle and the prevention of its zoonotic transmission.

2. LITERATURE REVIEW

2.1 Taxonomy of Mycobacteria

The genus mycobacterium is classified under the order actinomycetales and family mycobacteriaceae (Bergy, 1957 cited by Burrows, 1973). The generic name, mycobacterium was introduced by Lehman and Neuman in 1896 and it was given due to the mold like pellicular growth of these organisms in liquid media, myco = *fungus* and bacterium = *bacteria* (Bhatia and Ichhpujani, 1994).

The genus mycobacterium includes a number of species, some are pathogenic to man and animals, some are opportunistically pathogenic and others are essentially saprophytic living in water and soil (Freeman, 1979). The classic species of mycobacterium that cause disease in man and animals include: *M. bovis*, *M. tuberculosis*, *M. paratuberculosis*, *M. avium*, *M. leprae*, *M. lepraemarium*. Tuberculosis in mammals is caused by *M. tuberculosis* complex (*M. bovis*, *M. tuberculosis*, *M. microti*, *M. africanum*) and by *M. avium* in birds (Bhatia and Ichhpujani, 1994).

Mycobacterium species other than the *M. tuberculosis* complex that cause TB like diseases in man and animals are commonly called 'atypical mycobacteria' (Buxton and Fraser, 1977). They have been classified into four groups by Runyon in 1959 as, Photochromogenic, Scotochromogenic, Non chromogenic and Rapid growers (Annex 1) based on growth rate and formation of pigments (Carter and Chengappa, 1991). Atypical mycobacteria are not pathogenic to man and animals except in certain situations such as direct inoculation into wounds or introduction into immunocompromised host due to immunosuppressive therapy or due to HIV (Thoen, 1984); however, they are very important during diagnosis as they sensitize man/animals to tuberculin test (Carter, 1986).

2.2 Morphology and Staining

2.2.1 Morphology

Mycobacteria in the animal body are typically slightly curved rods, about 2 μm-4 μm long and 0.2-0.5 μm wide. The rods may be uniform width but more often appear beaded, with irregularly spaced, unstained vacuoles or heavily stained knobs (Bernard *et al.*, 1980).

Morphology in culture may vary between species, cells of *M. tuberculosis* are often arranged in "serpentine" cords, while those of *M. avium* are coccoid (Grange, 1990).

2.2.2 Chemical structure

The mycobacteria have high concentration of lipids, 20-40% dry weight, which is thought to be in part responsible for their resistance to humoral defense, chemical and physical environment such as disinfectants, acid and alkalis (Carter and Chengappa, 1991). The chemical structure of the cell wall is complex which is rich in mycolic acid, peptidoglycan, arabinogalactans and a variety of lipids including mycosides, cord factor and sulpholipids. These structures have a biological activity such as adjuvant activity and are responsible for its pathogenecity (Pritchard, 1988). The peptidoglycan is a net like structure which forms the backbone of the cell wall and it consists N-glycocylmuramic acid and N-acetyl glucosamine linked by amino acids. This net like structure can be cleaved by the enzyme lysozyme to produce muramyl dipeptide (MDP) which has powerful immunological adjuvant activity (Stewart-Tull, 1983, cited by Pritchard, 1988).

2.2.3 Staining

The tubercle bacilli are gram positive; however, due to the large amount of mycolic acid in the cell wall which renders them resistance to the penetration of dyes, they can not be easily stained with aniline dyes by the staining methods used for other bacteria. They are stained by steaming carbol fuchsin for some minutes and if once stained, they resist decolorization with acid, hence, they are known as acid fast bacteria (Freeman, 1979). Acid fastness is the capacity of biological material to form acid stable complexes with certain arylmethane dyes. The intact mycobacterial cell takes carbol fuchsin into its interior and binds the dye to the mycolic acid residues of the peptidoglycolipids of the outer cell wall. The binding is acid stable and the cell surface will be hydrophobic (Barksdale and Kim, 1977). The hydrophobicity may have an important role in resisting dehydration and ensuring the survival of the organisms under adverse conditions (Pritchard, 1988).

2.3 Cultural Characteristics

Mycobacterial species don't grow on simple laboratory media; they need a medium containing serum, potato and egg. The most commonly used media for the cultivation of mycobacterium are modified egg based media such as the Löwenstein-Jensen (LJ) that contain egg, glycerol, asparagine, mineral salt and malachite green. Glycerol suppresses growth of *M. bovis* and the malachite green inhibits growth of bacterial contaminants and provides a green background against which colonies of mycobacterium are more clearly visible. Stonebrink's medium is another commonly used culture medium for the isolation of *M. bovis* where the glycerol in LJ medium is replaced by pyruvate to enhance growth of *M. bovis* (Grange, 1990). They grow slowly with a generation time of 18 hrs (Buxton and Fraser, 1977). *M. bovis* grows more slowly than *M. tuberculosis*, which needs more than 8 weeks to appear on primary culture. Tubercle bacilli are obligate aerobes (Thoen, 1984), but growth of *M. tuberculosis* and *M. bovis* can be enhanced at 5-10% CO_2 (Vestal, 1981). The optimal growth temperature is 37°C except for *M. avium* which needs a temperature of 40-42°C. Growth in liquid medium is diffused, but on a solid medium *M. bovis* is dry, sparse, delicate and non luxuriant. *M. avium* is moist, slimy, glistening, luxuriant, frequently yellow or gray colour and *M. tuberculosis* is

dry, crumbly, luxuriant, colonies are yellowish with roughened surface (Carter and Chengappa, 1991).

2.4 Mycobacterial Species

There are several species of mycobacteria affecting various host species; the approved list of bacterial names includes 41 mycobacterial species (Skerman *et al.*, 1980) but several others have been introduced subsequently. The most important ones are the *M. tuberculosis* complex group which include *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum* and *BCG*. Members of this group are obligate pathogens which can't multiply outside the host.

Identification of pathogenic and saprophytic mycobacterial species has been extensively done based on several biological characteristics of the organisms, but Kubica (1973) has summarized 12 properties of mycobacterium that are used by many mycobacteriologists for identifying members of the genus (Barksdale and Kim, 1977) as shown on Annex 2.

2.5 Pathogenesis

2.5.1 Infection

The methods by which tubercle bacilli gain entrance to the animal body include: the respiratory, alimentary, genital, cutaneous and congenital routes (Gracey, 1986); the first two being the most commonly observed routes of infection resulting in pulmonary and extra-pulmonary forms of the disease, respectively. Calves are usually infected by suckling milk from cows with tuberculous mastitis (Barwinek and Taylor, 1996), humans most commonly acquire TB infection by inhaling aerosolized bacteria as droplet nuclei each containing 1-3 bacteria (Anderson, 1997). The infectious dose is very low; 1-3 viable bacteria are considered sufficient as infectious inoculum (Wiegeshaus *et al.*, 1989, quoted by Anderson, 1997).

Once infection occurs, the organism spreads in the body by two stages: primary complex and post primary dissemination. The primary complex is a lesion at the site of entry and associated lymph nodes which is commonly in the lung when infection is by inhalation, but it can also occur in the tonsils and intestine when infection is via the alimentary tract (Blood and Radostits, 1989).

Depending on the susceptibility of the individual, the course of the disease may range from self limiting, in which the primary infection is contained within 2-10 weeks, to fulminating disease with extensive tissue destruction. Miliary TB represents the most severe course of the disease with haematogenous spreading as a result of the lysis of macrophages that release bacteria into the blood from the primary foci and secondary seeding to various tissues (Andersen, 1997). In immunocompetent animals the body resists the invading bacilli thereby inhibiting the post primary dissemination and localizes them at the site of entry; when resistance is low the disease will spread to other organs either by local contiguity or through natural ducts, the lymphatic system or blood circulation. Entry of the tubercle bacilli to the systemic circulation results in acute miliary tuberculosis with millet seeds like lesions on the lung, spleen, bone marrow, liver, kidney, adrenals, testis, ovary, udder and meninges (Jubb and Kennedy, 1970).

2.5.2 Lesions

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within 8 weeks of bacterial entrance (Blood and Radostits, 1989). This is usually in the lung where mycobacteria are taken by the alveolar macrophages, pass through lining of the bronchioles, enter circulation, carried to lymph nodes and lung parenchyma and start to multiply within the macrophages after a lag period of few days. Cellular responses attempting to control the disease results in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion referred as a tubercle (Thoen and Bloom, 1995).

The macrophages attempt to kill the organism but virulent tubercle bacilli possess the ability to resist or to escape killing due to ingestion of the bacilli into phagosomes or intracytoplasmic vacuoles. The reactive oxygen radicals which have killing effect on other bacteria have little effect on virulent bacilli (Thoen and Bloom, 1995). Pro-inflammatory cytokines (Interleukin-1, IL-1; IL-6; Tumor Necrosis Factor, TNF-α) and chemokines such as macrophage inflammatory protein-1 and interferon inducible protein-10 are secreted from the infected macrophages and lead to the recruitment of monocytes and lymphocytes and development of inflammatory process (Andersen, 1997). This first phase of infection is considered as a symbiotic relationship between the host and the parasite in which the host stays unaffected by the infection and the macrophages have not yet been activated to inhibit microbial growth.

The cell mediated immunity (CMI) emerges 10-14 days after infection and triggers the release of cytokines from T-lymphocytes that activate the bacteriostatic effect of macrophages and accelerate the recruitment of additional blood-borne mononuclear cells into the site. This results in delayed type hypersensitivity reaction, that contributes to cell death and tissue destruction (caseous necrosis) and granuloma formation to localize the lesion. As the process progresses, monocytes mature into epitheloid cells and multi-nucleated giant cells of the langhan's type (Turk JL, 1982 cited by Anderson., 1997) that form the center of the young tubercle which will be surrounded by lymphocytes, plasma cells, monocytes and an outer boundary of fibrous connective tissue (Dundgworth, 1985).

TNF-α is a cytokine of major importance in the process of granuloma formation and neutralization of TNF-α results in lack of granuloma formation thereby leading to uncontrolled bacterial multiplication (Andersen, 1997). Caseous necrosis results from the delayed type hypersensitivity reaction. The gross appearance of the tubercle is usually, firm yellow and on section a yellowish caseous necrotic material or calcified tissue is observed (Neil *et al.*, 1994).

2.5.3 Virulence

Mycobacterium are intra-cellular organisms and their virulence appears to be related to their ability to survive and multiply within the macrophages. The mechanism for such survival is poorly understood and may vary from species to species. For example, *M. tuberculosis* inhibits the fusion of lysosomes with the phagosome thereby inhibiting exposure of tubercle bacilli to the hydrolytic lysosomal enzymes. The putative inhibiting factors produced by the bacilli are polyglutamic acid, ammonia, cyclic AMP, sulpholipids. The sulfur-containing glycolipid (sulpholipids) commonly called as sulfatides play the major role in inhibiting phagolysosome formation. *M. avium* survives within the fused phago-lysosome by a virtue of

their capsule like coating material of mycosides (Lowrie, 1979 and Nishiura, 1977 cited by Grange, 1985). *M. bovis* eludes the bacteriocidal activities of macrophages by escaping from fused phagolysosomes into non fused vacuoles in the cytoplasma (McDonough *et al.*, 1993). In addition to these survival mechanisms, an important aspect of pathogenecity of mycobacteria is their ability to subvert the protective immune response (Grange, 1985).

A characteristic feature of virulent strains of mycobacteria is that they form cords when they grow in a liquid culture media whereas the avirulent strains develop as clumps. Lipids present in the cell wall of virulent tubercle bacilli appear to contribute to the formation of "rope-like" cords in parallel form (Thoen and Boom, 1995); the cord factor is a glycolipid that inhibit leucocytic migration and has also toxic effect on leukocytes (Buxton and Fraser, 1977).

2.6 Immunity to Mycobacterium

Both humoral and cell mediated immune responses can be induced to mycobacterial infection, but the cell mediated immunity is generally accepted to have the most significant role in protection (Neill *et al.*, 1994). Cell mediated immunity (CMI) occurs following T-cell recognition of processed mycobacterial antigens in association with major histocompatibility complex (MHC) products (Kaufmann, 1990). Several cell types are involved but the macrophages have a central role, involved in processing and subsequent presenting of mycobacterial antigens to antigen-specific T-lymphocytes (Newell and Hewinson, 1995). The subsequent interaction of macrophages and lymphocytes with specific mycobacterial antigens stimulate the release of soluble substances (lymphokines) that attract, activate and increase the number of mononuclear cells at the site of infection (Thoen and Bloom, 1995) which either inhibit or destroy the mycobacterium.

The bactericidal or bacteriostatic effect of macrophages is mediated by cytokines (*INF-γ*, TNF) that trigger the production of reactive oxygen radicals and nitrogen intermediates such as superoxide anion and nitric oxide, respectively (Thoen and Bloom, 1995; Andersen, 1997).

T-helper (T_H) lymphocytes may respond to infection by supporting cellular immune response such as delayed-type hypersensitivity in T_H1-type response or by helping B lymphocytes to produce antibodies in T_H2-type response (Hutchings and Wilson, 1995). The T_H1 cells are important in the control of tuberculosis infection due to their ability to produce *INF-γ*, TNF-α and IL-2 which are directly involved in macrophage activation, but the T_H2 cells promote infection with intracellular pathogens by releasing IL-4 and IL-10 which have down-regulator effect on the T_H1-type response (Andersen, 1997). Cytokines released in one type of immune response are inhibitory to lymphocytes of the other type, so individuals tend to respond immunologically to a specific infection by one or the other type.

The T-cell sub-population changes following mycobacterial infection whereby the γδ T-cells increase in the first few hours of infection acting as first line of defense followed by an increase in CD4 T cells characterized by a significant increase in CD4 to CD8 ratio. In the advanced stage of infection CD4 to CD8 ratio decreases due to increased production of CD8 that may have a vital protective role when cells that do not express MHC class II molecules become infected (Pollock *et al.*, 1996; Doherty *et al.*, 1996). As in case of the beneficial cell mediated immunity, delayed type hypersensitivity (DTH) may also inhibit tubercle bacilli (Pritchard, 1988); but in this case macrophages containing replicating organisms are destroyed

releasing the organisms from their protective intracellular environment and exposing it to phagocytosis by a new, activated mononuclear phagocyte (Neill *et al.*, 1994).

Protective immunogens released by actively growing tubercle bacilli give rise to a protective cell mediated rather than the humoral immunity. The genes responsible for the production of these "protective" antigens are being cloned and transferred to suitable mycobacterial vectors by means of the newly developed "shuttle plasmid" technique (Collins, 1994), this can be the first step in preparing more effective vaccine against TB. These immunogens are termed as short-term culture filtrate (ST-CF) as they can be easily filtered from culture media containing actively growing mycobacteria (Andersen, 1997; Ravn *et al.*, 1997). The ST-CF is a mixture of released and secreted proteins which contain several antigens that constitute important targets for the protective immune response and stimulate the release of *IFN-γ* as well as the CD4+ mediated antigen specific cytotoxicity (Ravn *et al.*, 1997).

Various culture filtrate antigens have been purified including the *AG 85* complex(30-32kDa), *MPT51*, *MPT64*(27,26kDa) and the *ESAT-6*(6kDa) molecules (Andersen, 1997); however, the major antigen which is unique to pathogenic mycobacterium is the low mass secreted protein, *ESAT-6*, that dominates during the early stage of infection. It is an important antigen for the *IFN-γ* producing cells activity (Andersen, 1997; Pollock and Andersen, 1997a). This antigen could form the basis for a specific diagnostic test as it can differentiate between cattle infected with pathogenic mycobacteria and cattle sensitized by environmental bacteria (Pollock and Andersen, 1997b) or be a component of sub-unit vaccine (Orme, 1997).

2.7 Epidemiology of *M. bovis* Infections

M. bovis combines one of the widest host ranges of all pathogens with a complex epidemiological pattern which involves interaction of infection among human beings, domestic animals and wild animals (Grange and Collins, 1987); however, only little is done particularly in developing countries on the epidemiology of this organism and the epidemiological requirement for its control.

2.7.1 Risk factors

2.7.1.1 Agent factor

Mycobacteria are resistant to various physical influences and chemical disinfectants, also fairly resistant to acids and alkalis; this is partly due to the presence of lipid in their cell wall. Drying is only effective if they are also exposed to direct sun light but they may survive for several weeks even months in a dark and moist environment; freezing temperature has little if any effect, they are also fairly resistant to acids and alkalis.

M. bovis is an obligate pathogen, but can survive for substantial periods in the environment under favorable conditions (Morris *et al.*, 1994). Duffield and Young (1985) showed that *M. bovis* can survive for four weeks in non sterile dry and moist soils under 80% shed, in darkness; but re-isolation was not possible at 4 weeks from any of the substrates exposed to sun light. They can also retain their viability in putrefying carcasses and in moist soil for 1-4 years (Carter and Chengappa, 1991). Persistence in cadaver depends on the speed of decomposition and degree of environmental protection given to the carcass and this may act as source of infection for scavengers and cattle grazing around the site of decomposition

(Morris *et al.*, 1994). Cosivi, *et al.* (1995) have summarized the survival time of *M. bovis* in different climatic conditions and exposure to sunlight (Table 1) where the highest survival time was in a covered cow dung.

Table 1. Survival time of *M. bovis* under different environmental conditions

Contaminated material	Condition	Survival time
purulent emulsion	Direct sunlight	>10 h but <12 h
	Diffuse sunlight	at least 30 days
cattle dung	Direct sunlight	> 6 h but <37 h
	Diffuse sunlight	15-150 days
	Covered	365-730 days
pasture	temperate climate	7-63 days
water	experimentally contaminated	28 days

Source: Cosivi *et al.*, 1995

2.7.1.2 Host factor

Genetic resistance of hosts to *M. bovis* infection has never been conclusively demonstrated (Morris *et al.*, 1994), but Radostits *et al.* (1994) have stated that Zebu (Brahman) type of cattle are thought to be much more resistant to tuberculosis than the European cattle and effects on these cattle are much less severe. On an abattoir survey in India out of the 1,268 animals examined which include pure exotic, cross breed and local cattle, TB lesions were found in 25.97% of the pure exotic breeds, 9.7% of the cross breeds and in only 7.1% of the Zebu animals (O'Reilly and Dabron, 1995); in contrary to this, Seifert (1996) reported that cattle of the traditional pastoralists in Madagascar were affected up to 60%.

2.7.1.3 Environment and management factors

Housing predisposes to the disease, the closer the animals are packed together the greater is the chance that the disease will be transmitted, for example the disease incidence is higher in intensive dairy farms than in beef ranches (Morris *et al.*, 1994). In beef cattle the degree of infection is usually low because of the open range condition under which they are kept. Feeding and housing conditions are important risk factors for BTB. O'Reilly and Costello (1988) have demonstrated that cattle kept under good nutritional and husbandry conditions may not, irrespective of the nature and extent of the lung lesions, excrete *M. bovis* in sufficient number to infect contact cattle in the open grazing for a period of 4 to 9 months. Farm management practices such as feeding and nutrition, standards of fencing at farm boundaries, slurry disposal methods, cattle trading practices, presence of wild life are also significant risk factors for the occurrence and spread of TB in a given farm (Griffin *et al.*, 1992).

The ubiquitous distribution of *M. bovis* in farmed and to a large extent in the wild animal population, the trend towards intensification of animal production to meet the increase in demand for milk and meat, gathering of animals at watering points, markets and in corrals over night and lack of control measures in most African countries are some factors which are likely to facilitate *M. bovis* infection in the African animal population (WHO, 1993).

2.7.2 Host species affected

M. bovis has an exceptionally wide host range, but under natural conditions it infects mainly cattle. Abattoir surveys in North-eastern Nigeria indicated that TB prevalence was high in cattle, very low in sheep and goats and no report on camel (Alaku and Moruppa, 1993). Buxton and Fraser (1977) have indicated that human, swine and occasionally horses can be infected. Infection in other domestic animals such as deer (Clifton-Hadely and Wilesmith, 1991), dogs (Seifert, 1996), and cats (Isaac *et al.*, 1983) is also frequently reported.

Tuberculosis in sheep and goats is uncommon but goats are quite susceptible if they are maintained in close association with infected herds of cattle in which incidence may reach as high as 28 % (Blood and Radostits, 1989). Sheep have always been considered to be resistant, but a report from New Zealand has indicated that incidence can be as high as 5% due to high prevalence of the disease in local cattle and possums (Radostits *et al.*, 1994). Disease levels in pigs also reflects those in local cattle and an increased level of the diseases in cattle can result in high prevalences up to 20% in local pigs (Blood and Radostits, 1989).

Self-maintaining infection in wildlife has also been recognized in various countries, notably the European badger in UK and Ireland (Nolan and Wilesmith, 1994), the Australian brush-tailed possum in New-Zealand (Tweddle and Livingstone, 1994) and various species of ungulates such as feral buffalo, feral bison and feral pigs (Griffin and Buchan, 1994).

2.7.3 Transmission

2.7.3.1 Source of infection

Tuberculous cattle themselves are a major source of infection for cattle and other domestic animals (Neil *et al.*, 1989); all tuberculin positive cattle should be considered as potential source of infection which excrete the bacilli either through the expired air or through the swallowed sputum which leads to excretion of the tubercle bacilli in the feces. Cattle manure from TB restricted herds is therefore considered as an important source of infection (Hahesy *et al.*, 1992). The excretion is often in waves and occurs long before clinical symptoms are observed. Experimentally infected cattle may shade *M. bovis* in respiratory secretions at early stages of infection before any primary lung foci can be detected, but excretion in cases of naturally acquired BTB seem to begin around 87 days after infection (Neil *et al.*, 1989).

Wild life are also important sources of infection to cattle and may act as maintenance or spill over hosts. Badgers and possums maintain the infection by pseudo-vertical transmission from mother to young and horizontal transmission linked to breeding activity (Morris *et al.*, 1994).

2.7.3.2 Route of infection

The possible routes of infection in cattle include respiratory, alimentary, congenital, cutaneous venereal and the teat canal (Seifert, 1996); but the major route of infection, 80-90%, is the aerogenous route (Collins and Grange, 1983; Pritchared, 1988). Even though a large number of the organisms are excreted in feces, infection from pasture is very low due to the short survival time of the infective dose on the fomites and because animals are not commonly exposed to a dose high enough to be infective by the alimentary route (Morris *et al.*, 1994). Francis (1947) cited by Morris *et al.* (1994) has reported that incidence remained low in heifers which were grazing in the same pasture with heavily infected dams until they enter the

cow shed. Another experiment by Chaussi (1913) cited by Morris *et al.* (1994) showed that less than 5 bacilli would produce lesions in the lung of sheep if introduced as a fine spray, but 13 million bacilli would not always infect sheep by oral route.

Venereal transmission is possible if either the male or the female sexual organs are tuberculous (Neill *et al.*, 1994); Thoen *et al.* (1977) isolated *M. bovis* from the prepuce of herd bulls. Congenital transmission may occur if a calf is born from a cow with metritis originating from peritonitis, external genitalia or most commonly from hematogenous spread of the bacilli (Morris *et al.*, 1994; Seifert, 1996); only 1% of calves were found to be infected congenitally and the primary focus appears in the liver of the new born (Seifert, 1996). Udder infection due to hematogenous spread occurs only in 1-2% of the tuberculous cows and results in tuberculous mastitis (Morris *et al.*, 1994).
Iatrogenic infection of the udder through the teat canal due to infusion with contaminated materials is also common, and calves can be infected orally by drinking milk from the tuberculous udder (Neill *et al.*, 1994).

Transmission from wild animals to cattle is mainly through inhalation and principally occurs when there are interactions, due to atypical behavioral changes, between the excreting wild life host and domestic animals; but the badger is exceptional in excreting the bacilli in a large amount in the urine hence contaminating the pasture. Therefore transmission from badger to cattle is more by the oral route than by the respiratory route (Nolan and Wilesmith, 1994). Transmission from man to animals is probably mainly airborne, but spread via urine which contaminates the cow shade is also mentioned to be important by Huitema (1969) in Holland.

2.7.4 Distribution

Tuberculosis occurs in every country of the world. Out of the 1 billion cattle population all over the world one third are in areas where BTB is under control, another third are in areas where the disease is widespread but the incidence unknown and the remaining third are in regions where the prevalence is high (Steele, 1995).

In North America the status of bovine TB was summarized by Essey and Coller (1994) and stated that Canada is anticipating total eradication of the disease in the next few years while USA will face problems due to the imported steers and presence of the disease in farmed deer. BTB is still endemic in Latin America; Argentina and Brazil with huge livestock population have an estimated prevalence of 1%, but Cuba with 5 million cattle has eradicated BTB in 1984 (DeKantor and Ritacco, 1994).

Most countries in Europe have eradicated bovine tuberculosis by the test and slaughter policy, but reports show high herd prevalences of up to 10.8% in Spain, 8.8% in Ireland and 3.7% in Italy (Caffrey, 1994). The high prevalence in Ireland is due to the presence of the disease in reservoirs hosts other than cattle mainly the badgers and the deer. Australia has markedly reduced prevalence of BTB from 3.04% in 1981 to 0.3% in 1990 and will declare free of BTB in the near future, but New Zealand which follows similar eradication program as Australia, will stay with the problem due to the presence of significant wildlife (brush tail possum) that hindered the eradication of BTB (Tweddle and Livingstone, 1994).

In developing countries, especially in Africa where *M. bovis* infection appears to be high in a number of animal species, there is a substantial lack of knowledge of the distribution, epidemiology and zoonotic importance of BTB (WHO, 1992). Out of the 56 African countries

44 officially recognize the presence of BTB in their animal population, 10 do not report or do not have available information on BTB. Kenya has never reported and Namibia recorded its last cases in bovine and swine population in 1985 and 1965, respectively (WHO, 1993).

2.8 Diagnosis

Data on bovine tuberculosis from developing countries is always underestimated mainly due to lack of diagnostic facilities (WHO, 1993). There are several diagnostic techniques used to diagnose BTB both on live and dead animals which are described here below.

2.8.1 Clinical examination

Because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection, tuberculosis is difficult to diagnose on clinical examination (Radostits *et al.*, 1994); but it is usually followed by weight loss, decrease in milk production, weakness, enlargement of superficial lymph nodes and coughing.

2.8.2 The allergic skin test

The standard test recommended by Office International des Epizooties (OIE) to diagnose BTB in cattle is the allergic skin test where purified protein derivative (PPD) is injected intradermally to provoke an allergic reaction with localized inflammation and swelling of the skin if the animal is infected with homologous mycobacterium. The swelling is measured using caliper after three days. The test relies on the response of the animal to the injection of tuberculin which is classically described as delayed type hypersensitivity (DTH) response.

Tuberculin was used for the first time by Robert Koch in 1891 in his effort to develop a treatment to TB, since then it has been used to diagnose BTB in the control and eradication programs in many countries (Wood and Rothel, 1994). It is produced from most of the slow growing mycobacteria that grow in liquid media as a culture filtrate which is further processed by either heat concentration (Old Tuberculin, OT) or chemical fractionation (Purified-Protein-Derivative, PPD). The standardized tuberculin in use today is the purified protein derivative (PPD) in which bovine PPD is produced from *M. bovis*, AN5 or vallee strains and the avian PPD from *M. avium*, D4ER or TB 56 strains (Monaghan *et al.*, 1994). These days BCG can also be used for tuberculin test to avoid the risk of infection in the laboratory, but Nader *et al.* (1988) described that the potency is very low to use it for routine diagnosis.

There are several types of allergic skin tests: for screening purposes the single intradermal test (SIDT) has been widely used at a dose of 0.1 or 0.2 ml of bovine or human PPD in the cervical or caudal area (De Kantor *et al.*, 1987). In Europe, according to the Council Directive 80/219/EEC, 0.1 ml bovine PPD is inoculated intradermally at the middle of the neck after the hair is shaved and the area disinfected; but in America, Australia and New Zealand the caudal fold is widely used as it is easy and fast to apply (Barwinek and Taylor, 1996).
Stormont test is used in problem herds where inconclusive reactions are obtained. In this method 0.1 ml PPD (2 mg/ml concentration) is injected intradermally on the cervical region and the dose repeated on the same site 7 days later. An increase of 5 mm or more in thickness of the skin after 24 hours is a positive reaction (Lessilie, 1976 cited by Barwinek and Taylor, 1996). In order to distinguish animals infected with *M. bovis* from those sensitized by other atypical mycobacteria, particularly *M. avium*, the intradermal comparative tuberculin test

(IDCT) has been developed whereby 0.1 ml of bovine and avian PPD are inoculated simultaneously on the same side of the neck 12-15 cm apart, and cattle infected with bovine bacilli will show greater allergic reaction to the homologous PPD (Lessilie, 1976 cited by Barwinek and Taylor, 1996).

2.8.2.1 Problems associated with tuberculin test

Sensitivity and specificity of the test have been determined in various studies that have reported different values. Data summarized by Monoghan *et al.* (1994) suggested that the sensitivity varies from 68-95% while specificity is as high as 96-99%; the sensitivity and specificity of the IDCT in deer was found to be 65% and 98.5% respectively (WHO, 1994). There are certain conditions which can affect the sensitivity and specificity of tuberculin test and result in false positive and false negative test results.

Anergy is a condition in which infected animals give false negative results. The reason for this is poorly understood (Monaghan *et al.*, 1994), but Blood and Radostits (1989) stated that recently infected animals until 6 weeks after infection and advanced cases of tuberculosis show false negative result. Lepper *et al.* (1977b) have also indicated that animals with generalized severe tuberculosis are *anergic*.

Animals also fail to respond to tuberculin due to desensitization resulting from injection with PPD during the preceding 60 days (Radunz and Lepper, 1985; Radostits *et al.*, 1994) and also due to immunosuppression during the early postpartum period (Kehril *et al.*, 1989). About 30% of cattle in the periparturient period give false negative result returning to a positive state 4-6 weeks later. The reason for this may be due to depletion of T-lymphocytes from the skin into the circulation and then into colostrum (Blood and Radostits, 1989).

Apart from the failure to respond to tuberculin, there are few animals which are positive to tuberculin test, but fail to show evidences of infection during postmortem which are known as non visible lesion (NVL) reactors (Monaghan *et al.*, 1994). Such reaction occurs only in small proportion of cattle, hence, specificity of tuberculin test is high which may reach up to 96-98.8% (Wood *et al.*, 1991). Calves born from infected cows may have colostral immunity for about 4 weeks and react false positive in tuberculin test (Barwinek and Taylor, 1996). To avoid this false positive result the allergic skin test is usually conducted on animals older than 3 months of age.

2.8.3 Postmortem inspection

Tentative diagnosis of BTB can be made following the macroscopic detection of TB lesions during necropsy. In cattle 86% of the cases with single lesions can be identified by examination of 3 pairs of lymph nodes (mediastinal, medial retropharyngeal and bronchial) together with the lung. Examination of 3 additional pairs (parotid, caudal cervical and superficial inguinal) as well as the mesenteric lymph nodes enabled the detection of 95% of the cases (Corner *et al.*, 1990). During the BTB eradication program in the Federal Republic of Germany (FRG), tuberculin test results and postmortem findings were identical in 80-90% of the cases in highly infected herds and in only up to 60% in less infected herds (Rolle, 1984 cited by Barwinek and Taylor, 1996). On the other hand various workers have reported that sensitivity of meat inspection is very low; DeKantor *et al.* (1987) have reported that meat inspection in Argentina was able to detect only 33% of infected carcasses (Sensitivity=33%),

in Turkey it was reported that for one TB case found at meat inspection 10 remain undetected (Barwinek and Taylor, 1996).

2.8.4 Bacteriology

2.8.4.1 Differential staining

Final confirmatory diagnosis of tuberculosis depends on isolation and identification of the bacteria, but preliminary examination of stained smears from lesions, sputum, milk, urine, pleural and peritoneal fluids, uterine discharge and feces is very important (Vestal, 1981). To be detected microscopically, there must be between 5,000-10,000 bacilli in 1 ml of sputum (Grange, 1990). In the smear the organisms appear red in Ziehl-Neelsen staining and yellow with fluorescing dyes (Carter and Chengappa, 1991).

2.8.4.2 Culture

The definitive diagnosis of bovine TB depends on the isolation and identification of the mycobacterium (Pritchard, 1988). The success of *M. bovis* isolation depends on the type of media used, procedures applied for decontamination and concentration of the specimen as well as the incubation conditions (Corner, 1994).

The most commonly used media are either the egg based (Löwenstein-Jensen and Stone brink's media) or agar based medium enriched with blood and/or serum such as the modified middle brook 7H11 (Peterson *et al.*, 1989) and the tuberculosis blood agar medium called B83 (Cousins *et al.*, 1989). Corner (1994) indicated that growth on the agar media is much faster than on the egg based media with mean time to the first appearance of colonies being 27 days and 28 days on B83 and 7H11 respectively compared to 36 days on Stonebrink's medium. However, the agar medium is highly liable to contamination even after decontamination of the specimen. In a study with 362 clinical specimens, 3.9% of the specimens on the agar medium were contaminated compared to only 0.3% on the egg based medium (Corner, 1994).

Digestion-decontamination is very important in order to release the organism from body fluids and cells as well as to isolate *M. bovis* from contaminated specimen. This is based on the relative resistance of *M. bovis* to mild acids, alkalis and to certain disinfectants (Thoen, 1984). The ideal decontaminant should be toxic to other bacteria but less toxic to mycobacterium. Corner (1994) has examined the toxicity of four decontaminants for *M. bovis*: hexadecylpyridinium chloride (HPC) at a concentration of 0.075% and 0.75% w/v, oxalic acid 5% w/v, benzalkonium chloride (Zepharin) 0.25% w/v and sodium hydroxide 2% w/v. He found that HPC 0.075% was the least toxic and 2% NaOH the highest, but the commonly used chemicals for digestion-decontamination purpose in most laboratories are the N-Acetyl-L-cysteine (NALC) and 2-4% NaOH (Vestal, 1981).

2.8.4. 3 Differentiation of mycobacterial species

Differentiation of the pathogenic tubercle bacilli mainly *M. bovis* and *M. tuberculosis* is done based on the stimulation of growth on a medium containing pyruvic acid or glycerol. Glycerol inhibits the growth of some *M. bovis* strains; a Swedish group found that the use of pyruvate enriched, glycerol-free media is critical in isolating *M. bovis* (WHO, 1994). In addition to this biochemical tests such as niacin production, nitrate reduction, urease and pyrazinamidase activity and drug sensitivity tests are also commonly used in the identification of

mycobacteria (Vestal, 1981). Appart from these, laboratory animals (Carter, 1986), immunoassays (Wood *et al.*, 1992) and molecular techniques (genetic finger printing and PCR) are also being used to differentiate the two species of mycobacteria and in the epidemiological studies of tuberculosis.

Table 2. Criteria used for differentiation of *M. tuberculosis* complex

Species	Variants	TCH	NO_3	O_2	PZA	Niacin
M. tuberculosis	Classical human	R	+	A	S	+
	Asian human	S	+	A	S	+
M. africanum	Type I	S	-	M	S	variable
	Type II	S	+	M	S	variable
M. bovis	Classical bovine	S	-	M	R	-
	BCG	S	-	A	R	-
M. microti						

Source, Collins and Grange, 1987

S = Susceptible, R = Resistant
A = Aerobic, M = Micro-aerophilic
TCH = Susceptibility toThiophen-2-carboxylic acid hydrazide
NO_3 = Nitratase activity
O_2 = Oxygen preference
PZA = Susceptibility to Pyrazinamide
Niacin = Niacin production test

2.8.5 Animal inoculation

Laboratory animals such as rabbits, guinea pigs and chicken are used for diagnosis and identification of mycobacteria (Thoen, 1984). Rabbits die within 4-5 weeks from generalized infection with the bovine type if given intravenous but survive from human type, guinea pigs are highly susceptible to both types, chicken are not affected by both human and bovine type but die from the avian type (Buxton and Fraser, 1977).

2.8.6 In vitro cellular assay

2.8.6.1 Lymphocyte proliferation assay

The lymphocyte stimulation assay has been found to be a popular *in vitro* correlate of delayed type hypersensitivity response and was extensively used to detect cellular reactivity to tuberculin antigen (Wood *et al.*, 1990), but they are not used for routine diagnosis as they are time consuming which needs incubation for 3-5 days in a complex tissue culture for the isolation of T lymphocytes (WHO, 1993). They also need the use of radioactive nucleosides (thymidine) to detect the level of lymphocyte proliferation (Wood *et al.*, 1992).

2.8.6.2 Gamma interferon (γ-IFN) assay

The *γ-IFN* assay is recently developed technique which is simple and rapid, 24 hours, whole blood *in vitro* cellular assay that measures the release of lymphokine (*γ-IFN*) from the sensitized T-cells in response to specific antigen. A sandwich ELISA which utilizes two monoclonal antibodies to bovine *γ-IFN* is used for the detection of the *γ-IFN* released by

sensitized T-lymphocytes (Rothel *et al.*, 1990). The sandwich ELISA used in *γ-IFN* test is found to be with higher sensitivity and specificity than an indirect ELISA which indicates high humoral antibody associated with advanced cases of mycobacterial infection (Ritacco *et al.*, 1991). As it is carried with whole blood the *γ-IFN* test avoids the time consuming task of isolating T-lymphocytes (Wood *et al.*, 1990). It is also much better than the IDCT except for the cost of the reagents which is expensive, $6 per animal as compared to $0.12 for IDCT (Barwinek and Taylor, 1996).

2.8.7 Serology

Several serological tests have been used to diagnose bovine tuberculosis: Yugi and Nozaki (1972) compared the passive haemagglutination test, kaolin agglutination and complement fixation test and found the kaolin agglutination test to be the most reliable in detecting tuberculous cattle. Another comparison made by Vardman and Larsen (1962) has shown that high percentages of positive animals were found using CFT (77.6%). Lepper *et al.* (1977a) used indirect immunofluorescent antibody test (IFA) to measure the antibody level produced by artificial inoculation of *M. bovis* and only 38 out of 61 serum samples were positive.

ELISA is the most commonly used serological test to diagnose BTB. Ayanwale (1987) has tested 40 local and exotic breed animals in Nigeria using ELISA and found a sensitivity and specificity of 98% and 65.5% respectively. Ademe (1991) used ELISA to discriminate *M. bovis* from other mycobacteria obtained from artificially infected mice. Various workers used different antigens mainly PPD and phosphatide (Hanna *et al.*, 1989; 1992) for serological tests. Neill *et al.*(1994) have also used PPD and phosphatid antigens; they found a sensitivity of 4.8% and 27% and specificity of 97% and 88.5%, respectively. MPB70 which is the *M. bovis* specific antigen is also used as an antigen for ELISA (Wood *et al.*, 1992).

The role of serological tests in routine diagnosis of BTB is limited because of the low sensitivity they have, due to the large number of protein antigens present in mycobacterium and due to the variable response to mycobacterial infections (Wood and Rothel, 1994). They are only used as a supplement to pick out some of the anergic cattle which failed to respond to the single intradermal test (Radostits *et al.*, 1994) or to complement the in vitro cellular assay.

Wood *et al.* (1992) have compared the sensitivity and specificity of ELISA, IFN-γ, SID, and combination of IFN-γ and SIDT; the best results (sensitivity of 90.9% and specificity of 95.8%) were obtained from the combination test. Another work in New Zealand has found a higher sensitivity (95%) from the combined test of SIDT and ELISA compared to individual sensitivity of 82% and 85% respectively.

A recently developed technique by the British Company, Biotech Diagnostic, is found to be very fast, giving result in 10 hours; cheap, costing 50 cents per test and highly sensitive, detecting 100 bacilli per ml of sample.

The invention is called BiophaB in which the patients sputum is mixed with BiophB reagent that contains bacteriophage which specifically attacks the tubercle bacilli and replicate inside if the bacillus is present in the sample. The specimen is treated with a special reagent that kills all virus outside the bacteria and incubated to allow replication of the virus inside the bacteria. If there are no bacteria in the sputum, all virus will die. Presence of virus in the culture can be easily detected by the patches they form on the culture media. The number of patches formed

can give as an approximate number of mycobacteria in the sputum. (EVA news letter, 1997 extracted from New Scientist, Aug. 1996).

The method can also help in identifying drug resistant strains of mycobacteria in 2-3 days. To this effect, specimens can be treated with the antibiotic of interest and then tested for the presence of viable bacilli using the BiophB method. In addition to this, this technique has also an advantage of killing the potentially dangerous bacilli during the process.

2.9 Zoonotic Importance of *M. bovis*

2.9.1 Occurrence

One of the main reasons for the interest in tuberculosis of cattle is the susceptibility of man to disease due to *M. bovis* (Kleeberg, 1984). Schwabe (1984) has stated that *M. bovis* is causing almost all the non-pulmonary as well as a varying proportion of the pulmonary cases in human tuberculosis; but the statement given by Robert Koch in 1901 that "The human subject is immune against infection by bovine bacilli or is slightly susceptible that I do not consider it necessary to take any measures to counteract the risk of infection" has influenced a lot of medical personnel to neglect the importance of *M. bovis* in human TB cases. However, McFadyean forwarded strong evidences that milk from tuberculous cattle posed a hazard to human health and concluded that "we ought not to concede to the milkman the right to sell us tubercule bacilli!"(Grange, 1995).

In Europe in the 1930s and 1940s bovine TB was considered to be a significant zoonosis. Scrofula (tuberculous cervical lymphadenitis) was noted to be much common in infants who were fed cows' milk than in those who were breast fed (Grange and Yats, 1994). *M. bovis* infection in cattle is still endemic in developing countries and some epidemiological conditions for the spread of *M. bovis* infection between animals and humans are very similar in Africa today to those in Europe in the 1930s (Cosivi *et al.*, 1995).

The proportion in which *M. bovis* contributes to total tuberculosis cases in humans depends on the prevalence of the disease in cattle, socio-economic conditions, consumer habit, practiced food hygiene, medical prophylaxis, etc. With high BTB prevalence in cattle and insufficient milk hygiene, the proportion is usually estimated to be about 10% (Denes, 1981). The effect of BTB on human health is also increased due to the impact of HIV/AIDS pandemic; out of the 12 million people infected with HIV, 4 million are also concurrently infected with TB and nearly 80% of them live in sub-Saharan Africa (Dabron and Grange, 1993). In Monze district of Zambia 70 % of the TB patients were also HIV positive (Cook *et al.*, 1996).

2.9.2 Transmission

Both pulmonary and mammary TB are source of infection to man (Kleeberg, 1984). BTB is mainly pulmonary in cattle and only 1% (Grange and Yates, 1994) up to 4% (Rolle, 1984 quoted by Barwinek and Taylor, 1996) of the tuberculous cattle excrete the organism in their milk; but enough number of bacilli to contaminate a milk from 100 cows are excreted from a single cow (Kleeberg, 1984). Transmission of tuberculosis from cattle to man is mainly through ingestion of raw milk from tuberculous cattle. *M. bovis* was isolated from raw milk in Ethiopia (Kenfe and Eshetu, 1987; Teshome, 1993). Milk products such as yoghurt and cream prepared from unpasteurized milk are also found to contain tubercle bacilli 14 days after

contamination and butter up to 100 days (Kleeberg, 198-). Aerogenous transmission of *M. bovis* between cattle and man is also possible (Grange and Yates, 1994) which is more common in rural areas than in urban areas where both live in close association (WHO, 1994).

In addition to the isolation of *M. bovis* from mil of tuberculous cattle, *M. tuberculosis* has been also isolated from milk of cattle indicating the possibility of transmission of the disease from man to cattle. Out of the 113 isolates of mycobacteria by Boulahbal *et al.* (1978) in Algeria, 7 (6.2%) were *M. tuberculosis* isolates. Although transmission of *M. tuberculosis* from man to cattle and back to man is possible, no overt disease is seen in cattle (Grange and Yates, 1994); but the important aspect is the ability of farm workers with TB due to *M. bovis* to infect their herds (Collin and Grange, 1987). Apart from cattle monkeys are the only species likely to infect man (Kleeberg, 1984).

Berhrend (1893) as quoted by Pritchard (1988) acknowledged the risk of tuberculous meat for man and recommended effective meat inspection, but due to the effective meat inspection methods and thorough cooking of meat before consumption, it is unlikely meat to be a major source of *M. bovis* infection. On the other hand the tradition of consuming raw meat in countries like Ethiopia may draw attention that meat can be source of infection. Another route of infection, though not common, is through skin cuts. Butchers, pathologists and slaughter house workers are at high risk to skin infection, hence, the lesion is termed as "Butchers Wart" (Grange and Collins, 1987).

2.9.3 Organs Affected in man

In the early part of this century TB in man due to *M. bovis* was common and the most frequently involved organs were the lymphnodes, usually in the cervical region, the bones, joints, the abdomen and skin (scrofulodermia or lupus vulgaris). In Europe in the 1930s and 1940s bovine TB was responsible for more than 50% of the cervical lymphadenitis cases in children (Cosivi *et al.*, 1995); but the urogenital and the pulmonary type were very rare at that time. In contrast to earlier days, the pulmonary and urogenital tuberculosis are now the most usual sites of *M. bovis* infections in man (Grange and Yates, 1994), maybe due to reactivation forms which frequently involve the lung and other organs.

2.10 Control of Bovine Tuberculosis

The test and slaughter policy is likely to remain the mainstay of the bovine tuberculosis control program. However, it can not be applied to all cattle in all areas of the developing countries as they can't refund their farmers and can't control reinfection from wild life (WHO, 1994). Pritchard (1988) has described various options of the test and slaughter scheme such as Bang's and Ostertage method and the method is well defined by Radostits *et al.* (1994) in which he recommended to test all animals over 3 months of age using the intradermal tuberculin test and to dispose positive animals according to local legislation. Re-test of the herd should be done every 3 months until negative test is obtained, then one final test is required after 6 months to declare the herd as negative. When the overall prevalence rate is less than 5% compulsory test and slaughter is the only satisfactory method for eradication (Radostits *et al.*, 1994); but when incidence of BTB is high like in the developing countries test and slaughter program will not be accepted both socially and economically.

Alternatively vaccination may be used as a temporary measure to reduce the prevalence in preparation for eradication program (Radostits *et al.*, 1994). Newell and Hewinson (1995) indicated the importance of vaccination for the control of TB in Badgers. BCG is the only safe vaccine which can be given to calves immediately after birth, but the problems associated with the vaccine are the interference with tuberculin testing, so hindrance for control and the creation of a carrier state that can excrete the bacteria and act as source of infection to man and to domestic animals (Pritchard, 1988). Due to this the WHO/FAO had at times recommended to stop the use of BCG vaccine, however the problem of high prevalence of BTB in developing countries has not been amendable by test and slaughter methods and so attention has turned again to BCG (Pritchard, 1988). Advances in molecular genetics of mycobacterium and in the understanding of protective responses may lead to the development of an effective vaccine in the future.

The use of chemotherapy in animals is limited due to the long period drug administration required which makes it expensive, due to the multidrug resistance developed to *M. bovis* as well as the side effect of drug residues in milk and meat for man (WHO, 1994). A study in the USSR, where treatment of BTB was permitted, have shown that 4 out of the 16 *M. bovis* strains were multi-drug resistant (Kassich *et al.*, 1988 cited by Barwinek and Taylor, 1996).

More than 50% of African cattle are found in countries without any BTB control measures and only 10% are found in countries where the BTB is notifiable (WHO, 1993). 30 out of the 56 African countries apply some measures of BTB control. In 16 of these countries BTB is a notifiable disease, however only 6 of the countries where BTB is notifiable carry out the test and slaughter policy.

2.11 Economic Importance of Bovine Tuberculosis

The costs associated with BTB fall into two main categories: costs associated with losses in animal production and costs associated with human TB cases contracted from cattle.

2.11.1 Costs associated with losses in animal production

Besides to its zoonotic importance BTB has an adverse effect on the livestock production. In the FRG in 1952 with cattle population of 11.5 million and BTB prevalence of 38.5% loss of production was estimated to be DM 275 million which was around DM 62 per infected animal (Meyn, 1953 cited by Barwinek and Taylor, 1996). In Madagascar losses in weight gain of the Zebu cattle was found to be 12% at intensive fattening of 100 days and 28.7% in pasture fattening of 200 days (Barwinek and Taylor, 1996 quoted from Blancou *et al.*, 1974). In Hungary the estimated weight loss was 15 kg in cows and 25 kg in beef cattle (Denes, 1981).

Losses in meat inspection due to organ or whole carcass condemnation is considerably high particularly in countries with high prevalence of BTB and with strict laws of meat hygiene. In Ethiopia the estimated cost of organs and carcasses condemned during meat inspection of 1.2 million cattle slaughtered in six export abattoirs was 600,842 birr equivalent to US $300,000 at that time (Gezahegn, 1991). Loss due to decreased milk production is also high when infected cattle develop tuberculous mastitis; 1-2% of infected cows may develop tuberculous mastitis (Morris *et al.*, 1994). In Hungary Denes (1981) has reported that infected cows produce 10-12% less milk than healthy ones, similarly in Poland the milk production of infected cows was calculated to be 30% bellow the normal production (Lis, 1980).

Infertility is another problem in tuberculous cows, 5-10% of the infected cows may develop tuberculous metritis (Morris, *et al.*, 1994) out of which 5% may become infertile (Denes, 1981). It is also reported that 1% of the calves from tuberculous dams infected congenitally leading to death or retarded growth (Seifert, 1996). Such cows may be culled before they finish their production life that incurs additional loss. In Hungary the production life of infected cows was reduced on the average, by 1.5 lactations (Denes, 1981).

2.11.2 Costs associated with human tuberculosis due to *M. bovis*

This includes cost of treatment, hospitalization, loss of working days and payment for sick leave. Even though difficult to quantify, it was estimated to be 6.2 billion Lira in Italy between 1965 and 1978 (Caporale, *et al.*, 1980 cited by Barwinek and Taylor, 1996).

2.12 Status of Human and Bovine Tuberculosis in Ethiopia

2.12.1 Bovine tuberculosis

Bovine tuberculosis is endemic in Ethiopia; FAO (1967) as cited by Solomon (1975) indicated a prevalence of 0.2-3% based on the intradermal tuberculin test and 4% based on abattoir survey. Solomon (1975) has collated a two year (1972-73) data from 8 municipality abattoirs and found an overall prevalence of 0.54% and a prevalence of 0.05% (200 out of 418100 slaughtered cattle) based on whole carcass condemnation. Based on lung condemnation he found highest prevalence in Makale 3.1% and the lowest in Gondar 0.02%. Gezahegen has also compiled a five year (1985-90) abattoir data from six export abattoirs. Out of the 1,240,572 cattle slaughtered 978 (0.08%) were totally condemned; based on lung condemnation prevalence was 1.13% (2,852 out of 252,242 animals slaughtered). The latest report was by Teshome (1993) that indicated a prevalence of 0.4% based on the 250,000 cattle slaughtered at Addis Ababa abattoir.

Table 3. Prevalence of BTB based on carcass and organ condemnation in Ethiopia

place	year	animals examined	No./ % of organs affected		
			lung	others organs	whole carcass
Asmara	1972-73	43753	328(0.75%)		
	1985-90	124160	782(.63%)	----------	------
Makale	1972-73	65544	1549(2.36%)		
	1985-90	----------	----------	----------	-------
Gondar	1972-73	12525	3(0.024%)		
	1985-90	20496	21(0.1%)	66(0.32%)	7(0.03%)
Dridawa	1972-73	28926	194(0.67%)		
	1985-90	35731	241(0.67%)	163(0.46%)	9(0.025%)
Kombolcha	1972-73	47077	211(0.45%)		
	1985-90	42570	352(0.83%)	947(2.2%)	63(0.15%)
Wondo	1972-73	58239	255(0.44%)		
	1985-90	88941	337(0.38%)	1105(1.24%)	102(0.11%)
Addis Ababa	1972-73	94032	217(0.23%)		
	1985-90	854170	3417(0.4%)	------	51(0.006%)
Debre Zeit	1972-73	3934	7(0.18%)		
	1985-90	64504	1907(2.96%)	631(0.98%)	746(1.16%)

Adapted from Solomon, 1975; Gezahegn, 1991 and Teshome, 1993

Survey on selected state dairy farms was done in 1984 by Shola Veterinary Laboratory where 4,838 animals were tuberculin tested and showed an average prevalence of 16.8% with the highest prevalence, 77%, in Mojo state farm followed by Kuriftu (50.6%). Another study in 1985 was conducted on 3,352 animals of which 817 (24.4%) reacted to bovine tuberculin with highest reactors being in Mojo followed by Kuriftu and a prevalence of 35.5% was found at Debre Zeit state farm (MOA, 1987). In 1991/92 a survey was made whereby 2,000 animals of various breeds were tuberculin tested and the highest prevalence (71.6%) was found at Debre Zeit state dairy farm (Teshome, 1993). Gobena has also conducted SIDT in 1996 on 486 dairy animals in five different farms and obtained an average prevalence of 50% with the highest prevalence (87%) being in Debre Zeit state dairy farm.

In Ethiopia, relevant data on mycobacterial zoonosis is scarce: for the first time, Kinfe and Eshetu (1987) reported that out of the 5 mycobacterial isolates from 100 milk samples taken from tuberculin positive cows, 2 were identified as *M. bovis* indicating the potential hazard of raw milk to man particularly to children. In a similar study *M. bovis* was isolated from 7 of 486 raw milk samples from cattle and 1 of 247 sputum samples (Teshome, 1993). However, the significance of such a finding in terms of public health has not yet been studied in the country.

2.12.2 Human tuberculosis

In Ethiopia tuberculosis has long been recognized as a major cause of morbidity and mortality; however, the extent of the problem is not yet estimated accurately due to lack of reliable data. According to Ministry of Health TB ranked 12th with 2.8% as a cause of outpatient morbidity, 3rd with 6.2% as a cause for hospitalization and 1st with 14.3% as a cause of death in hospitalized patients (MOH, 1992).

Based on the latest National PPD survey carried out between 1987 and 1990 in 16 districts including Addis Ababa, the annual risk of infection is estimated to be around 1.5%. The incidence of infectious cases in Ethiopia with a population of 50 million calculated based on the 1.5% annual risk is expected to fall between 41,000 to 70,000 per year; including the smear negative and extrapulmonary cases the total annual incidence may range between 90,000 to 154,000. The prevalence, assuming to be roughly twice the incidence, is estimated to fall between 180,000 and 308,000. Approximately 50% of the infectious case (20,000-35,000) may die every year (MOH, 1992).

2.13 Control of Tuberculosis in Ethiopia

2.13.1 In cattle

There is no official regulation for tuberculosis control or eradication program in the country. The test-and-slaughter policy applied by several developed countries can not be adopted in Ethiopia due to lack of infrastructure, financial limitations, cultural and geographical reasons. Test and isolation of reactors as well as pasteurization of milk are some of the control measures taken by government dairy farms to prevent spread of infection to negative animals and to human beings, respectively.

2.13.2 In humans

Tuberculosis in man can be effectively controlled by chemotherapy, chemoprophylaxis and BCG vaccination. The national tuberculosis control program is aiming to cure 85% of all new smear positive cases using the six essential anti-tuberculosis drugs: isoniazid and rifampicine are the major bactericidal, streptomycin and pyrazinamide have a complementary bactericidal action and thiacetazone and ethambutol being bacteriostatic prevent the emergence of drug resistance bacilli.

Chemotherapy can be either the short course or the standard regimen. The short course regimen is treatment of tuberculosis for six months with rifampicine and isoniazid given throughout the whole period of 6 months, supplemented by pyrazinamide and streptomycin or ethambutol administered during the first two months (intensive phase). This is replaced in some places by the 8 months regimen consisting of rifampicine, isoniazid, pyrazinamide and streptomycin or ethambutol for the first two months and thiazina (TB 450) for the following 6 months.

The standard (12 months) regimen consists of an initial intensive 2 months treatment with streptomycin and thiazina and a continuation phase of 10 months with thiazina. This is indicated to adults with smear negative pulmonary tuberculosis and with mild forms of extra-pulmonary tuberculosis as well as for children with tuberculosis lymphadenitis. Children under 5 years of age receive the 6 months chemotherapy as prophylaxis if they are in contact with pulmonary smear positive tuberculosis patients. Non infected children are preferably given BCG vaccine intradermally at the deltoid area of the left arm at a dosage of 0.05 ml for children under one year and 0.1 ml for children aged one year or over.

Health education is also given regularly in TB clinics aiming at sensitizing and increasing the awareness of the patient and his family about the disease, its means of transmission and preventive methods most importantly the regular drug intake.

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted around Debre Zeit and Addis Ababa, Ethiopia. The majority of the field work was done in Debre Zeit where three dairy farms and a TB clinic were selected for sampling. Debre Zeit is the town of Ada woreda, 45 km south-east of Addis Ababa and located 9° N and 40° E in the central highlands with an altitude of 1900 masl. The dairy farms were selected based on the previous history of high TB prevalence and the clinic was selected as a site to study the relationship between human TB patients and their household/associated cattle.

All laboratory work was done at the Armauer Hansen Research Institute (AHRI) in Addis Ababa. AHRI was established in 1970 in the compound of the All Africa Leprosy Rehabilitation and Training (ALERT) with the aim of immunological and bacteriological research on leprosy; recently it shifted to tuberculosis research also using the good facilities for the culturing and immunological research of mycobacterium.

TB clinic in ALERT hospital and a village around the hospital from where the majority of the TB patients are coming were also used as a study site for sample collection both from human TB patients and associated cattle. This hospital is located south-west of Addis Ababa and gives service for leprosy patients. It is also treating TB patients, particularly the leprosy people and their families infected with *M. tuberculosis*. Patients in this hospital come from all over the country, but more than 80% are from a village around the hospital. This suburb was established at the same time with the hospital by leprosy patients coming from all over the country for treatment. The majority of these people own cross-breed dairy cattle and supply milk directly to consumers in the city.

3.2 Study Subjects

The study was conducted both on human TB patients and on cattle associated with them as well as on dairy cows from selected dairy farms and on workers of these farms. A total of 788 animals and 85 people were used as study populations. Out of the 788 cattle 302 were local, 280 crosses and the rest 206 pure exotic breeds. The crosses and the exotic cattle were dairy cows kept for milk production, while the local animals were mainly oxen kept for draft purpose. Of the 85 people sampled, 57 were TB patients from both clinics and the remaining 28 were dairy farm workers from the three dairy farms in Debre Zeit.

3.3 Study Design

3.3.1 Cross-sectional study

A cross-sectional tuberculin survey was conducted on cattle in 1997 to study the prevalence of bovine tuberculosis and to investigate the effect of risk factors associated with it.

3.3.2 Case study

Human TB cases were followed back to their home in an attempt to trace back the source of infection and to investigate if relationships exist between human TB patients and TB cases in their household cattle. Dairy workers associated with tuberculin positive cows were also followed to study the zoonotic importance of *M. bovis*.

3.4 Sampling

3.4.1 Human

Sputum samples were collected from human TB patients in both clinics for mycobacterial isolation; all patients were also interviewed for their identity (address), profession, status of livestock ownership and history of contact with livestock (Annex 3). Based on the information obtained from the first interview patients with close association to livestock (owners, attendants, dairy/feed lot workers, farmers, abattoir workers) were selected for further interview and were followed up. Selection was made based on accessibility of their homestead and interest of the patients to participate in the study. The questionnaire for this group included information on livestock management mainly on housing, watering, milking and feeding conditions, use of livestock and livestock products, degree of physical contact with livestock, etc. (Annex 4).

Specimens were collected before the first antimicrobial therapy by using sterile, leak proof, disposable plastic materials labeled with the patient's code number, type of specimen and date of collection. Transportation of specimen to the laboratory (AHRI) was done immediately after collection, but if this was not the case samples were stored at 2-8°C. The predominant sample collected was sputum; 5-10 ml morning sputum taken for three consecutive days were collected and sputum which gave positive result for acid fast bacilli on smear was taken for culture. Peritoneal fluid was also taken from a single abdominal TB case.

3.4.2 Cattle

All animals used in the study were thoroughly observed for their body condition. Local animals were scored according to the guidelines established by Nicholson and Butterworth (1986): nine scores were used in which the three main scores (Fat, Medium, and Lean) were divided into three categories each having F^+, F, F^-; M^+, M, M^- and L^+, L, L^-. Each score was given a number from 1 (L^+) up to 9 (F^-) by looking at the structure of the tail head, transverse processes of the lumbar vertebrae, the ribs, the hump, the hips, the brisket, etc. Scoring of exotic breeds was done based on the lecture notes issued by the Faculty of Veterinary Medicine, FU Berlin (1996). In addition to the body condition score other relevant data such as age, sex, breed, physiological status (milking, dry, pregnant) were also collected for each animal before sampling.

Samples were collected both from live and slaughtered animals; milk samples (50 ml) were taken aseptically from all tuberculin positive cattle towards the end of milking using a sterile disposable plastic container and transported immediately to the laboratory. For the *γ-INF* assay, 10 ml blood was collected from the jugular vein using heparinized tubes, in addition to this, 15 tuberculin positive animals were also slaughtered for postmortem inspection; after a thorough inspection of the carcasses for gross lesions samples were taken aseptically in a sterile leak proof container for isolation and in 10% buffered formalin for histopathology.

3.5 Diagnosis

3.5.1 Tuberculin test

The comparative intradermal tuberculin test was conducted on 788 animals of which 38.3%, 35.5%, 26.2% were local, cross and exotic breeds, respectively. All materials used in the test were purchased from RHÔNE MERIEUX GMBH, Germany.

All animals above 3 months of age were included in the study and inoculated with 50,000 IU bovine PPD (AN_5 strain, Bovituber, Rhône-Merieux) and 25,000 IU avian PPD (D4 ER strain, Avituber, Rhône-Merieux). For inoculation two sites at the middle of the neck were shaved about 12 cm apart from each other and the thickness measured with a 0.01 mm graduated caliper; then 0.1 ml of each PPD was injected intradermally into each site using an automatic syringe which constantly injects 0.1 ml tuberculin. The injection sites were examined for swelling and skin thickness was measured again after 72 hours.

The difference in skin thickness before and after injection at both sites was used for the interpretation of results. When differences in skin thickness were greater at the site of injection for avian PPD than for bovine PPD, then the animal was considered positive for *M. avium* or other atypical mycobacteria; but when the change in skin thickness was increased at both

injection sites, difference in thickness of the two sites was considered and results interpreted according to the standards set by the manufacturer of the PPDs.

The picture below shows inoculation of bovine and avian PPDs on the middle of a neck about 12 cm apart from each other. The sites were disinfected and shaved before injection.

Interpretation: av_2-av_1 = avd and bv_2-bv_1 = bvd
then, bvd-avd < 2 mm = negative
bvd-avd between 2 and 4 both values inclusive = doubtful
bvd-avd >4 mm = positive

Key: av = injection site for avian PPD
bv = injection site for bovine PPD
avd = skin thickness difference before and after injection of avian PPD
bvd = skin thickness difference before and after injection of bovine PPD

Picture 1. Injection of avian and bovine PPDs on the middle of the neck during CIDT

3.5.2 Direct microscopy

Sputum and tissue samples were subjected to direct microscopic examination before processing for culture. The sodium hypochlorate concentration technique was used for sample processing for staining in which 1-2 ml specimen was mixed with equal volume of 100% sodium hypoclorate and incubated at room temperature for 15 minutes, then 8 ml distilled water was added and shaked well. The mixture was centrifuged at 3,500 rpm for 15 minutes at 4°C; finally a smear was prepared from the sediment and stained with the Ziehl-Neelsen staining technique.

3.5.3 Culture

All specimens collected from human TB patients and from tuberculin positive cattle were processed and prepared for mycobacterial culture at the AHRI laboratory in a biological safety cabinet. Picture 2 shows sample processing for culture inside the biological safety cabinet at AHRI TB laboratory.

3.5.3.1 Sample processing

All samples detained for isolation were digested and decontaminated using 2% NaOH in order to initiate the release of mycobacteria organisms from body fluids and cells and reduce bacterial contaminants. The method used to process specimens was more or less similar to all types of samples except for some modifications.

3.5.3.2 Sputum culture

Sputum samples were collected from 57 TB patients and 28 dairy farm workers and processed for culture by mixing with 2% NaOH (1:3 ratio), agitated in a vortex mixer and decontaminated by thorough shaking for 15 minutes at room temperature. They were centrifuged at 3,500 rpm for 15 minutes at 4°C. The supernatant was taken off and the sediment neutralized with H_2SO_4 plus bromocresole purple until the colour changed to yellow. It was then centrifuged again at the same speed and time; the supernatant decanted and the sediment was inoculated into two slants of Löwenstein-Jensen media, one with pyruvate and the other without.

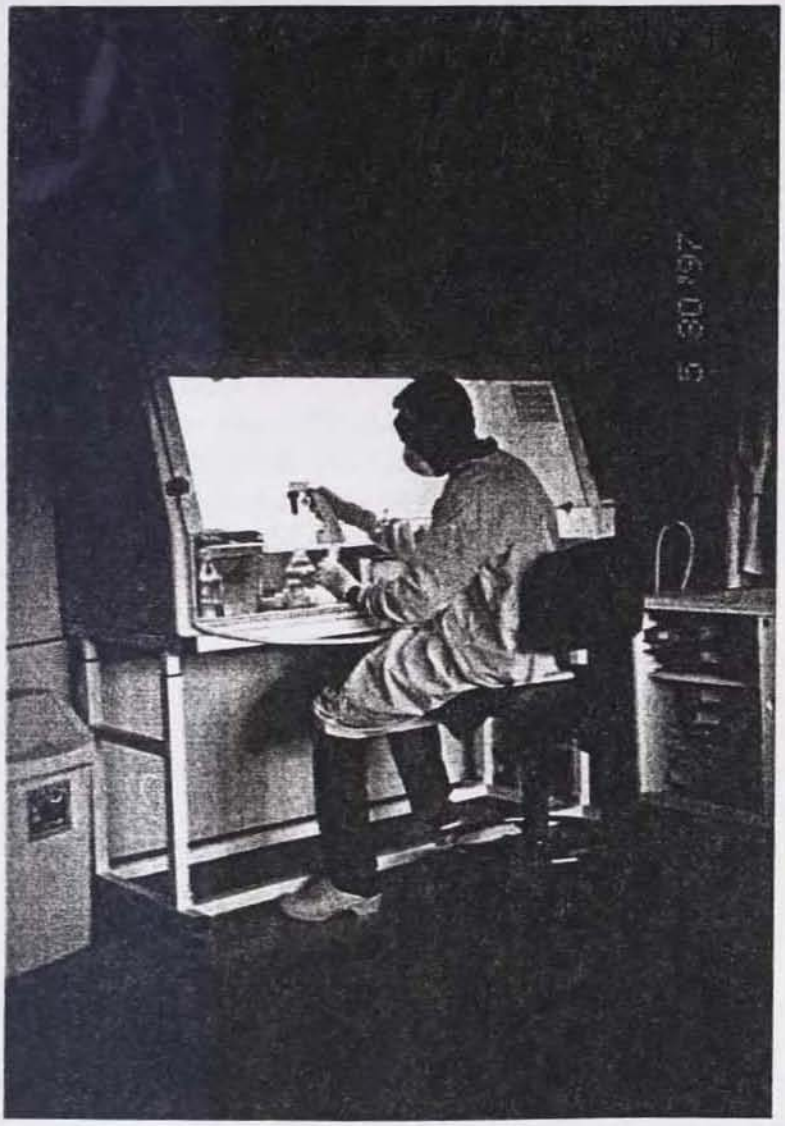
3.5.3.3 Milk culture

Milk samples were collected from 157 tuberculin positive milking cows and processed for culture. 50 ml milk was taken from the four quarters of each cow towards the end of milking and centrifuged immediately at 3,500 rpm for 15 minutes at 4°C. The cream was removed with a sterile spatula, the supernatant taken off and the sediment decontaminated with 2% NaOH (1:1). Then it was processed as for the sputum.

3.5.3.4 Tissue culture

Twenty-two tissue samples taken from 15 tuberculin positive animals slaughtered for postmortem examination were similarly processed. Each of them was grounded in a sterile homogenizer with 2-4 ml physiological buffered saline (PBS) pH 7.3; then 1-2 ml of the fluid was taken and treated the same as sputum. All tubes with all types of samples were incubated at 37°C in a horizontal position for one week to achieve uniform distribution of the specimen all over the slant and then in upright position for 7-11 weeks. Observation for growth of

mycobacteria was done every week; whenever colonies were seen Ziehl-Neelsen staining was done to confirm the presence of acid fast bacilli.



Picture 2. Sample processing for culture inside the biological safety cabinet

3.5.4 Identification

Slants with colonies of acid fast bacilli were subjected to mycobacterial species identification using their growth characteristics and reaction to some biochemical tests.

3.5.4.1 Growth intensity and colony form

Eugonic growth which was relatively rapid, seen in 2-3 weeks with luxuriant, dry, cauliflower like, yellowish colonies indicated the primary culture of *M. tuberculosis*; whereas dysgonic growth which was slow, seen after 4 weeks with small, roundish, whitish and moist colonies mostly underneath the pyruvate enriched media was indicative for *M. bovis*. Fast growing colonies that appear in a week time and which were mostly yellow/deep orange in colour were considered as atypical mycobacteria.

3.5.4.2 Niacin production test

All mycobacteria produce nicotinic acid during growth, but *M. tuberculosis* and some isolates of *M. simiae* and *M. chelonae* do not metabolize the nicotinic acid further; it therefore accumulates in the media which can be detected during niacin test. The test was conducted only on 3-4 weeks old pure sub-cultures grown on Löwenstein-Jensen media which showed heavy growth, in order to avoid false negative results.

For the test, 1.5 ml sterile distilled water or saline was added into a 3-4 weeks old culture slant with heavy growth (at least 50-100 colonies) of mycobacteria and the media was stabbed with a sterile loop to allow extraction of niacin into the surface. The slant was incubated at 37°C in a horizontal position for 30 minutes and vertically for 10 minutes, then 0.6 ml fluid was taken and placed on a sterile labeled, screw capped 13 x 75 test tube. 0.5 ml of aniline and 0.5 ml of cyanogen bromide were added to the test tubes to see a colour change immediately (yellow if positive). Alternatively the Bacto-TB niacin test strips were dropped with arrows downward using flamed forceps into each test tube including the controls, then the tubes were incubated at room temperature with frequent shaking at regular intervals for a maximum of 30 minutes. A positive result was indicated by the appearance of a yellow colour in the extracts of a test culture and on the positive control but no colour in the negative control.

A niacin positive test result on non-chromogenic mycobacteria was considered strongly indicative for *M. tuberculosis* and an attempt was done to classify the niacin negative isolates as *M. bovis* and atypical mycobacterium according to the morphology of their colonies, growth rate and pigment production as no other biochemical test could be conducted on these isolates due to time shortage. However, final identification of these species will be done in the future using other biochemical tests, drug susceptibility tests and molecular techniques.

3.5.5 Gamma interferon (*γ-INF*) assay

The test was conducted only on 20 animals which were also tested with the CIDT in order to compare the sensitivity and specificity of the two tests used. However, as the animals could not be slaughtered before the end of this study it was impossible to establish a gold standard. The result of the *γ-INF* assay hence, was just used to establish the level of agreement between the two tests. All materials used were supplied together with the *γ-INF* test kit. (Annex 5)
Blood samples were collected directly from the jugular vein and transported immediately to the laboratory to be processed within 8-16 hours of collection. One ml blood from each animal was dispensed into three different wells of the 24 well cell culture plate; 20 µl of avian and bovine PPD were added into well 1 and 2 respectively and nothing was added into well 3. The plate was incubated at 37°C and 5% CO_2 for 24 hours. The supernatant was collected gently from each tube and subjected to the monoclonal antibody (*Mab*) based sandwich ELISA in order to detect the production of gamma interferon from pre-sensitized lymphocytes of an exposed animal. Results were given as a mean optical density after measuring the absorbance value of the wells at 650 nm and interpretation was according to the guidelines provided by the manufacturer of the *γ-INF* test kit together with the kit. (Annex 5)

3.5.6 Postmortem examination

Fifteen animals which were also tuberculin tested were slaughtered and examined thoroughly for gross pathological lesions with special emphasis given to the lung, mediastinal and bronchial lymph nodes as well as abdominal organs such as liver, kidney, spleen and mesenteric lymph

nodes. Each organ was visualized, palpated and incised for the presence of lesions. Whenever lesions were detected samples were taken for culture and histopathology.

Specimens taken for histopathology were fixed in 10% formalin and taken to the pathology laboratory of the Faculty of Veterinary Medicine in Debre Zeit for processing. A total of 50 slides were prepared from various organs and stained with Ziehl-Neelsen and hematoxylin-eosin stains. The presence of histological lesions such as cellular infiltration with lymphocytes and giant cells, lesions of necrosis and calcification as well as the presence of acid fast bacilli in the cells were taken as indicatives for tuberculosis infection.

3.6 Data Analyses

The raw data was entered into MS EXCEL spread sheets and analyzed using the Microsoft Excel 5.0, Winepiscope 1.0 (Ortega *et al.*, 1996), Epi info 6.0 (Dean *et al.*, 1994) and Statgraphics Plus 2.1 statistical softwares.

Descriptive statistics in MS Excel and statgraphics Plus were used to describe the rate of occurrence of tuberculosis due to *M. bovis* and *M. tuberculosis* both in cattle and humans.

Chi-square test in Epi info program was applied to test if statistically significant associations exist between risk factors such as age, sex, breed, management and tuberculin test positivity in cattle as well as to determine the association between risk factors such as age, sex, occupation, address, cattle contact, habit of raw milk drinking and the type of TB in human tuberculosis patients.

Odds Ratio (OR) in Winepiscope was utilized to measure the degree of association between risk factors and the disease both in humans and cattle. In addition to this Mantel-Haenszel stratified analysis was also used to correct the confounding effect of management on breed and breed on body condition.

4. RESULTS

4.1 Intradermal Tuberculin Test

Results of the skin test are shown on table 4. Out of the total 788 cattle subjected to the comparative intradermal tuberculin test 234 animals reacted to the bovine PPD resulting in an average prevalence of 29.7% (95% CI=26.5-32.9), among this 46 (5.84%) animals were doubtful reactors and the remaining 188 (23.9%) were positive reactors.

Table 4. Results of comparative intradermal tuberculin test in different farms/sites

farms/sites	tuberculin test results			positive * + doubtful	95% CI	total
	negative	doubtful	positive			
Karakore	214 (84.3%)	5 (1.9%)	35 (13.8%)	40 (15.8%)	11.3 - 20.2	254 (32:2%)
Ada woreda	250 (94.7%)	8 (3%)	6 (2.3%)	14 (5.3%)	2.6 - 8.0	264 (33.5%)
Debre Zeit State Dairy Farm	14 (9.2%)	29 (19.1%)	109 (71.7%)	138 (90.8%)	86.2 - 95.4	152 (19.3%)
Military Eng. College	8 (17 %)	2 (4.3%)	37 (78.7%)	39 (83%)	72.2 - 93.7	47 (6.0%)
Agricultural Research Center	68 (95.8%)	2 (2.8%)	1 (1.4%)	3 (4.2%)	0 - 8.9	71 (9.0%)
Total	554 (70.3%)	46 (5.8%)	188 (23.9%)	234 (29.7%)	26.5 - 32.9	788

* Doubtful reactors were added to the positive reactors in calculating the overall prevalence due to the fact that sensitivity of tuberculin test is low which may miss some infected animals and underestimate the actual prevalence of BTB.

Prevalences between farms differ significantly (χ^2=456, df=4, p<0.001) the highest being in Debre Zeit state dairy farm (90.8%) followed by the Military Engineering College (78.7%) as shown on table 4.

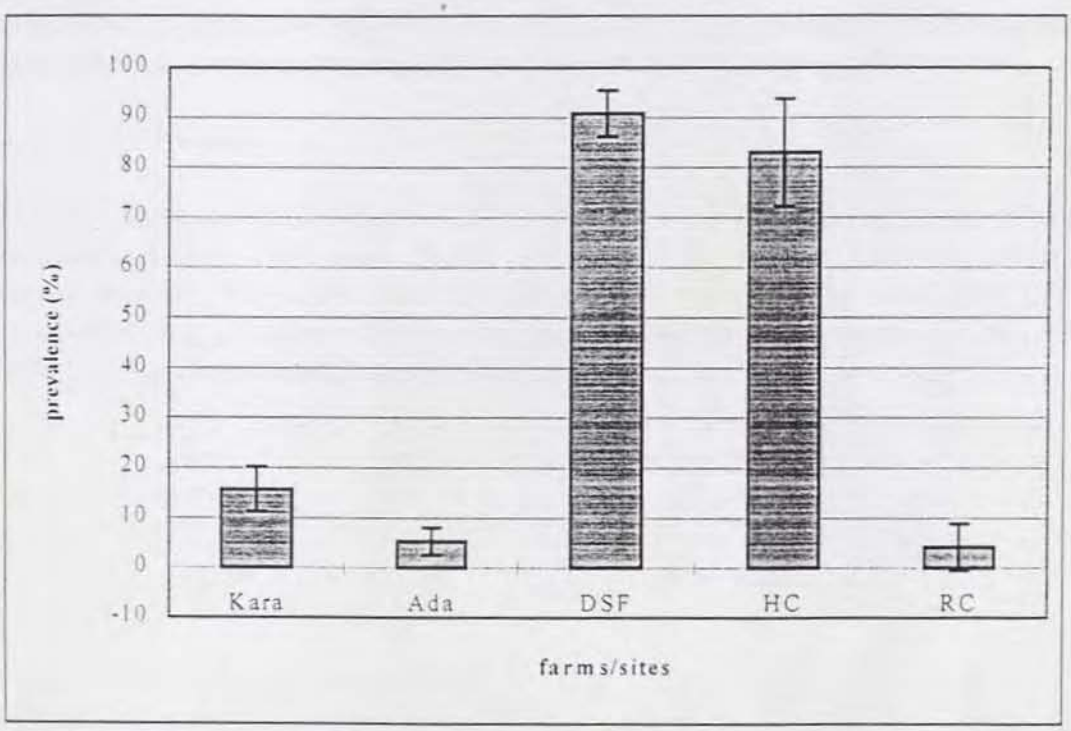


Figure 1. Prevalence of bovine tuberculosis in different farms with 95% CI

4.1.1 Effect of risk factors on prevalence of BTB

4.1.1.1 Management

The farms were classified into two management categories as good and poor based on farm cleanness, stocking rate, waste disposal, aeration, feeding and watering facilities. Clean farms with good aeration, low stoking rate of animals per cow shade, with good drainage system and with a separate feeding and watering equipment for each herd/group of animals in each barn were categorized as farms with good management system; the contrary holds true for farms under poor management system.

Table 5. Relationship between management and tuberculin test positivity

Management	tuberculin test result		95% CI for positive	total
	positive	negative		
poor	209 (51.2%)	199 (48.8%)	48.91-58.83	408 (51.8%)
good	25 (6.6%)	355 (93.4%)	4.09-9.09	380 (48.2%)
Total	234 (29.7%)	554 (70.3%)		788

The effect of management on the prevalence of BTB is shown in table 5; the results indicate that there was a significant association between management and prevalence (χ^2=187.9 df=1, p<0.001). OR was calculated to measure the strength of association and was found to be 14.9 with 95% CI (9.5-23.4) indicating that cattle under poor management system were 15 times more likely to develop tuberculosis than cattle under good management system.

4.1.1.2 Breed

Figure 2 indicates prevalences of BTB with 95% CI in different breeds. Out of the 788 animals included in the study 38.3% were local, 35.5% crosses and the remaining 26.2% exotic animals. Prevalence was high in exotic breeds (86.4% with 95% CI=81.5-91.1); relatively lower prevalence rates were found in cross and local breeds, 13.9% and 5.6% with 95% CI= 9.8-18 and 3.0-8.2, respectively.

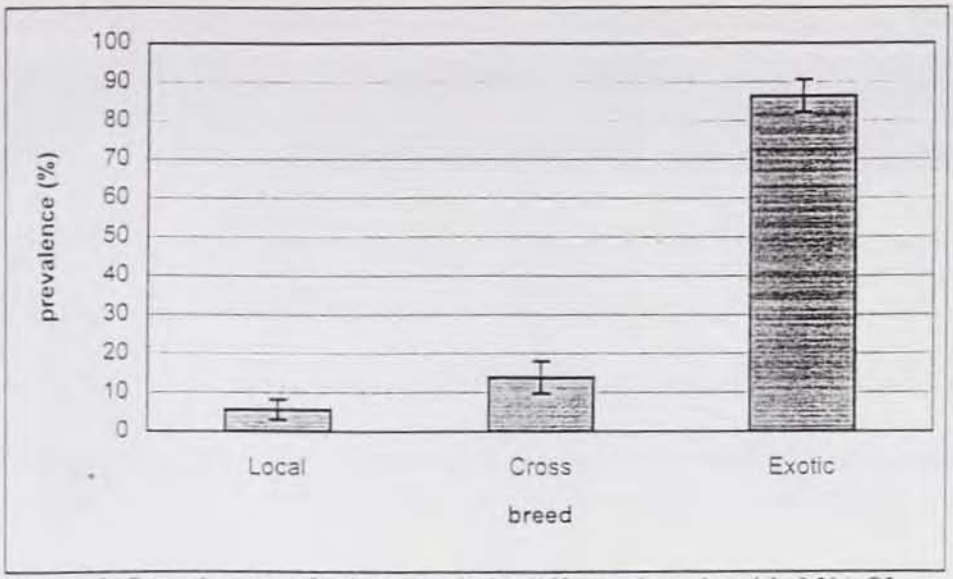


Figure 2. Prevalences of tuberculosis in different breeds with 95% CI

Breed did exert a highly significant effect on TB prevalence ($\chi^2 = 434.5$, df = 2, $p<0.001$). The strength of association (OR) was calculated considering breed as risk factor where crosses and exotic breeds were compared against the local breeds, accordingly crosses and exotic breeds were 2.7 and 106.6 times more likely to develop tuberculosis than local breeds, respectively. The exotic breeds were also more affected than the cross breeds (OR=51.4); but as 96.6% of the exotic breeds were under poor management system it is necessary to consider management as a potential confounding factor and control its effect using the Mantel-Haenszel stratified analysis for OR.

Table 6. Interaction between breed and management and their effect on the prevalence of BTB

Breed	Management			
	Poor		Good	
	positive	negative	positive	negative
local	5	48	12	237
cross	27	129	12	112
exotic	177	22	1	6
total	209	199	25	355

Even after controlling for the effect of management, breed still had an influence on the prevalence of bovine tuberculosis (pooled $OR_{M-H}=2.7$, 95% CI=1.6-4.6).

4.1.1.3 Age

Animals were grouped into 4 age groups as calf (<1 yr.), heifer/bull (1-3 yr.), adult (3-6 yr.) and old animal >6 yr.). The distribution of tuberculous cattle among different age groups is shown on Table 7. Prevalence increases with age; however, the association between age and prevalence was not statistically significant ($\chi^2=2.6$, df=3, P value=0.46).

Table 7. Age distribution and prevalence of tuberculosis in cattle with 95% CI

Age group	tuberculin test result			95% CI for positive
	positive	negative	total	
<1	26 (28.6%)	65 (71.4%)	91 (11.5%)	19.3-37.9
1-3	49 (25.4%)	144 (74.6%)	193 (24.5%)	19.3-31.5
3-6	89 (31.6%)	193 (68.4%)	282 (35.8%)	26.2-37.03
>6	70 (35.5%)	152 (64.5%)	222 (28.2%)	29.2-41.8
Total	234	554	788	

4.1.1.4 Body condition

The method used to rank body condition of local and exotic animals was different; however, all could be grouped into two main categories as poor and good condition. Accordingly, 320 (40.6%) animals were graded as poor with a prevalence of 24.7% (95% CI=20.1-29.5) and the remaining 468 (59.4%) were under good body condition with a prevalence of 33.1% (95% CI=28.8-37.4) as shown on Figure 3.

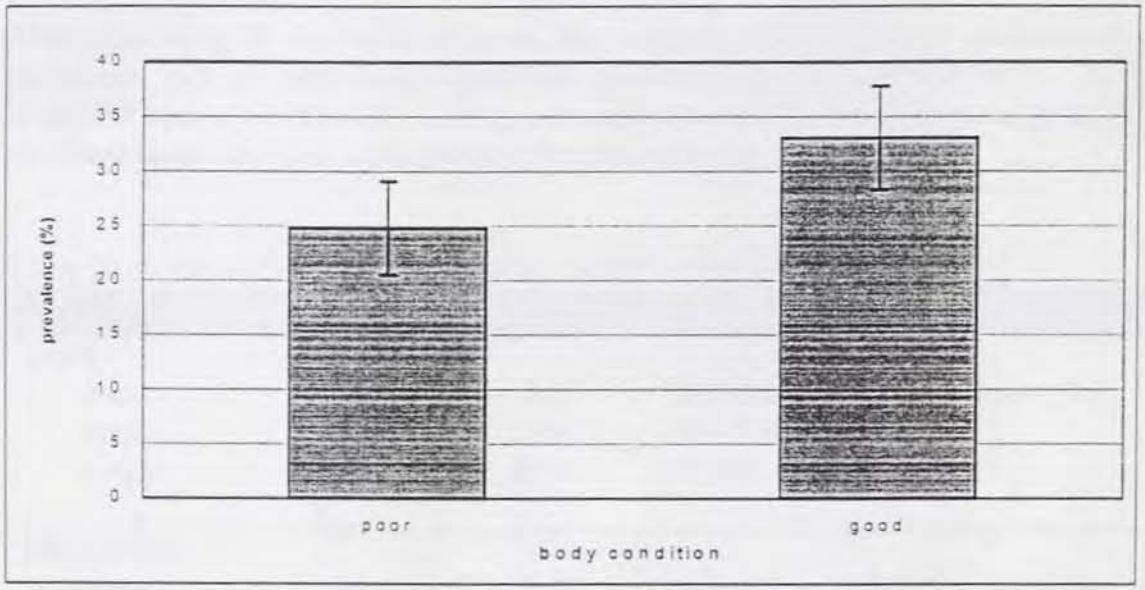


Figure 3. Body condition and reaction to tuberculin test with 95% CI

The association between body condition and prevalence was analyzed and found to be significant (χ^2=6.24, df=1, P<0.05). The OR calculation for degree of association indicated that animals with good body condition were more likely to react to bovine PPD (OR=1.5, 95% CI=1.1-2.1).

Table 8. Distribution of breed and body condition score

body condition	breed			total
	local	cross	exotic	
good	132 (43.7%)	193 (68.9%)	143 (69.4%)	468
poor	170 (56.3%)	87 (31.1%)	63 (30.6%)	320
total	302	280	206	

Table 8 shows distribution of breeds in different body condition scores. About 70% of the exotic breeds which were found to be with high prevalence of BTB were under good body condition and more than 56% of the local animals which were with low prevalence of BTB were under poor body condition, therefore to show the net effect of body condition on prevalence it is very important to control the effect of breed using the Mantel-Haenszel stratified analysis for OR as shown on Table 9.

Table 9. Stratification of tuberculin test results according to breed and body condition

Body condition	Breed					
	local		cross		exotic	
	positive	negative	positive	negative	positive	negative
good	10	122	26	167	119	24
poor	7	163	13	74	59	4
Total	17	285	39	241	178	26

After controlling for the effect of breed, the association between body condition score and prevalence was not statistically significant (pooled OR_{M-H}=0.8, 95% CI=0.5-1.3); but the crude OR was 1.5 (95% CI=1.1-2.1), indicating association between prevalence and body condition score when the confounding effect of breed was ignored.

Table 10. Summary of effect of risk factors on BTB prevalence

Risk factors	No. tested	Preval. (%)	95% CI	χ^2 value	p value	OR
Breed						
local	302	5.6	3.03-8.2	434.5	p<0.001	1
cross	280	13.9	9.9-18.0			2.7
exotic	206	86.4	81.7-91.1			106.6
Management						
poor	408	51.2	48.9-58.8	187.9	p<0.001	14.9
good	380	6.6	4.1-9.1			
Age group (yr)						
<1	91	28.6	19.3-37.9	2.6	p=0.46	----
1-3	193	25.4	19.3-31.5			
3-6	282	31.6	26.2-37.0			
>6	222	35.5	29.2-41.8			
Body condition						
good	468	33.1	28.2-37.3	6.2	p<0.05	1.5
poor	320	24.7	20-29.4			

4.2 Bacteriological Findings

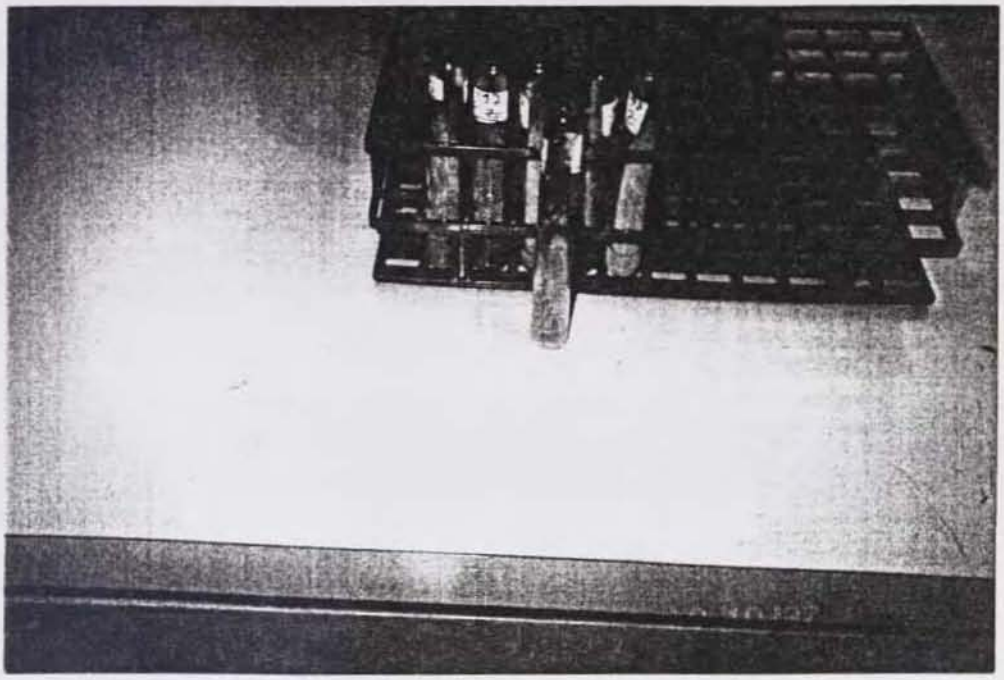
4.2.1 Primary culture

A total of the 265 human sputum and peritoneal fluid as well as cattle milk and tissue samples were cultured on Löwenstein-Jensen media with and without pyruvate. Results on a primary culture revealed that 102 (38.5%) samples were positive for bacterial growth (Table 11), of which 81 isolates were found to be acid fast bacilli on subculture and subjected to niacin test.

Table 11. Results of primary culture on Löwenstein Jensen media

Type of sample	Growth on L-J media		95% CI for positive	Total
	positive	negative		
Milk	28 (17.8%)	129 (82.2%)	11.84-23.82	157
Tissue	14 (63.6%)	8 (36.4%)	43.53-83.73	22
Peritoneal fluid	0	1		1
Sputum	60 (70.6%)	25 (29.4%)	60.9-80.26	85
Total	102 (38.5%)	163 (61.5%)		265

Picture 3 shows typical colonies of *M. tuberculosis* grown on Löwenstein-Jensen media after the 8th weeks of incubation and the typical acid fast appearance of *M. tuberculosis* is shown on Picture 4. The smear was prepared from a sputum sample of dairy farm worker and stained with Ziehl-Neelsen staining technique.



Picture 3. Colonies of *M. tuberculosis* on Löwenstein-Jensen media

Picture 4. Acid fast bacilli on a sputum smear from a dairy farm worker

4.2.2 Niacin production test

The 102 isolates which were positive for growth on primary culture were subcultured on two slants of Löwenstein-Jensen media without pyruvate for about 3-4 weeks. Those slants (81 isolates) which were positive for pure colonies of acid fast bacilli and with heavy growth on the subculture were niacin tested for identification of species. Results of the niacin test conducted on isolates obtained from different sources of samples are shown in table 12. Out of the 81 isolates, 36 (44.4%) were positive indicating *M. tuberculosis* and the remaining 45 (55.6%) were niacin negative indicating *M. bovis* or other atypical mycobacteria.

Table 12. Niacin test results conducted on isolates from various types of specimens

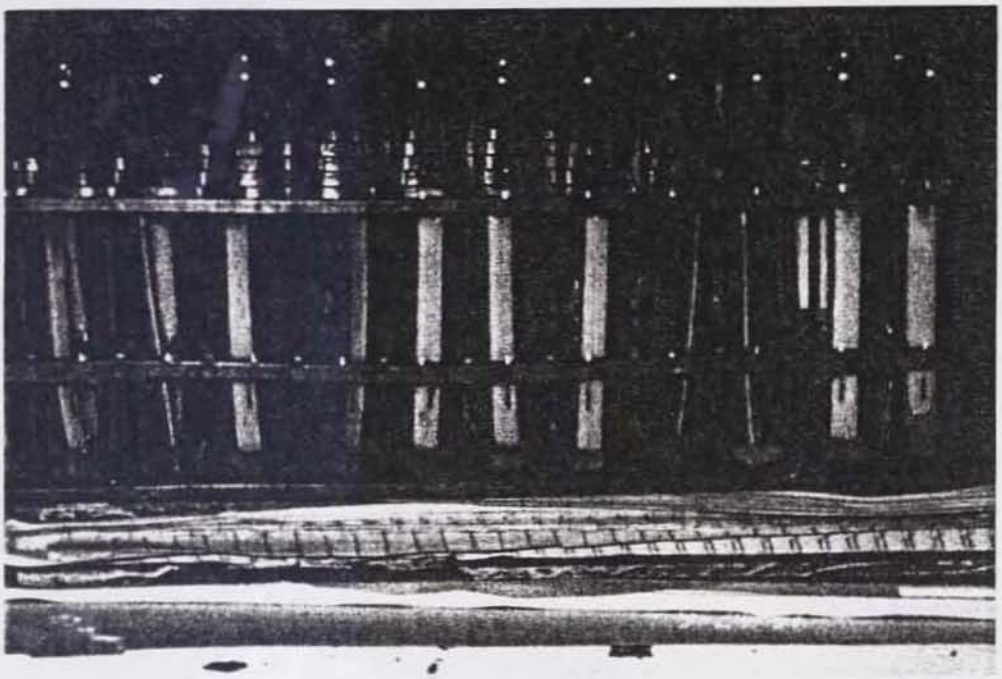
Type of sample	niacin test		
	positive	negative	total
Milk	2 (10.5%)	17 (89.5%)	19
Tissue	0	14 (100%)	14
Sputum	34 (70.8%)	14 (29.2%)	48
Total	36 (44.4%)	45 (55.6%)	81

Out of the 85 sputum samples cultured, 28 were from dairy farm workers and 57 from TB patients in both hospitals; of these 9 (32.1%) and 51 (89.5%) were positive for growth on primary culture respectively. Of the total 60 culture positive sputum samples, 48 (80%) were positive for acid fast bacilli on subculture and subjected to niacin test resulting in 14 (29.2%) niacin negative and 34 (70.8%) niacin positive isolates (Table 12). The rest 12 (20%) were not tested due to poor growth or contamination.

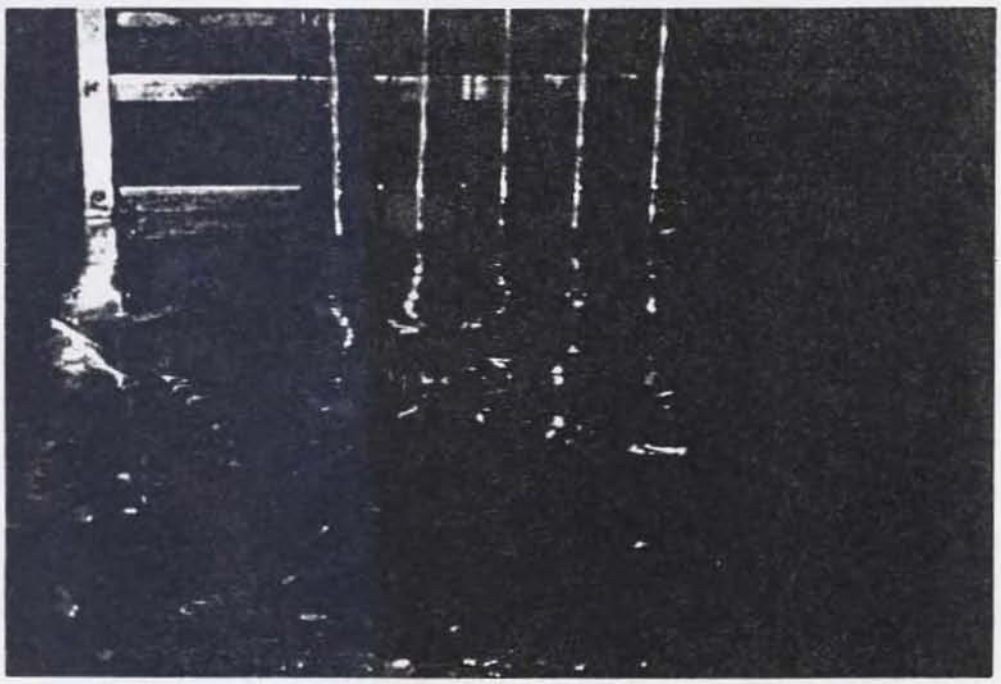
A total of 22 tissue samples taken from 15 slaughtered animals were cultured and 14 (63.6%) were culture positive for niacin negative mycobacteria. Out of the 15 animals slaughtered 10 were tuberculin positive and 5 negative; on culture 11 of them were positive. 28 of the milk samples which were positive for growth on primary culture were also subcultured for niacin test; however, only 19 were subjected to niacin test resulting in 2 positive and 17 negative isolates (Table 12). The remaining 9 isolates were discarded as they were negative for acid fast bacilli on subculture.

Pictures 5 A and B show niacin test results conducted using the Bacto-TB niacin test strips (A) and solutions of aniline and cyanogen bromide (B). Yellow colour indicates a positive result.

Picture 5. Niacin test results of mycobacterial isolates from different samples.



Picture 5 (A) Strip method



Picture 5 (B) Method which uses solution form of the test reagents.

Table 13. Summary of culture and niacin test results of the mycobacterial isolates

Type and source of sample		Results
sputum from TB patients, n=57	acid fast bacilli positive 51 (89.5%)	niacin positive = 32 (80%) niacin negative = 8 (20%) niacin not tested = 11 (21.6%)
	acid fast bacilli negative 6 (10.5%)	
sputum from dairy farm workers, n =28	acid fast bacilli positive 9 (32.1%)	niacin positive =2 (25%) niacin negative = 6 (75%) niacin not tested = 1 (11.1%)
	acid fast bacilli negative 19 (67.9%)	
milk from tuberculin positive cows, n=157	acid fast bacilli positive 19 (12.1%)	niacin positive =2 (10.5%) niacin negative = 17 (89.5%) niacin not tested = ------
	acid fast bacilli negative 138 (87.9%)	
tissue from slaughtered cows n= 22	acid fast bacilli positive 14 (63.4%)	niacin positive =0 (0%) niacin negative = 14 (100%) niacin not tested = ------
	acid fast bacilli negative 8 (36.4%)	
total n= 264 (peritoneal sample not included)	acid fast bacilli positive 93 (35.2%)	niacin positive =36 (44.4%)* niacin negative = 45 (55.6%)* niacin not tested = 12 (12.9%)
	acid fast bacilli negative 171 (64.8%)	

*Denominator used in calculating the percentage is 81 not 93 as only 81 of the isolates were subjected to niacin test. These numbers should be taken with caution as the final identification of the mycobacterial species is not yet done.

Based on the niacin test result we can consider the niacin positive organisms as *M. tuberculosis*, due to the fact that all niacin positive organisms are *M. tuberculosis* except for some isolates of *M. simiae* and *M. chelonae* which can be easily identified from the morphology and appearance of their colonies.

4.3 Postmortem Examination

4.3.1 Gross lesions

During postmortem examination of the 15 animals slaughtered for diagnosis and evaluation of tuberculin test, 11 of them showed gross pathological lesions out of which 5 were affected by generalized TB with miliary lesions in various organs of the body. Millet seed like small tubercles approximately 0.5-3 cm in size and gray to white in colour were found in the inner surface of the ribs, omentum, liver and kidney; bigger tubercles with pus were also found on the uterus, ovary and mammary gland of one cow.

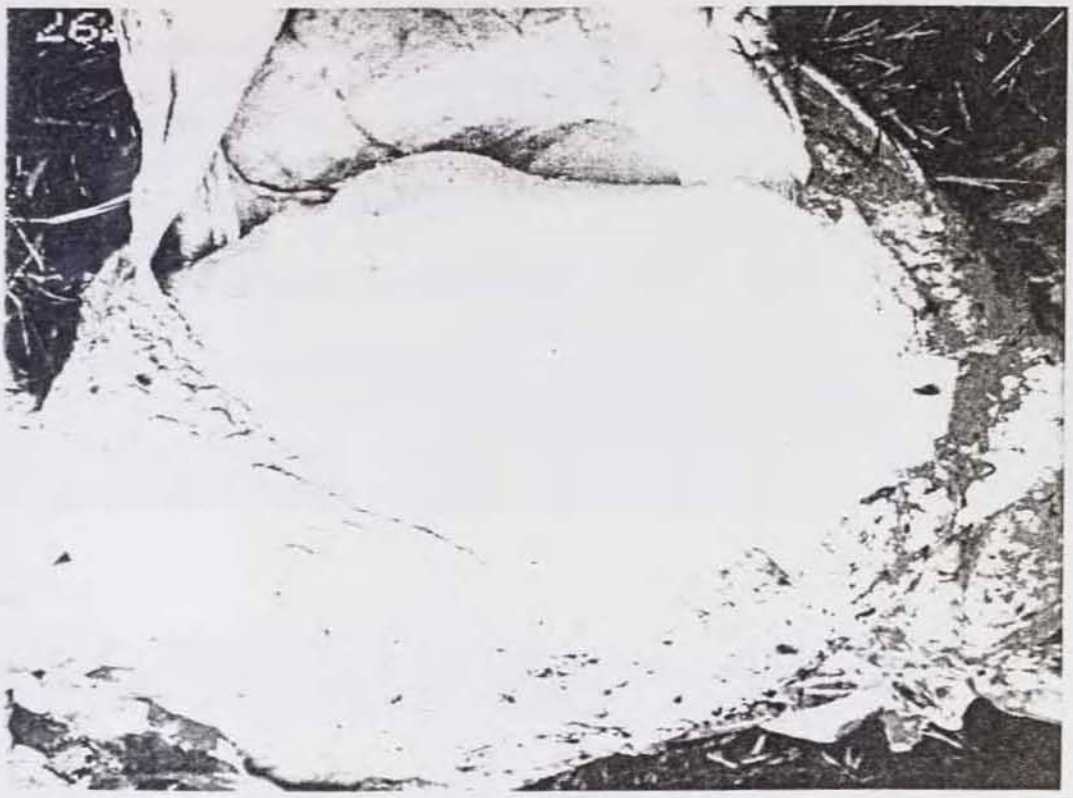
Localized lesion on the lung, bronchial, mediastinal, retropharyngial and mesenteric lymph nodes were also found in 6 of the cows. Tubercles in the lung and associated lymph nodes were bigger in size and mostly with pus upon incision, but lesions on the mesenteric lymph nodes were calcified and dark in colour. In addition to these one big encapsulated lesion was also found on the muscle of the hind leg, upon incision it was full of calcified lesions with pus. No gross postmortem lesion was found in 4 of the cows even though one was positive for tuberculin test.

Gross pathological lesions found on the abdominal cavity of a cow with miliary tuberculosis is shown on pictures 6.

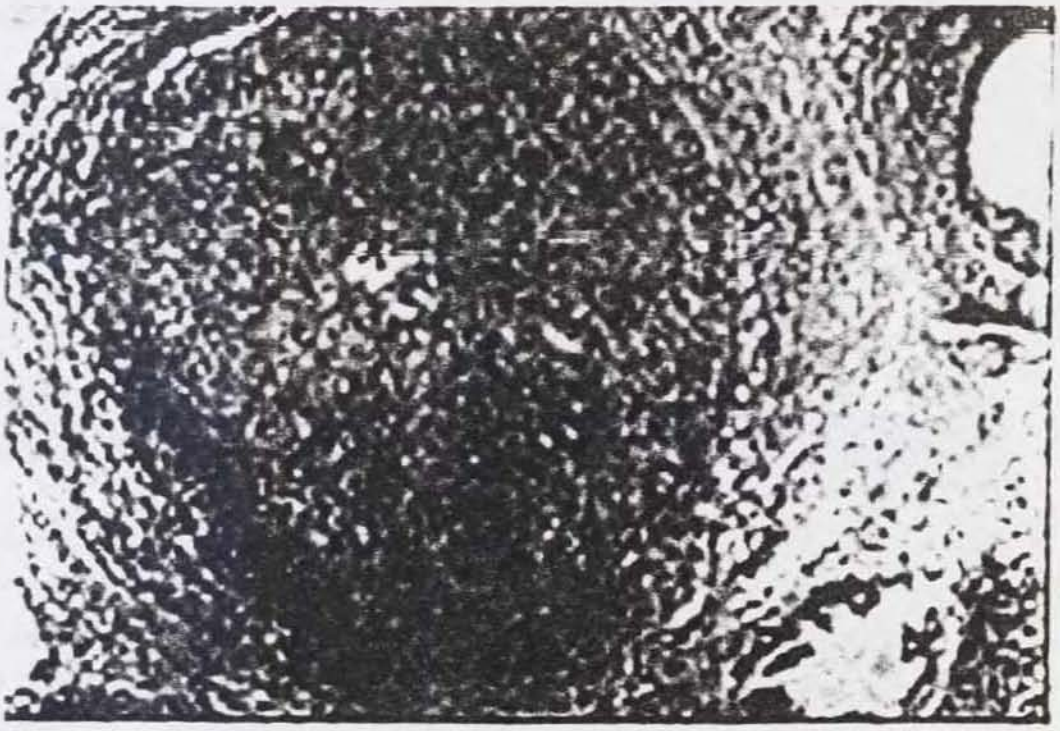
4.3.2 Histopathology

25 samples were taken from different organs and tissues (both normal and with gross lesion) and 50 slides were prepared; half stained with hematoxylin-eosin and another half with Ziehl-Neelsen stain. Results of the hematoxyline-eosin stain prepared from tissues with gross lesions revealed the presence of giant cells, epitheloid cells, necrosis and calcification. Acid fast bacilli were also found in some of the slides stained with Ziehl-Neelsen stain, which appeared as faint pink in colour inside the macrophages, but those taken from tissues with no gross lesion were negative in both cases.

Picture 7 shows histological lesion of a tubercle with a central necrosis and calcification surrounded by lymphocytes and an outer boundary of fibrous connective tissue. The specimen was taken from a uterus of a cow with generalized TB.



Picture 6. Gross pathological lesions of BTB in the abdominal cavity of a cow slaughtered for postmortem examination.



Picture 7. Histological lesion of a tubercle prepared from uterine tissue and stained with hematoxylin eosin stain.

4.4 Test for Agreement and Evaluation of Different Tests

Evaluation of tests was not one of the objectives of this study; however, as results for the CIDT, *γ-INF* assay and culture of few animals were obtained, an attempt was done to compare results of CIDT with the other two tests. In addition to this sensitivity and specificity of CIDT were evaluated based on the culture results as gold standard, but as the sample size was so small results obtained may not be conclusive.

4.4.1 Gamma Interferon (*γ-INF*) assay and CIDT

As shown in Table 14, twenty tuberculin tested animals were also subjected to *γ-IFN* assay test resulting in 3 (15%) positive and 17 (85%) negative. Of the 3 *γ-IFN* test positive animals 2 were also positive in tuberculin test and 1 negative; similarly out of the 17 *γ-IFN* test negative animals 2 were tuberculin test positive and the remaining 15 were negative in both tests.

Table 14. Results of tuberculin test and *γ-INF* assay

tuberculin test result	*γ-INF* pos.	*γ-INF* neg.	total
positive	2	2	4
negative	1	15	16
Total	3	17	20

Test for agreement between these two tests revealed a moderate agreement with *Kappa coefficient*=0.483, (95% CI=0.02-0.99, p<0.05).

4.4.2 Culture and CIDT

Test for agreement between these two tests was conducted based on the culture results of 15 slaughtered animals. Of these 15 animals, 10 were tuberculin positive and 11 positive both on postmortem and culture as shown in Table 15.

There was a moderate agreement between these two tests with *Kappa* value of 0.53, (95% CI =0.056-0.996, p<0.05).

Table 15. Results of tuberculin test and culture

result on culture	tuberculin pos.	tuberculin neg.	total
positive	9	2	11
negative	1	3	4
Total	10	5	15

Sensitivity and specificity of CIDT test were also determined using the culture/postmortem results as "gold standard", accordingly the following results were obtained.

Sensitivity = 81.8, 95% CI = (69.025-104.610)

Specificity = 75. 0, 95% CI = (32.565- 117.44)

4.5 Questionnaire Survey

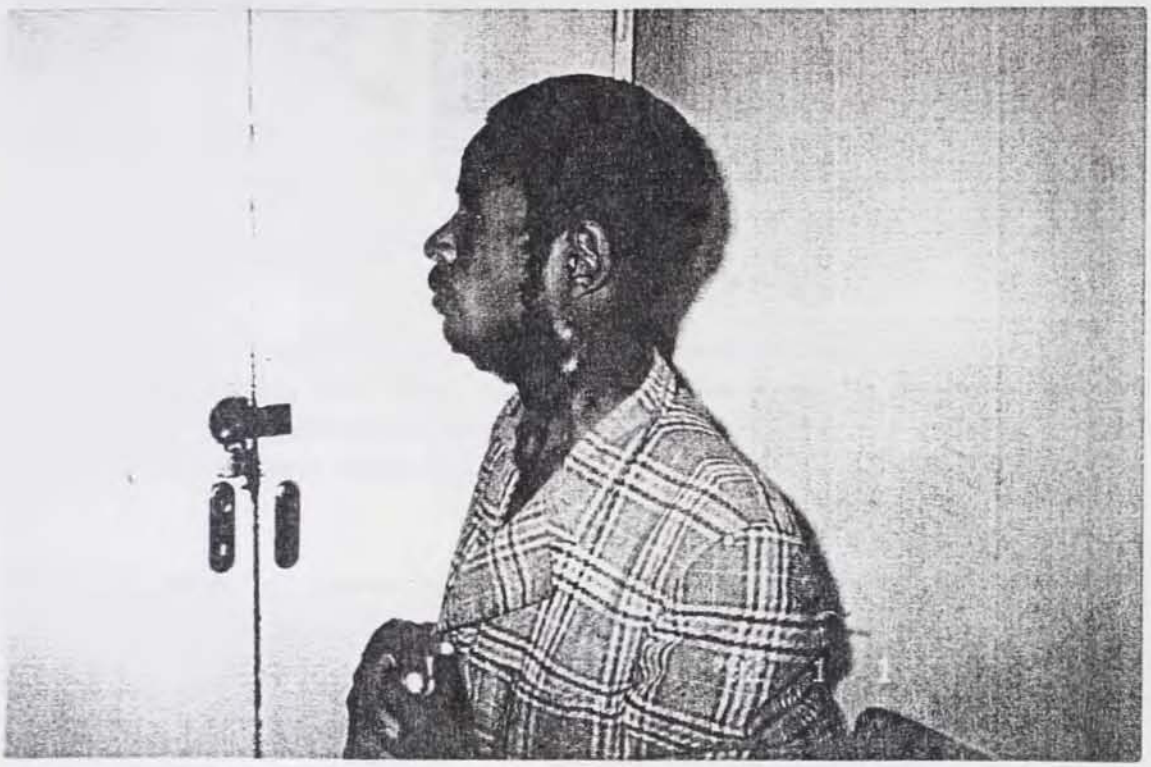
A total of 138 TB patients were interviewed during the study period in both hospitals out of which 38.4% (95% CI=30.3-46.5) were with extrapulmonary tuberculosis (EP TB). The results of the survey revealed that risk factors such as sex, age, address, occupation, habit of milk consumption and degree of cattle contact seem to have an influence on the type of TB; accordingly high proportion of patients with EP TB were found in females (though statistically not significant), in the young age groups, in farmers, in those who frequently drink raw milk and those who have close contact with cattle. Results of the questionnaire survey are summarized in Table 16.

Sex has no influence on type of TB (χ^2 value=1.4; df=1, p=0.24). Age and type of TB were significantly associated (χ^2=16.4, df=2, p<0.001); comparing to the older age group, the younger age group (<15 yr. and 15-45 yr.) were more likely (OR=25.7 and 4.5, respectively) to develop EP TB.

Occupation and type of TB were also significantly associated (χ^2=17.6, df=3, p<0.001) indicating that farmers followed by students (OR= 6.3 and 2.6, respectively) were more likely to be affected by EP TB than civil servants and people in other occupation. Patients from rural area had significantly higher proportion of EP TB (χ^2=11.7, df=1, p<0.001) and they were 3.6 times more likely to develop EP TB than patients from urban areas. Those with close contact to cattle and those who frequently drink raw milk were also with significantly higher proportion of EP TB (Chi square test, p<0.001 and p<0.05, respectively).

Table 16. Results of questionnaire survey and effect of risk factors on the type of TB (N= 138)

risk factors	no. of patients	type of TB		χ^2 value	p value	OR
		EP(%)	P(%)			
Sex						
female	59	44.1	55.9	1.4	p=0.24	
male	79	34.2	65.8			
Age group(yr.)						
<15	14	78.6	21.4	16.4	p<0.001	25.7
15-45	100	39	61			4.5
>45	24	12.5	87.5			1
Origin of the patient						
rural	44	59.1	40.9	11.7	p<0.001	3.6
urban	94	28.7	71.3			
Occupation						
farmer	39	64.1	35.9	17.6	p<0.001	6.3
civil servant	18	22.2	77.8			1
student	21	42.9	57.1			2.6
others	60	25	75			1.2
Raw milk consumption						
frequently	108	48.1	51.9	5.9	p<0.05	3.1
rarely	30	23.3	76.7			
cattle contact						
yes	74	58.1	41.9	26.2	p<0.001	7.5
no	64	15.6	84.4			



Picture 8 Extrapulmonary tuberculosis on the cervical lymph nodes of a 38 years old man

4.6 Association of TB in Man and in Cattle

In an attempt to ascertain the association of tuberculosis in human TB patients and their cattle, three dairy farms with pure exotic and cross breed cattle and 518 local and cross breed animals were investigated. Dairy farms with high prevalence of TB in their cattle were also found to have relatively high numbers of TB cases in their workers indicating association between tuberculosis in man and in cattle.

During the study period there were 4 cases of tuberculosis in the workers of Debre Zeit state dairy farm. One of these died before sputum sample was taken, two started treatment before the study was commenced, hence, sputum sample was collected only from the fourth case which was found to harbor niacin negative acid fast bacilli. This patient was a defaulter who started therapy some time back and defaulted. In addition to this there was also a history of tuberculosis in 3 other workers, of which one woman died and the other 2 treated and cured some time back. Similarly there were also two (one death and one recovered) cases of tuberculosis in the dairy farm of the Military Engineering College.

Table 17. TB cases in cattle and dairy farm workers

farms	TB prevalence in cattle	No. of *workers	History/Case of TB in dairy farm workers			
			sick	cured	died	total
State Dairy Farm	90.8%	29	2	2	3	7
Military Engineering College	82.9%	10	1	1	1	3
Agricultural Research Center	1.4%	31	0	0	0	0
Total			3	3	4	10

* Includes workers which retired in the last few years.

Table 17 indicates the possible association that may exist between tuberculosis cases in man and tuberculin positive dairy breed cows. On the other hand, the association between TB patients from both hospitals and the local cattle they keep was not statistically significant (χ^2=0.01, df=1, p =0.9) as shown in Table 18.

Table 18. Proportion of tuberculin positive cattle owned by house holds with and without TB

house hold TB	tuberculin test result in cattle			95% CI for positive
	positive	negative	total	
positive	26 (10.5%)	221 (89.5%)	247	6.7-14.4
negative	28 (10.3%)	243 (89.7%)	271	6.7-13.95
Total	54 (10.4%)	464 (89.6%)	518	

4.7 Hospital Data

A 5 year hospital data (1993-1997) revealed that a total of 4,930 TB patients were treated in both hospitals. 513 (10.4%) patients were from ALERT hospital and the remaining 4,421(89.6%) from Debre Zeit hospital; the proportion of males (62.7%) was significantly higher than females (37.3%). In addition to this the age distribution of the patients indicated that 71.8% were from the active, working age group (15-45 years).

Table 19. Age and Sex distribution of TB patients treated in Debre Zeit and ALERT hospitals between 1993 and 1997

Age group (years)	Sex		Total
	male	female	
<15	388 (56.8%)	293 (43.2%)	681 (13.8%)
15-45	2234 (63.1%)	1309 (36.9%)	3543 (71.9%)
<45	468 (66.3%)	238 (33.7%)	706 (14.3%)
Total	3090 (62.7%)	1840 (37.3%)	4930

Out of the 513 patients treated in ALERT hospital 27.9% were with EP TB. HIV test result for 182 TB patients treated in the same hospital revealed that 19.7% of the TB patients treated in the hospital were test positive and 15.8% negative; however the result for the remaining 64.5% was not known.

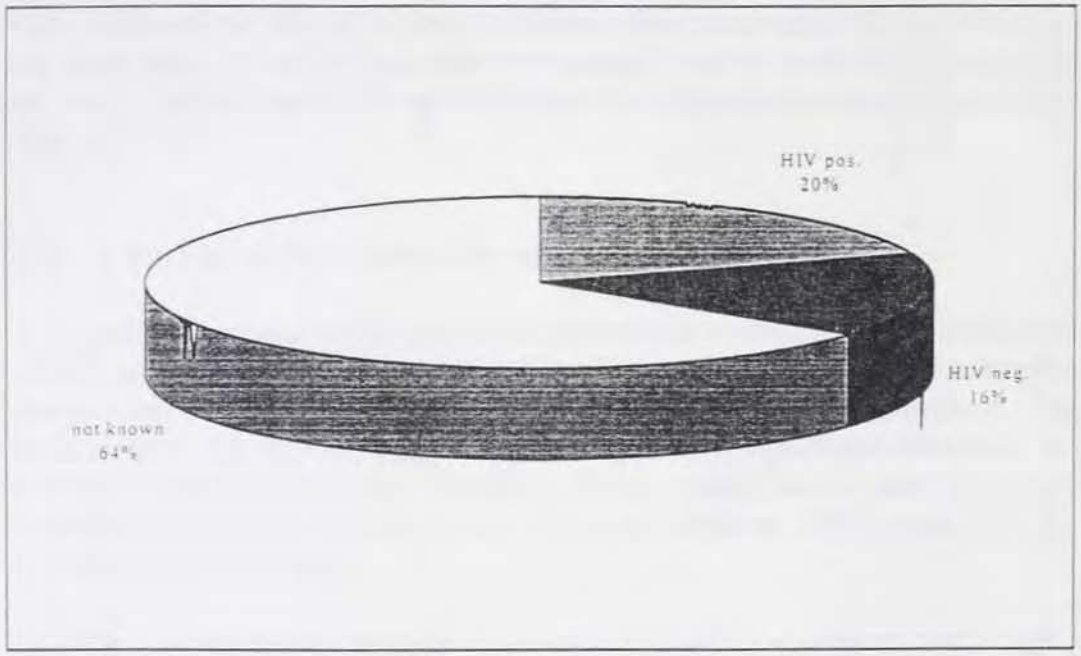


Figure 4. HIV test results of tuberculosis patients in ALERT hospital.

Similar to the questionnaire survey, analysis of risk factors on the type of TB for patients in ALERT hospital has revealed that the association between sex and type of tuberculosis was not statistically significant (χ^2=0.3; df= 1, p =0.6), however, the effect of age on type of TB was found to be significant (χ^2=9.2, df=2, p < 0.05). The association between HIV and type of tuberculosis was not statistically significant (χ^2=0.76, df=1, p=0.4).

Table 20. HIV test results and type of TB for 182 tuberculosis patients in ALERT hospital

HIV test result	Type of TB		Total	95% CI for EP
	EP	P		
positive	15 (14.9%)	86 (85.2%)	101 (19.7%)	11.3-18.4
negative	16 (19.8%)	65 (80.3%)	81 (15.8%)	14-25.5
Total	31	151	182	

5. Discussion

Human tuberculosis in Ethiopia is markedly increasing together with the HIV/AIDS pandemic. Tuberculosis in cattle is also endemic in the country, however the extent of BTB and its role in the increment of human tuberculosis is not yet known as no nationwide epidemiological study has been conducted: on the other hand WHO (1993) reported that human tuberculosis due to *M. bovis* is increasing the world over. Therefore, the aim of this study was to gather preliminary information on the epidemiology of BTB both in exotic and local cattle and the role of *M. bovis* in human tuberculosis cases. To this effect exotic cattle in big dairy farms as well as local and cross animals kept by small scale farms were included in the study, besides human TB patients from two hospitals and dairy farm workers were also sampled.

5.1 Comparative Intradermal Tuberculin Test

The result of comparative intradermal tuberculin test conducted on 788 cattle has indicated an overall prevalence of 29.7% including the 5.8% doubtful reactors; for convenience doubtful reactors were considered as positive in calculating the overall prevalence. The prevalence ranging from 4.2 to 90.8 indicated that there was a significant difference in prevalences between farms/sites (χ^2=456, P<0.001). These results are in line with previous studies conducted on the same farms (MOA, 1987 and Teshome, 1993) except for the increase in prevalences in state farms.

In Debre Zeit state dairy farm the increase in prevalence was significantly high compared to the prevalence in 1987 (χ^2=53.5, p<0.001). This could be partly due to the fact that it was used as an isolation site for tuberculin positive cattle coming from various dairy farms for slaughter in Debre Zeit abattoir 10 years ago. In a similar study conducted by Gobena (1996) prevalence obtained was higher than this study. This may be due to the low specificity of the single intradermal test used by the investigator. Prevalence in local animals has also increased compared to the abattoir based prevalence reported by Solomon (1975) and Gezahegn (1990). In fact there were much higher reactor animals than what is reported in this study; but as the differences in skin thickness were below the standard, they were considered as negative. This may raise a question whether the standards given by the PPD manufacturers also work for local cattle?

Analysis for the effect of risk factors on prevalence has revealed that there was a significant association between management and BTB (χ^2=187.9, p<0.001); cattle under poor

management were 15 times more likely to be infected by BTB (95% CI for OR=9.5-23.4) than cattle under good management system. Published literature by O'Reilly and Costelo (1988), Griffin *et al.* (1992) and Morris *et al.* (1994) could explain this result. Similarly there was a statistically significant association between breed and prevalence (χ^2=434.5, p<0.001); the local animals were compared against the cross and exotic breeds and it was found that these two breeds were more likely to react to bovine PPD than the local cattle (OR=2.7 and 106.6 respectively). This finding is in line with other published studies (Radostits *et al.*, 1994; O'Reilly and Dabron, 1995) which reported lower cases of BTB in Zebu animals followed by their crosses.

As 96.6% of the exotic and 55.7% of the cross breeds were under poor management system, management was considered as confounding factor and its effect controlled using the Mantel-Haenszel stratified analysis and breed still had an influence on prevalence (R_{M-H} =2.7, 95% CI= 1.6-4.6) which further proves the finding.

Animals were grouped into 4 age categories based on their physiological status as calf (<1), heifer/bull (1-3), adult (3-6) and old (>6) years. The association between age and prevalence of BTB was not statistically significant (χ^2=2.6, df=3, p=0.46); which contradicts with other published literature (Dehorty *et al.*, 1996; Gobena, 1996) that justify reaction to tuberculosis increases with age due to the chronic nature of the disease. This deviation could be due to the presence of large number of calves under one year of age that reacted to tuberculin in one of the farms with high prevalence of BTB. The calves might have contracted the bacilli soon after birth through the pooled milk they are being offered.

Body condition scoring in local and exotic animals was different; however, all were grouped again into two categories, good and poor, based on the results of the standard scoring system for both breeds. The crude analysis for association showed that body condition had a significant effect on prevalence (χ^2=6.2, df =1 p<0.05), indicating that cattle with good body condition were more likely to react to bovine PPD (OR=1.5, 95% CI=1.1-2.1). This could be justified by the fact that animals under good body condition are with good immune status that can respond to any foreign protein better than those with poor body condition which can be immuno-compromised due to other diseases or malnutrition. However, as more than 70% of the animals with good body condition were the exotics with high TB prevalence, controlling the confounding effect of breed was necessary; accordingly the pooled OR was calculated and revealed that the association between body condition and prevalence was not statistically significant when the effect of breed was controlled.

5.2 Bacteriological Findings

A total of 265 bovine milk and tissue as well as human peritoneal fluid and sputum samples were cultured resulting in 102 (38.5%) positive isolates on primary culture. Complete identification of the bacilli was not done due to time limitation and hence identification was done depending only on niacin test results, but all isolates are stored properly for further use. Out of the 157 milk samples taken from tuberculin positive milking cows 28 (17.8%) were positive for growth on primary culture, but only 19 (12%) of these were positive for acid fast bacilli on subculture and considered for niacin test. On niacin test 2 (10.5%) of the 19 isolates were positive indicating *M. tuberculosis*. This finding is comparable with Boulahbal (1978) who identified 7 *M. tuberculosis* out of 113 isolates in Algeria; similarly Idrisu and Schnurrenberger (1977) have also isolated *M. tuberculosis* from milk in Nigeria. This is the

first report in Ethiopia which needs further investigation to prove if it could also be due to contamination of milk from the TB positive workers during the study period in that particular farm. With the rapidly increasing incidence of human tuberculosis in the country, this finding suggests the possibility of cattle to act as a reservoir host for human tuberculosis.

Published literature has indicated that only 1% of tuberculous cattle excrete mycobacterium in the milk (Grange and Yates, 1994) but in this study it was significantly higher suggesting that some of the isolates could be niacin negative bacilli other than *M. bovis*. If we assume that 4% of the tuberculin positive cattle in this study were excreting tubercle bacilli in their milk, then a maximum of 6 isolates are expected to contain *M. bovis* which is comparable with findings of Kinfe and Eshetu (1987).

Sputum samples were collected from smear positive TB patients and dairy farm workers. Out of the 85 sputum samples 60 (70.6%) were positive for growth on a primary culture; on subculture 12 of these were either contaminated or with poor growth, hence, not considered for the niacin test. Of the 48 isolates subjected to niacin test 34 (70.8%) were niacin positive indicating *M. tuberculosis* and the rest 14 (29.2%) were negative indicating *M. bovis* or other niacin negative atypical mycobacterium. We expect few isolates of these niacin negative cultures to be due to *M. bovis* which suggests a high risk for man to acquire even pulmonary TB from cattle.

Out of the 22 tissue samples collected from 15 slaughtered animals 14 were positive for niacin negative mycobacterium which are more likely *M. bovis* from the morphology and appearance of their colonies and from the gross pathological lesions seen on the tissue specimens taken. During postmortem examination, 11 of the 15 slaughtered animals were with gross lesions; 5 being with generalized miliary tuberculosis, 6 with local lesions in the lung and associated lymph nodes and the remaining 4 showed no lesions. Lesions found in the lung and other organs were typical TB lesions more or less similar to what is described in pathology text books (Jubb and Kennedy, 1970). Lung lesions were more frequent which were found in all positive animals indicating that the respiratory route is the major route of infection in cattle. This finding is in line with abattoir reports by Solomon (1975) and Gezahegn (1990). The lesion which was found on the muscle of the hind leg of a bull was encapsulated and with pus which if opened could contaminate the carcass and pose a risk of infection particularly when raw meat is consumed.

Histopathology results of these tissue samples revealed that samples taken from tissues with gross lesions were also positive for histological lesions in hematoxylin-eosin stain; acid fast bacilli were also found in some of the slides stained with Ziehl-Neelsen which were faint pink in colour inside macrophages. This was similar to what was reported by Croner (1994) and Gobena (1996).

5.3 Test Agreement

This was not included in the objectives of this study. However, results of CIDT, culture and *γ-INF* were available that initiated analysis for test agreement between CIDT and *γ-INF* as well as between postmortem and CIDT; in addition to this the sensitivity and specificity of CIDT was evaluated using results of culture as gold standard. Moderate agreement was found between CIDT and culture as well as CIDT and *γ-INF* assay with *Kappa* coefficient of 0.53 and 0.48, respectively, $p < 0.05$ in both cases. The sensitivity and specificity of CIDT were

81.8% and 75% respectively which do not coincide with the findings of other workers who mostly found a specificity greater than 90% (Monogahan *et al.*, 1994; WHO, 1994). The lower specificity of this study may be due to the low sample size we used as we could slaughter only 15 tuberculin tested animals.

5.4 Questionnaire Survey

Questionnaire survey was conducted on TB patients in both hospitals to assess risk factors associated with EP TB. The assumption was that the majority of the EP TB cases in man are due to *M. bovis* (Cosivi *et al.*, 1995; Schwabe, 1984). The survey was aimed at identifying groups of the population which are at higher risk of being infected with *M. bovis*. The results obtained from the 138 patients interviewed in both hospitals revealed that, 38.4% (95% CI =30.3-46.5) were with EP tuberculosis, a finding which contradicts a report from one of the big hospitals in Addis Ababa that indicated more than 50% of the TB patients were with EP TB (MOH, 1992), but our finding is comparable with the findings of workers in Tanzania and Somalia who reported 30% to 31% EP cases in human TB patients.

In an attempt to assess the association of risk factors and type of TB, sex was found not to be associated (χ^2=1.4, p=0.24); whereas other risk factors: age, origin of patient, occupation, cattle contact and habit of raw milk consumption were significantly associated with the type of TB with χ^2 value ranging from 5.9 to 26.2 and $p<0.05$ for habit of raw milk consumption and $p<0.001$ for all the other factors.

Patients who frequently drink raw milk were 6 times more likely to develop EP TB. This could be due to the fact that they can acquire *M. bovis* through the raw milk which usually causes EP TB. Similarly the younger age group (<15 yr.) were more likely to develop EP TB than the other two groups (OR=5.7 and 25.7 respectively) which can be explained due to the fact that children frequently drink raw milk and hence are more exposed to the risk of being infected with *M. bovis*. This could be further justified by Kleeberg (1984) who indicated more than 50% of the EP cases in children are due to *M. bovis* contracted from raw milk drinking. Patients from rural areas, farmers and those in close contact with cattle were also found to be more frequently affected by the EP (OR=3.6, 6.3, 7.5 respectively) which could be due to the close association of these people with cattle even sharing watering holes and due to the habit of the community to consume raw milk and meat that may expose them to *M. bovis* infections.

5.5 Hospital Data

A five year hospital data from Debre Zeit and ALERT hospitals has indicated that there were significantly higher proportions of male than female TB patients (χ^2=637, $p<0.001$). This is in line with other reports and also with the result of the questionnaire survey of this study. This can be due to the common practice of men to share/exchange cigarettes, Gaya (local tobacco) and glasses during drinking of local beer. More than 70% of the patients were in the active age group (15-45 yr.) which is in agreement with reports of WHO (1992, 1993 and 1994); this suggests that tuberculosis is affecting the national economy due to loss in working days and payment for sick leave.

In ALERT hospital HIV test result was known only for 182 TB patients and 19.7% of the tuberculosis patients treated in the hospital were also test positive for HIV. This proportion of

HIV test positive TB patients is very low for countries in sub-Saharan Africa where the majority of TB patients are also concurrently positive to HIV; for example in the Monze district of Zambia 70% of the TB patients were also positive for HIV (Cook *et al.*, 1996). This could be due to the fact that HIV test results are confidential and hence majority of the positive results were not registered in the TB register book. In addition to this, patients treated in ALERT hospital are not representative of the population as the hospital gives service only to leprosy patients living in a suburb near to the hospital.

6. CONCLUSION AND RECOMMENDATIONS

When compared to results obtained some years back, incidence of bovine tuberculosis is increasing gradually both in local and exotic breed animals. This increment is mainly in parastatal dairy farms with exotic and cross breed animals; even though management, intensification, could contribute a lot to the spread of the disease, exotic dairy breeds were found to be at high risk of infection which needs special attention in controlling the disease.

Isolation of niacin negative mycobacterium from milk, sputum of TB patients and dairy farm workers is indicative for the public health importance of *M. bovis* particularly in children and in people with direct or indirect contact to cattle (farmers, abattoir and dairy farm workers). In addition to this, the presence of niacin positive mycobacterium in milk indicates the possibility of cattle to act as a reservoir host of *M. tuberculosis*, therefore, source of infection to man.

Tuberculosis in humans is also increasing in an alarming rate and affects mainly the active, working age group (15-45 years old) which may have a significant influence in the national economy. More than 30% of the patients were with EP TB which were mainly in people with direct or indirect contact to cattle and those who frequently consume raw milk and meat. This suggests the possible association that may exist between EP TB and *M. bovis*. As people and cattle in Ethiopia are living in close association and due to the fact that raw milk and meat are consumed to a large extent, the rate of human tuberculosis due to *M. bovis* would be much higher than what is reported. Therefore any control program against tuberculosis in humans should be designed parallel to control strategies in cattle.

The most effective method of controlling tuberculosis in cattle is the test and slaughter policy. However, due to economic, cultural and infrastractural problems prevailing in the country, it can not be practiced in Ethiopia; therefore the alternate and possible recommendations are:

1. Nationwide epidemiological study of bovine tuberculosis to identify high risk areas to focus on during any control program.

2. Regular tuberculin testing of animals under high risk area and isolation of reactors to concentrate them in a particular concentration camp until they finish their production life. After identifying infected farms strict control on the sale of animals and pasteurization of milk from these farms should be implemented.

3. Isolation of calves born in infected farms soon after birth and rearing of a replacement stock in a separate disease free farm. Workers in this farm should not come from infected farms and they have to be tested regularly for TB.

4. Stringent meat inspection in abattoirs and proper disposal of positive organs/carcass is very important to prevent spread of the disease among livestock and to man. Abattoir data should be used properly in the epidemiological investigation of the disease; slaughterhouse surveillance and trace-back of animals to herds of origin is most appropriate in the epidemiological study, as it is technically and economically feasible.

5. Public education to increase the awareness of the community about the potential risk of raw milk and expansion of milk pasteurization is the easiest and effective method of controlling the zoonotic aspect of *M. bovis*.

6. Attention should be given, by the medical profession, to the importance of *M. bovis* as a public health hazard; in addition to this strong collaboration of medical and veterinary personnel is paramount important in investigating and controlling the zoonotic importance of *M. bovis*.

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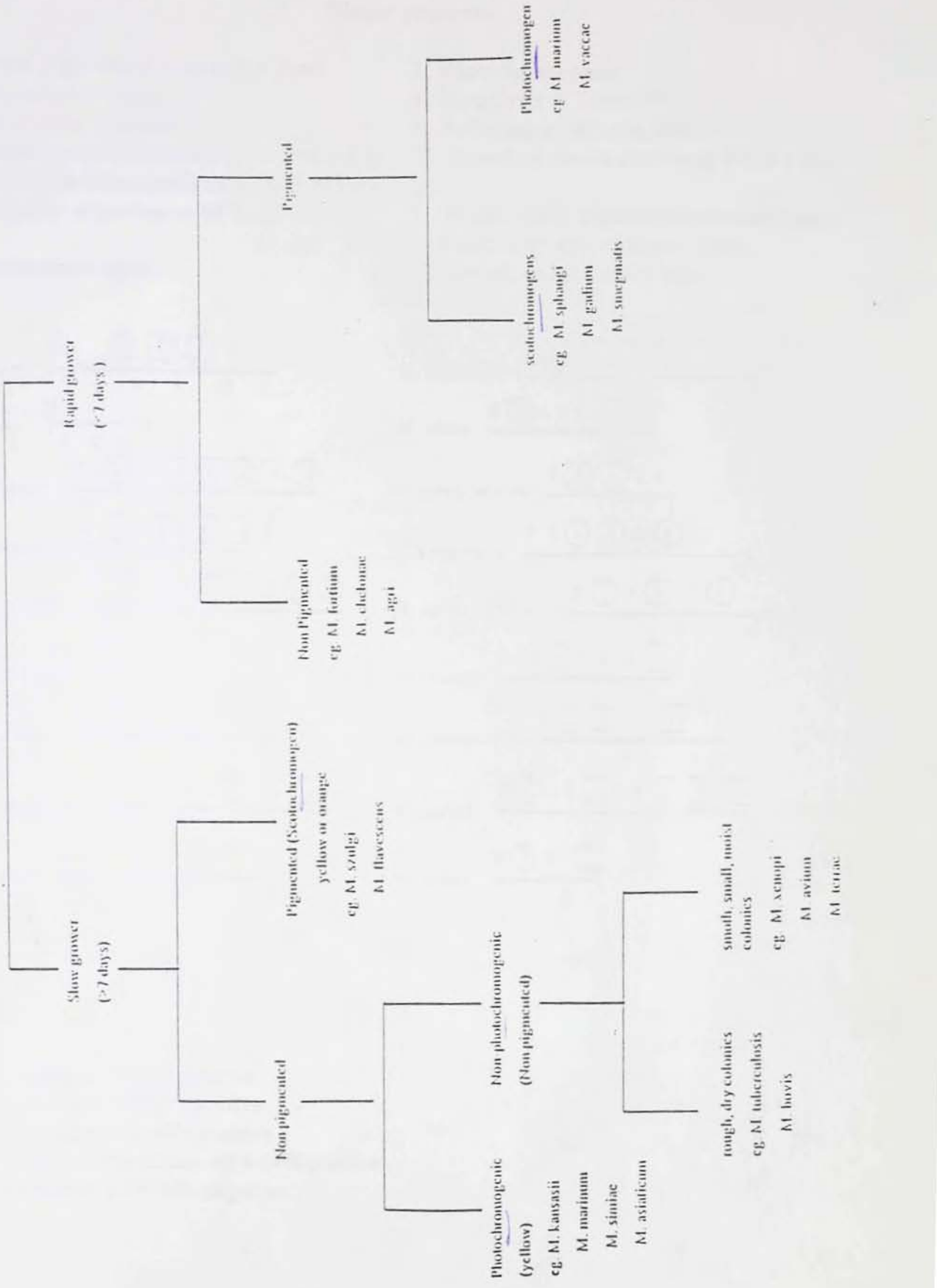
8. ANNEXES

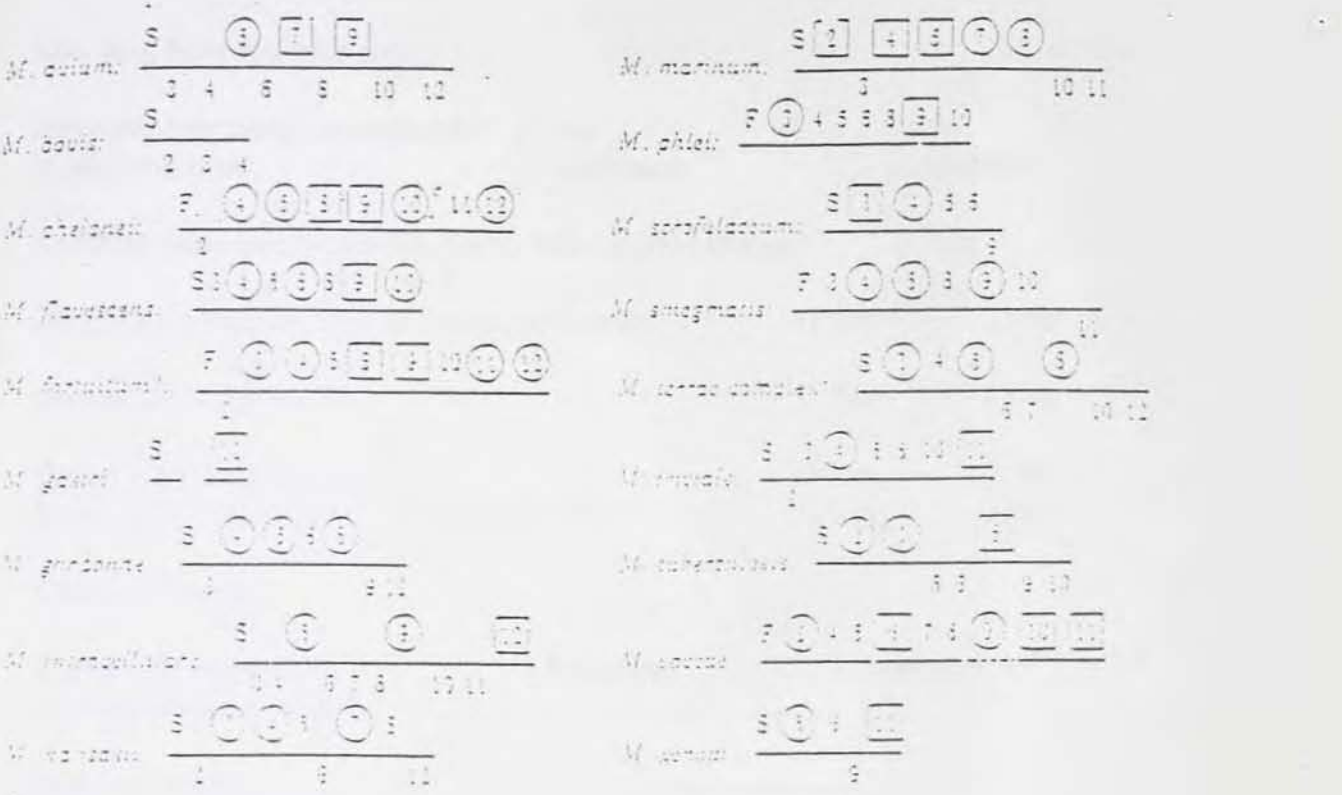
Annex 1. Classification of mycobacteria



Annex 2. Identification of mycobacterium according to the twelve properties examined by Kubica (1973)

Twelve properties

1. Rate of growth (S = slow; F = Fast)
2. Secretion of niacin
3. Reduction of nitrate
4. Semiquantitative test for hyperproduction of catalase (column of gas bubbles >45 mm
5. Stability of catalase to 68°C, 20 min.
6. Scotochromogenic
7. Photochromogenic
8. Hydrolysis of Tween 80
9. Reduction of tellurite, 3 days
10. Growth on media containing 5% w/v NaCl
11. Hydrolysis of tripotassiumphenolphthalein disulfate by aryl sulfatase, 3 days
12. Growth on McConkey agar



Key

1. No. Unboxed = 100% positive
2. No. in circle = 70-99% positive
3. No. in square = 15-69% positive
4. No. under horizontal line =0,4-14% positive
5. No number at all =100% negative

Annex 3. Questionnaires for TB patients (Part one)

Date..... Code/Case No.....

Patient's Name.....

Age (yrs.) 1) <15 2) 15-45 3)>45
Sex 1) Male 2) Female

Address: Urban, City.....Woreda.....Kebele.....House No.....

Rural Woreda..... P/A.....Village.....

Occupation: 1) Farmer 2) Civil servant 3) Others.....

How long have you been sick? 1) More than a year 2) Less than a year

Have you ever taken any treatment? 1) Yes 2) No
If yes, what type? 1) Traditional 2) Modern

Are there other members of the family with a similar disease? 1) Yes 2) No

Do you have /had any type of contact with cattle? 1) Yes 2) No

Do you drink raw milk? 1) Yes 2)No

Do you have cattle at present? 1) Yes 2) No
If yes, do you want them to be tested for TB? 1) Yes 2)No

Clinical Record

Type of TB suspected? 1) Pulmonary 2) Extrapulmonary
If extrapulmonary, specify?.....

Sample(s) taken:.....

Result on direct smear: 1) Positive 2) Negative

Result on culture: 1) Positive 2) Negative
If positive, type of Mycobacterium? 1) *M. bovis* 2) *M. tuberculosis*

Annex 4. Questionnaire for TB patients with Cattle (Part two)

Date..... Code No.....

Name of the Owner.....

What type of animal do you have? 1. Local 2. Cross 3. Exotic

How many cattle do you have? 1. Less than 10 2. 10-20 3. More than 20

What are the most common diseases affecting your cattle, in order of priority?

1).....
2).....
3).....

Were/are your animals coughing? 1) Yes 2) No

How do you mange cattle? 1) Free grazing 2) Stall feeding

Is there any contact of your cattle with wild animals? 1) Yes 2) No
If yes, which type, in order of priority? 1).....
2).....
3).....

Do you mix your cattle with other cattle? 1) Yes 2) No
If yes, where? 1) Watering points 2) Grazing fields 3) Market

Do you use the same watering point with animals? 1) Yes 2) No

Do you share the same house with your animals? 1) Yes 2) No

How frequent do you drink raw milk/its products? 1) Never 2) Rarely 3) Frequently

Annex 5. Gamma interferon assay test reagents and procedures

5.1 Reagents

- 96 well ELISA plate coated with anti-bovine *γ-INF*
- positive and negative controls
- conjugate: anti-bovine *γ-INF* + horse radish peroxides (HRPO)
- TMB substrate and TMB diluent
- wash solution
- stop solution
- avian and bovine PPDs

5.2 Reagent preparation

Controls: are reconstituted by adding 2 ml of distilled water into the positive and negative control vials and mixed through vortexing. Prepared control reagents can be stored at 2-7°C up to 3 months.

Wash solution: is prepared by diluting wash concentrate with distilled water at 1:10 ratio. For a single microtiter plate, 30 ml of wash solution and 270 ml of distilled water are mixed.

TMB substrate: is reconstituted by mixing equal volume of the stock substrate and TMB diluent. Reconstituted TMB should be used within 30 minutes. 6 ml of TMB concentrate and 6 ml of TMB diluent are mixed for a single ELISA plate.

5.3 Test procedure

- All reagents should be at room temperature for 10-15 minutes before use.
- Record the position of each sample and control on the IDEXX worksheet and each of them has to be dispensed in triplicates.
- Dispense 100 µl of each control and test plasma in to the designated triplicate wells.
- The plate is incubated at room temperature for one hour.
- The liquid content is taken off and the plate washed 4 times using the 300 µl wash solution.
- 100 µl conjugate is added into each well and incubated at room temperature for 30 min.
- After the liquid is aspirated the well is again washed 4 times as step 5.
- Dispense 100 µl of TMB substrate into each well and incubate the plate at room temperature for another 30 minutes.
- Finally 100 µl of stop solution is added into each well to stop the enzyme-substrate reaction.
- Absorbance value (OD) is measured using the spectrophotometer at 650 nm

5.4 calculations

- Mean of negative control (NC) = OD at 650 nm of NC wells (1+2+3) divided into three
- Mean of positive control (PC) = OD at 650 nm of PC wells (1+2+3) divided in to three.

  NB. NC mean should be always less than or equal to 0.25 and the difference between the two means (PC-NC) must be greater or equal to 0.5.

- Cutoff value = the OD of well 3 (control) for each sample plus 0.1; well 3 has plasma produced by inoculating an antigen other than the avian and bovine PPD in to whole blood of TB suspected animal.

5.5 Interpretation of results

1. When the OD value of well 1 and 2 is less than the cutoff value then the animal is negative both for avian and bovine *γ-INF*.

2. If the OD value of well one (avian PPD) is found to be higher or equal to the cutoff, then the animal is considered to be positive to *M. avium* or to other species of the bacilli. On the other hand if the absorbance value of well 2 (bovine PPD) is greater or equal to the cutoff value the animal is positive to *M. bovis*.

3. If OD value of both wells is greater than the cutoff, the animal is positive for both PPDs and interpretation of result is done by taking the ratio of the OD value of both wells. OD of well two divided by OD value of well one less than 0.7 indicates that the animal is preferably positive to *M. avium*. Alternatively Ratio grater than 1.8 indicates that the animal is positive for *M. bovis*.

9. Curriculum Vitae

1. Personal Data

Name	Tadele Kiros
Date of Birth	May 1, 1966
Place of Birth	Makale, Tigray
Marital Status	Single
Nationality	Ethiopian
Profession	Veterinarian
Occupation	University Lecturer

2. Educational background

Year	Institution	Award
1974-1980	Kasate Berhan elementary School	-
1981-1984	Atse-Yohannes Comp. Sec. School	Ethiopian School Leaving Certificate Examination GPA= 4.00 out of 4.00
1985-1991	Addis Ababa University, Faculty of Vet. Medicine	Doctor of Veterinary Medicine (DVM) CGPA= 3.56 out of 4.00 (Gold medal winner of the year)
Jan. 1996-Jan. 1998	Free University of Berlin	MSc in Tropical Veterinary Epidemiology

3. Work experience

Year	Institution	Responsibility
1990-1991	National Trypanosomiasis and Tse Tse Control Center	Research as undergraduate associate on tse tse control activities.
1991-1992	Ministry of Agriculture	Regional coordinator of the Pan African Rinderpest Campaign in Tigray province of Ethiopia.
1993-1995	Addis Ababa University	-Lecturer in the department of Microbiology, Infectious diseases and Veterinary Public Health. -Assistant Registrar
1997	Armauer Hansen Research Institute	Graduate associate dealing with human and bovine TB research mainly isolation and characterization of mycobacterium.

4. Language skill

Tigrigna:	Mother tongue
Amharic:	Writing and speaking
English:	Writing and speaking
Deutsch:	Fair knowledge

5. Membership

Member of the Ethiopian Veterinary Association

6. Research output

1) Major causes of carcass condemnation in abattoir and their economic significance.
(Seminar paper, 1990)

2) Efficacy test of two insecticides deposited on targets against Glossina morsitans.
(DVM thesis paper, 1991)

3) Epidemiology of small ruminant diseases around Debre Zeit: An example of Pox.
(Submitted for publication)

4) Characterization of Dermatophilus congolense, Ethiopian isolates.
(Submitted for short communication)

5) Epidemiology and zoonotic importance of bovine tuberculosis.
(MSc thesis)

7. References

1) Professor Sven Britton
Director, Armauer Hansen Research Institute
Addis Ababa, Ethiopia

2) Dr. Getachew Abebe, Associate Dean
Faculty of Veterinary Medicine, Addis Ababa University
Ethiopia

3) Professor Karl-H. Zessin
Course director, Tropical Veterinary Epidemiology,
Freie Universität Berlin
Germany

10. Signed Declaration Sheet

I the under signed, declare that the thesis is my original work and has not been presented for a degree in any University.

Name Tadele Kiros

Signature

Date of Submission 03/01/98

This thesis has been submitted for examination with our approval as University advisors.

Dr. Ademe Zerihun Dr. Ademe Zerihun

Dr. Lübke-Becker

Epidemiology & Zoonotic Importance of Bovine Tuberculosis in selected sites of Eastern Shoa, Ethiopia

Tadele Kiros