PATHOLOGICAL INVESTIGATION OF BOVINE TUBERCULOSIS EMPHASIZED ON TUBERCULIN HYPERSENSITIVITY TEST

A THESIS

BY

GANAPATI RAY

SEMESTER: MARCH – AUGUST/ 2011
REGISTRATION NO.: 1005027
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MASTER OF SCIENCE (M. S.)

IN

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DEPARTMENT OF PATHOLOGY AND PARASITOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND
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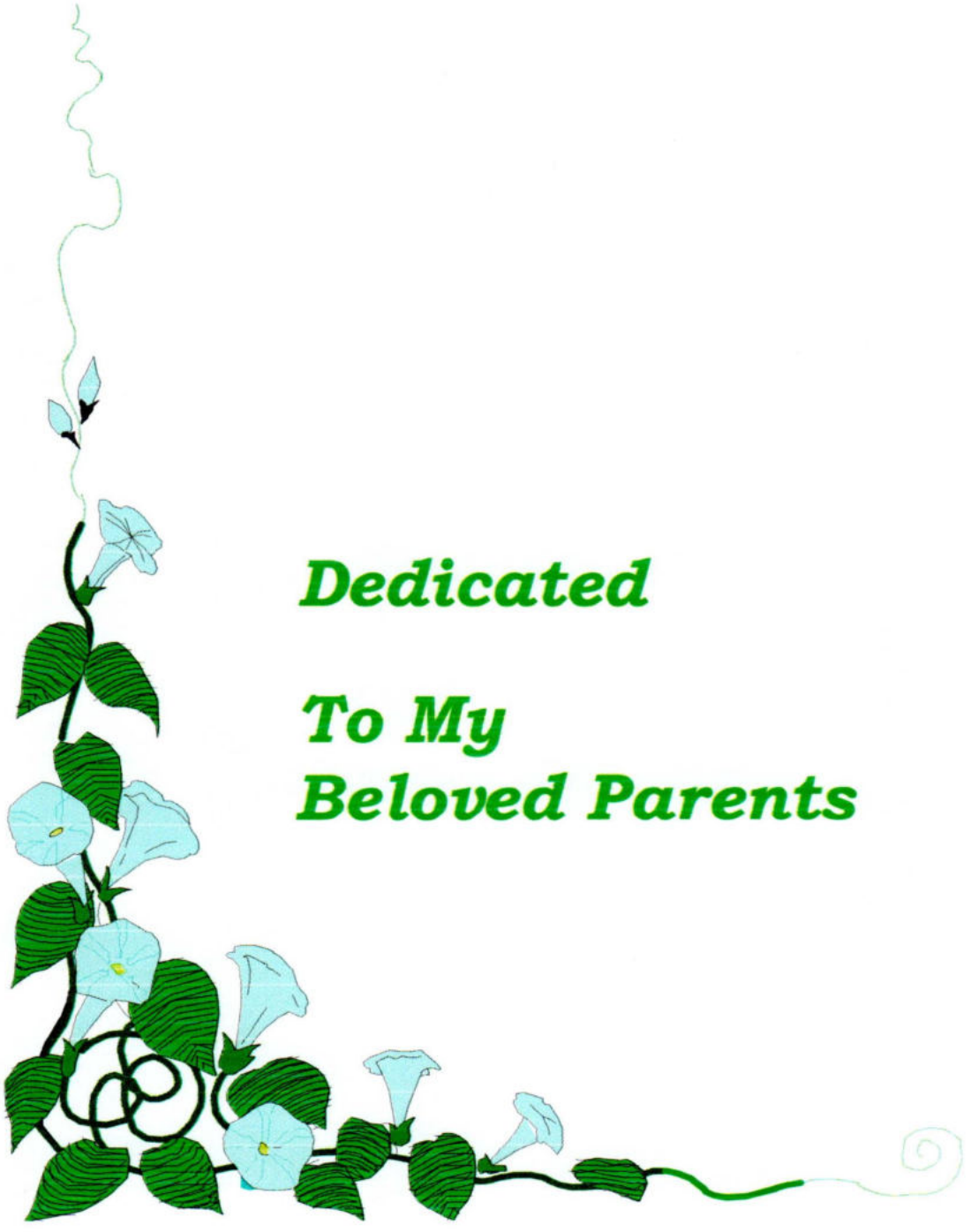

Dedicated

To My
Beloved Parents

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ABSTRACT

The study was designed to investigate the pathological conditions of bovine tuberculosis (BTB) emphasized on tuberculin hypersensitivity test in HSTU campus, Dinajpur and it's surrounding regions during March –December, 2011. A total of 150 cattle (over the age of 6 months) were selected for tuberculin test by mammalian PPD (purified protein derivative) at caudal fold area in intradermal route regardless of sex and breed. In the present study, 5 (3.33%) out of 150 cattle showed tuberculin positive reaction (distinct and visible swelling) and 6 (4.00%) cattle showed doubtful reaction (indistinct and invisible swelling). Hematological examination showed an increase in TLC; decrease in TEC, Hb, and PCV% in tuberculin positive animals compared to negative ones and an increase in Globulin content, decrease in total serum protein and albumin in tuberculin positive animals in case of biochemical examination but was statistically insignificant. Grossly congested, consolidated localized nodular type lesions found in the lungs and small nodule found also in liver and histopathologically granulomatous nodular lesions with purple coloured necrotic mass surrounded by various reactive cells and fibrous connective tissues were found in lungs pursued by necropsy of selected tuberculin positive animal. But acid fast bacilli were not found in aspirate smears of lungs and spleen by classic Ziehl–Neelsen staining. The study dictates a positive correlation among the tuberculin hypersensitivity test, gross and histopathological study of visceral organs, hematological and biochemical examinations.

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AFB	-	Acid-fast bacilli
bp	-	Base pair
BCG		Bacille Calmette-Guérin
BTB	-	Bovine Tuberculosis
cm^3	-	Centimeter
CFU	-	Colony forming unit
CFT	-	Caudal fold test
CFP	-	Culture filtrate unit
CFTCCT		Caudal fold test and Comparative Cervical test
DTH	-	Delayed type hypersensitivity
DNA	-	Deoxyribo nucleic acid
DW	-	Distilled water
EDTA	-	Ethylene di-amino tetra acetic acid
ELISA	-	Ezyme Linked Immunosorbent Assay
ESR	-	Erythrocyte sedimentation rate
et al.	-	And his associates
Etc.	-	Etcetera
Fig.	-	Figure
G	-	Gram
H and E	-	Hematoxylin and Eosin
Hb	-	Hemoglobin
hr	-	Hour
HSTU	-	Hajee Mohammed Danesh Science and Technology University
IFN-γ	-	Interferon gamma
IL	-	Interleukin
ITT	-	Intradermal tuberculin test
M.	-	Mycobacterium
mg	-	Milligram

MGIT	-	Mycobacterium growth indicator tube
Min	-	Minute
M-PCR	-	Multiplex Polymerase chain reaction
ml	-	Milli Liter
MS	-	Master of Science
MTC	-	Mycobacterial tuberculosis complex
N	-	Normal
nm	-	Nanometer
No.	-	Number
N-PCR	-	Nested Polymerase chain reaction
NTM	-	Non tubercular Mycobacteria
PBS	-	Phosphate buffer solution
PCR	-	Polymerase chain reaction
PCV	-	Packed cell Volume
PM	-	Postmortem
PNCEBT	-	Plan of national control and eradication of bovine tuberculosis
PPD	-	Purified protein derivative
RBC	-	Red blood cell
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
rpm	-	Rotation per minute
sq	-	Square
TB	-	Tuberculosis
TEC	-	Total erythrocyte count
TLC	-	Total leukocyte count
TU	-	Tuberculin unit
WBC	-	White blood cell
%	-	Percent
µg	-	Micro gram

CHAPTER I

INTRODUCTION

Bovine tuberculosis (BTB) is an important zoonotic disease caused by intracellular infection with the acid-fast bacilli *Mycobacterium bovis.* This bacterial infection is found in a broad host range (Wedlock *et al.*, 2002; Ameni *et al.,* 2007). In cattle, exposure to this organism can result in a chronic disease that jeopardizes animal welfare and productivity and in some countries leads to significant economic losses (Kranss and stuzewski, 1960). Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide (Lima *et. al.,* 2005). Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly important in developing countries (Anon, 1990; Cadmus, 2002; WHO, 2002; Ofukwu *et. al.* 2006).

BTB is a contagious disease, which can affect most warm-blooded animals, including human being (Blood *et al.*, 1979; Radostits *et al.,* 2000). Cattle, goats and pigs and the domestic livestock are most susceptible to infection, while horses are relatively resistant to infection.

This disease has socio-economic or public health importance and is of great significance to international trade of animals and animal products (OIE, 2004). It is a chronic debilitating disease of animals associated with progressive emaciation and tubercle (granuloma) formation that are mostly confined to respiratory system, primarily in the lungs, lymph nodes and occasionally in other organs (Menzies and Neill, 2000).

In addition to this, the causative organism not only produces tubercles but also affects hemato-biochemical parameters as various diseases have an adverse effect on the haematological parameters (Sattar and Mirza, 2009).

The disease is prevented and controlled in the most developed countries by regulations, due to its ability to infect humans and cause significant livestock production losses. The development process of the eradication of TB usually shows a tendency to establish epidemiologic surveillance systems by the systematic collection of tuberculous sample for their subsequent culture, disease confirmation and epidemiological trace back of the outbreak, which usually ends in the total culling of the herd that have tested positive by means of the agent isolation.

Animals can be diagnosed before death by TB screening test (Tuberculin hypersensitivity test), staining of lymphnode aspirates or other body fluids by acid fast stain, taking biopsies and culturing suspicious lesions. The overall routine diagnosis of this disease is made by intradermal tuberculin test, while the gold standard is histopathological examination of morbid tissues and culture isolation of organism along with IFN-γ (gamma interferon) based assays (Wood and Jones, 2001) and molecular diagnostic systems based on the real-time PCR technology (Q-PCR) (Sawyer *et al.,* 2007).

As it is a zoonotic disease (transmissible from bovine to man and vice versa), it is very essential to find out the prevalence of the disease in different regions.Tuberculin testing has traditionally been used to determine the prevalence of infection of TB in animals and human. Variation in the haemogram picture is a good aid in the diagnosis and prognosis of a disease and as such it has been a subject of study in several diseases of animals.

In the regions of Dinajpur district, no such study was performed to investigate the pathological conditions of bovine tuberculosis. Under these circumstances, it is necessary to explore a study to detect the prevalence and to investigate the pathological conditions of TB infection in animals. Rapid and accurate detection of *Mycobacterium sp.* is of paramount importance in the effective treatment, control and eradication of TB in man and animal.

However, the present study was conducted with aim and fulfillment of the following objectives:

- To determine the prevalence of BTB in the HSTU campus and surrounding regions;
- To detect the correlation between different hemato-biochemical features in the PPD (purified protein derivative) positive cattle with those of PPD non reactors;
- To study gross and histopathology of necropsy specimens collected from typically TB affected animals;
- To study acid-fast staining of lung and spleen aspirates from necropsy.

CHAPTER II

REVIEW OF LITERATURE

In this study, available and relevant literatures are reviewed emphasized on tuberculin test of cattle, hematological and biochemical examination of tuberculin positive and negative animals with gross and histopathological changes of visceral organs and classic Ziehl–Neelsen straining to detect acid fast bacilli. A number of studies have been conducted on tuberculin test of cattle highlighting different pathological explorations after a brief overview on the zoonotic importance, etiology, epidemiology, diagnosis and prevalence.

2.1. Tuberculosis

An infectious disease caused by the tubercle bacilli and characterized by the formation of tubercles in various tissues of the body; especially in lungs is called tuberculosis. Tuberculosis (TB) is a contagious disease of both animals and humans. *Mycobacterium bovis* is the specific cause of TB in cattle, can be transmitted from livestock to humans and other animals (Romano *et al.,* 1996; Ameni *et al.,* 2007).

The presence of bovine tuberculosis (*Mycobacterium bovis*) in the Kruger National Park (KNP) was determined for the first time in 1990 (Vos *et al.,* 2001). It was diagnosed in an African buffalo (*Syncerus caffer*) bull, which was found recumbent and in an emaciated and moribund state near the south-western boundary fence. This prompted an investigation into the bovine tuberculosis (BTB) status of the KNP, with emphasis on its epidemiological determinants and risk factors.

Bovine tuberculosis (BTB) is caused by *M. bovis*, which can infect a wide range of animal species and cause approximately 2000 human deaths per annum (6%) world wide (Parthiban *et al.,* 2007). Bovine tuberculosis has severe implications for animal health since it causes reduced productivity and premature death in cattle in affected farms, causing severe economic losses.

Tuberculosis lesions in cattle are most often found in organs rich in reticuloendothelial tissue, particularly the lungs and associated lymph nodes, as well as the liver (Pritchard *et al.,* 1988; Corner *et al.,* 1990). Tuberculous granulomas may be found in any of the

lymphnodes, or there may be generalized tuberculosis (Cvetnic *et al.,* 2005). The most common signs are difficulty in breathing, coughing and poor reproductive performance. Several showed respiratory signs and 3 showed central nervous signs including ataxia, blindness and circling. Post mortem examination revealed extensive lesions of tuberculous pneumonia (Omer *et al.,* 2001).

Cows with positive test results produced less milk than cows with negative test results after adjusting for variables biologically related to milk production (Hernandez *et al.,* 1998). Calving season was associated with significant reduction in milk production.

2.2. Classification of Tubercle Bacilli

Tuberculosis is caused by certain of the organism, which are curved or rod-shaped, sometimes filamentous, acid fast bacteria (grow in 2-12 weeks) (Wilkins *et al.,* 2008).

- *M. tuberculosis*- human tubercle bacillus
- *M. bovis* -bovine tubercle bacillus
- *M. africanum*-African tubercle bacillus
- *M. microti*-Vole tubercle bacillus

2.3. Bovine tuberculosis

Bovine tuberculosis is one of the most important infectious diseases of cattle (Gortazar *et al.*, 2008). It is caused by infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis (*Anon, 1990; Cadmus, 2002; WHO, 2002; Ofukwu *et al.*, 2006). While *Mycobacterium bovis* is a major cause of pulmonary tuberculosis in cattle, it is also the primary cause of extra-pulmonary tuberculosis in humans (Cotter *et al.*, 1996; Cousins and Dawson, 1999).

Their epidemiologies in developed and developing countries differ, owing to differences in the implementation of preventive measures (Mishra *et al.*, 2005). Identification and differentiation of these closely related mycobacterial species would help to determine the source, reservoirs of infection and disease burden due to diverse mycobacterial pathogens.

2.4. Epidemiology

2.4.1. Susceptible hosts

All age groups and species are susceptible but infection is predominantly found in cattle and pigs (Radostits *et al.,* 2000). No other TB organism has as great host range as bovine TB, which can infect all warm-blooded vertebrates (animals with a backbone). *Mycobacterium avium* can affect all species of birds, as well as hogs and cattle. *Mycobacterium tuberculosis* primarily affects humans but can also be transmitted to hogs, cattle, and dogs (Wedlock *et al.*, 2002; Evans *et al.*, 2007).

2.4.2. Source of infection

Infected cattle are the main sources of infection and primary source of infection for human is ingestion of unpasteurized milk and close association between man and animals but wild life reservoirs are important in some regions. Commonly entry is affected by inhalation or ingestion (Cotter *et al.*, 1996; Cousins and Dawson, 1999).

2.4.3. Mode of transmission

Portal entry is the lung; pathogen is carried as airborne particles (droplet nuclei); exposure to airborne bacilli from sputum of infected persons; direct invasion of mucous membranes or breaks in skin; bovine tuberculosis from exposure to infected cattle (airborne, ingestion of raw milk or dairy products, ingestion of meat from infected animals) (Neill *et al.,* 1994; Collins *et al.*, 1995); medical personnel at risk while performing autopsies, intubation, bronchoscopies or by dermal innoculation (Hancox *et al.*, 2006).

The link between drinking milk from diseased cows and the development of scrofula, cervical lymph node tuberculosis, was established mid-19th century when more than half of all cervical lymphadenitis cases in children were caused by *M. bovis*. Infection acquired through ingesting *M. bovis* is more likely to result in non-pulmonary forms of disease (Neill *et al.,* 1994).

2.4.4. Incidence of infection

The incidence of BTB detected by tuberculin test varies due to variation of place, season; breed, age, sex of animals. The incidence of TB caused by *M. bovis* of agricultural workers in Kazakhastan(USSR) was observed from 9.5% to 25% (Blagodernyi *et al.*, 1975) and overall 5.9% incidence of BTB was detected at Pabna (Bangladesh) milk shed areas under the cattle development project by intradermal tuberculin test (Pharo *et al.*, 1981) of which 3.4% showed positive reaction and 2.6% doubtful reaction. Incidence rates of TB in cattle have continued to increase from 0.3% in 1976 to as high as 7.3% in 2003 (Ofukwu *et al.*, 2006).

The epidemiology of tuberculosis (TB) in the United States is changing as the incidence of disease becomes more concentrated in foreign-born persons. *Mycobacterium bovis* appears to be contributing substantially to the TB incidence in some national communities with ties to Mexico. Some scientists conducted a retrospective analysis of TB case surveillance data from the San Diego, California, region from 1994 through 2005 to estimate incidence trends, identify correlates of *M. bovis* disease, and evaluate risk factors for deaths during treatment (Ramos *et al.*, 2002). *M. bovis* accounted for 45% (62/138) of all culture-positive TB cases in children (<15 years of age) and 6% (203/3,153) of adult cases. *M. bovis* incidence increased significantly ($p = 0.002$) while *M. tuberculosis* incidence declined ($p<0.001$). Almost all *M. bovis* cases from 2001 through 2005 were in persons of Hispanic ethnicity. Persons with *M. bovis* were 2.55x ($p = 0.01$) as likely to die during treatment as those with *M. tuberculosis.*

Cattle in larger herds and in herds under poor management conditions are more likely to give positive reactions (Asseged *et al.*, 2000). The risk of a positive reaction varied also with the animal's age/parity. Other factors such as: mixing, location, breed, physiological state and body condition scores did not appear to significantly contribute to tubercular infection in the study area.

2.4.5. Prevalence of bovine tuberculosis

The prevalence of tuberculosis caused by *M. bovis* in developing countries is largely unknown due to the complexities and prohibitive cost in differentiating between mycobacterial species (Cockle *et al.,* 2006). The organism is known to be widely distributed and the zoonotic importance of *M. bovis* is potentially a serious public health problem, particularly in areas badly affected by the HIV pandemic and where effective controls through pasteurization and the slaughter of infected animals are not applied.

Since implementation of the National Plan for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT) in Brazil, the prevalence of the disease was reported to range from 0.7 to 3.3% (Ribeiro *et al.*, 2003; Baptista *et al.*, 2004; Poletto *et al.*, 2004; Oliveira *et al.*, 2007).

The prevalence of BTB infection as determined by single comparative intradermal tuberculin test (SCITT) was 1.3%, whereas the non-specific infection prevalence was 6%. In the pastoral sector, the prevalence was 1 and 7%; under intensive systems, they were 2 and 6% for BTB and non-specific infections, respectively. The prevalence was significantly higher in the intensive than pastoral production systems (Shirima *et al.*, 2003). However, the prevalence of BTB infection was higher in the small-scale (3%) than in other production systems (0.6-1.1%). Non-specific infections were lowest in the small-scale dairy sector (4%) than in other dairy-production systems (6-11%).

A total of 626 animals (251 cows and 376 buffaloes) were tested for tuberculosis and Johne's disease using single intradermal test (Singh *et al.*, 2004). Prevalence of tuberculosis (9.09%) was found to be significantly higher ($P<0.01$) than paratuberculosis (2.71%).

Mycobacterium bovis PPD tuberculin test for bovine tuberculosis was conducted on 61 cattle and 13 water buffaloes from 45 households in 10 barangays in Los Banos, Laguna (Cataluna *et al.,* 2006). Skin thickness was measured before and 72 h after intradermal administration of 0.1 ml tuberculin using a sliding caliper. Eight (13%) cattle and 5 (38%) water buffaloes tested positive.

The prevalence of tuberculosis in buffaloes on the basis of comparative intradermal tuberculin test revealed it to be from as high as 8.48% (14/165) to as low as 2.45% (4/163) on the basis of positive reaction to bovine PPD.

However, a doubtful reaction was observed in 8.58% (14/163) of buffaloes at farm 2 with low prevalence. It was also observed that the reaction to bovine or avian PPD was much stronger in buffaloes compared with indigenous cattle (Javed *et al.,* 2010).

After the discovery of TB in two cows at meat inspection from an apparently healthy 57-cow herd in Barbados, 50 cows and one bull were submitted to the single intradermal comparative test of cervical skin using PPD tuberculin; 30 reactions were positive and seven suspicious (Wilson *et al.,*1980). Post mortem examination of these 37 animals revealed macroscopic lesions ranging from minute foci to entire mediastinal lymph nodes, which were tubercular. Tissue specimens were removed for histology, culture, biochemical, drug susceptibility and animal inoculation tests. All strains isolated were identified as *M. bovis*.

Four groups of six calves were infected experimentally with either a low dose of approximately 104 colony-forming units (cfu) or a high dose of approximately 106 cfu of *Mycobacterium bovis*. Each dose was delivered by the intranasal and intratracheal routes. More severe disease was observed in the groups inoculated with the high dose (McCorry *et al.,* 2005). Visible lesions were identified in 21 of the 24 animals, all of which also gave positive skin tests and interferon- gamma (IFN- gamma) responses. Nasal shedding was detected in 15 of the 24 animals and the frequency of shedding was influenced by both the route and the dose of infection; no shedding was observed in the group infected intratracheally with the low dose. Two of the 15 confirmed shedders had no visible lesions at postmortem examination; both of these calves gave IFN- gamma responses but only one was skin test positive.

2.5. Pathogen risk factors

The causative organism is moderately resistance to heat, desiccation and many disinfectants. It is readily destroyed by direct sunlight unless it is in a moist environment

(Radostits *et al.*, 2000). In warm, moist, protected positions, it may remain viable for weeks.

2.6. Pathogenesis and Pathology

Adult cattle are usually infected by inhaling invisible droplets containing the bacteria into their lungs while pre-weaned calves are more often infected by drinking contaminated milk. Since the bacterium is usually inhaled into the lungs in adult cows, the most common clinical signs are related to pulmonary tuberculosis. Once the bacteria enter the lungs, they begin to multiply and formation of tubercle (granuloma) that are mostly confined to respiratory system, primarily in the lungs, lymph nodes and occasionally in other organs ((Menzies and Neill, 2000; Kirk *et al.*, 2002). At this primary site of infection, the lesions can remain quietly inactive or develop further depending on the ability of the cow to fight off the infection. When the immune system of the cow is fully activated, the infection may remain limited to the lungs and could go undetected for the productive life of the cow. If the infection over-powers the body defenses, the bacteria may be carried to other locations in the body in the lymph or blood circulation. New areas of infection often occur in the lungs, kidneys, liver, spleen and the lymph nodes associated with these organs. In cases allowed to progress over an extended period of time, lesions may be present in the uterus or mammary gland. When calves are exposed to the tuberculosis bacteria in the milk, the most common lesions are noted in the lymph nodes at the junction of the neck and head around the throat. The main sign may be swelling of these nodes.

Indeed, most studies of naturally infected cattle describe the majority of lesions within the lungs (McIlroy *et al.,* 1986), pulmonary lymph nodes and cranial lymph nodes (Stamp, 1944; Lepper and Pearson, 1973).

Lesions of bovine tuberculosis are typically characterized by the presence of tubercles with central caseation and calcification. In the early stages of infection, these lesions are not encapsulated, but are surrounded by condensed alveolar tissue. Initially, there is the presence of epitheloid and giant cells at the center of the tubercle, and, as the disease

progress, they are surrounded by lymphocytes, plasma cells and monocytes, developing a peripheral fibroplasia and central caseous necrosis (Neill *et al.,* 1994).

Small lesions in the anterior lobes of the lungs of young sheep were divided macroscopically into band and localized types. Localized lesions were subdivided into red, red-grey, grey-red and grey categories. Histological examination revealed 4 types of lesions. Two major types were equally represented. One type showed atelectasis with little or no inflammation and the other was characterized by infiltration with eosinophils. Lesions with inflammation characterized by neutrophils and others with marked lymphoreticular cuffing of airways were seen infrequently. The range of lesion types supported a previous view that the small lesions may have numerous aetiologies. Most small lesions were not directly related histologically to larger consolidated lesions but might predispose the lung to infections that lead to them. Small lesions with inflammation characterized by neutrophil infiltration might possibly progress directly to larger consolidated lesions (Pfeffer *et al.* 1979).

2.7. Minimum Infective Dose of *Mycobacterium bovis* in Cattle

To determine the minimum infective dose of *Mycobacterium bovis* is necessary to stimulate specific immune responses and generate pathology in cattle. Four groups of calves (20 animals) were infected by the intratracheal route with 1,000, 100, 10, or 1 CFU of *M. bovis*. Specific immune responses (gamma interferon [IFN-γ] and interleukin-4 [IL-4] responses) to mycobacterial antigens were monitored throughout the study and the responses to the tuberculin skin test were assessed at two times. Rigorous post mortem examinations were performed to determine the presence of pathology, and samples were taken for microbiological and histopathological confirmation of *M. bovis* infection (Dean *et al.,* 2005). One-half of the animals infected with 1 CFU of *M. bovis* developed pulmonary pathology typical of bovine tuberculosis. No differences in the severity of pathology were observed for the different *M. bovis* doses. All animals that developed pathology were skin test positive and produced specific IFN-γ and IL-4 responses. No differences in the sizes of the skin test reactions, the times taken to achieve a positive IFN-γ result, or the levels of the IFN-γ and IL-4 responses were observed for the different *M. bovis* doses, suggesting that diagnostic assays (tuberculin skin test and IFN-γ test) can

detect cattle soon after *M. bovis* infection regardless of the dose. This information should be useful in modeling the dynamics of bovine tuberculosis in cattle and in assessing the risk of transmission.

2.8. Immune response against mycobacterial infections

After infection, there is an initial interaction between macrophages and mycobacteria, which define subsequent events and the consequences of exposure to tubercle bacilli (Pollock and Neill, 2002). Bacteria can be killed and eliminated from the host, lie dormant, lead to development of active tuberculosis, or reactivate from dormancy at some stage in the future (Welsh *et al.*, 2005).

Apparently, members of this genus may produce spores, as (Ghosh *et al.*, 2009) recently demonstrated with *Mycobacterium marinum.* However, the role of that characteristic on the development of the disease has not been elucidated.

It is well established that *M. bovis* causes a delayed hypersensitivity type (DTH) reaction; T-cell recognition of mycobacterium antigens may be the major immune response to tuberculosis (Alito *et al.*, 2003; Pollock *et al.*, 2005; Welsh *et al.*, 2005).The immune response against mycobacterial infections is dependant upon a complex interaction between T lymphocytes and macrophages in the context of the granuloma (Liebana *et al.*, 2007).

CD8+ T cells (CD8 cells) have been shown to respond to mycobacterial antigens in humans, cattle, and mice. To determine the role of CD8 cells in bovine TB in vivo, two groups of calves were infected with the virulent *M. bovis* strain AF2122/97. After infection, one group was injected with a CD8 cell-depleting monoclonal antibody (MAb), and the other group was injected with an isotype control MAb. Immune responses to mycobacterial antigens were measured weekly in vitro. After 8 weeks, the animals were killed, and postmortem examinations were carried out (Liébana *et al.,* 1999). In vitro proliferation responses were similar in both calf groups, but in vitro gamma interferon (IFN-γ) production in 24-h whole-blood cultures was significantly higher in control cattle

than in CD8 cell-depleted calves. Postmortem examination showed that calves in both groups had developed comparable TB lesions in the lower respiratory tract and associated lymph nodes. Head lymph node lesion scores, on the other hand, were higher in control calves than in CD8 cell-depleted calves (Ramos *et al.*, 2003). Furthermore, there was significant correlation between the level of IFN-γ and the head lymph node lesion score. These experiments indicate that CD8 cells play a role in the immune response to *M. bovis* in cattle by contributing to the IFN-γ response. However, CD8 cells may also play a deleterious role by contributing to the immunopathology of bovine TB.

2.9. Importance in Dairy Cattle

Tuberculosis, caused by *Mycobacterium bovis* is emerging as the most important disease affecting cattle. Furthermore, it results in a major public health problem when transmitted to humans (Romero *et al.,* 1995). Due to its difficult and non-specific diagnosis, *M. bovis* has been declared to be one of the etiologic agents causing significant economic loss in the cattle industry.

While *Mycobacterium bovis* is a major cause of pulmonary tuberculosis in cattle, it is also found in humans (Cotter *et al.*, 1996; Cousins and Dawson, 1999) where cow milk is usually consumed fresh and unpasteurized.

Tuberculosis in cattle causes substantial damages in the agricultural sector; it is nowadays subjected to expensive eradication programs in most EU countries, Spain among them (Parra *et al.,* 2008).

2.10. Zoonotic importance

Bovine tuberculosis is a zoonotic disease that not only causes huge economic losses but also poses an important risk for human infection (Anon, 1990; Cadmus, 2002; WHO, 2002; Ofukwu *et al.,* 2006).

The current increasing incidence of tuberculosis in humans particularly in immunocompromised humans, has given a renewed interest in the zoonotic importance of *M. bovis*, especially in developing countries. The case and frequency of the spread of

tuberculosis from animals to humans in an uncontrolled environment makes this an important zoonosis (Sreedevi *et al.*, 2003).

Infection in humans occurs largely through consumption of infected milk by children by but spread can also occur by inhalation (Radostits *et al.*, 2000). Transmission to humans can be significantly reduced by paseturization of milk but only complete eradication of the disease can protect the farmer and his family. Transmission from cattle to humans in developed countries is an unlikely event nowadays but still occurs. The widespread occurrence of tuberculosis in exotic animals maintained in captivity adds to the public health importance of these infections.

Mycobacterium tuberculosis can be transmitted from human to cattle that were confirmed by molecular typing of isolates (Ocepek *et al.*, 2005) IS6110-based restriction fragment length polymorphism analysis showed that the isolates from the cattle and farm worker who suffered from pulmonary tuberculosis 1 year prior to this case were the same strains.

2.11. Diagnosis

Bovine tuberculosis is a major problem in many countries; hence, new and better diagnostic tools are urgently needed (Agaard *et al.*, 2006). The diagnosis of tuberculosis in the living animal may be based on clinical findings, the tuberculin test (TB screening test) and demonstration of the organisms in exudates or excretions (Jones *et al.*, 1997).

There are different methods used for the diagnosis of tuberculosis in animals and man (Raval *et al.*, 2006).

Table 1: Recommended tests for diagnosis of TB

Tests	References
Tuberculin test	Monaghan *et al.*, 1994; Collins *et al.*, 1994; Sidibe *et al.* 2003; Bo Norby *et al.*, 2004; Brasil, 2006
Isolation and identification of organisms	Thorel, 1994; Salfinger and Pfyffer, 1994; Rinsho *et al.*, 1998; Kekkaku *et al.*, 2003; Rodriguez *et al.*, 2004
Histopathology and Immunohistological examination	Wards *et al.*, 1995; Lilenbaum *et al.*, 1999; Bai *et al.*, 2004; Pollock *et al.*, 2005; Miranda *et al.*, 2007; Purohit *et al.*, 2007; Liebana *et al.*, 2008
Serological test	Silva, 2001; de la Rua Domenech *et al.*, 2006; Lilenbaum *et al.*, 2001; Fonseca, 2006; McNair *et al.*, 2006
Haematological and Biochemical Parameters	Samad and Rahman, 1986; Amin, 1989; Rao *et al.*, 1992; Kumar *et al.*, 1994; Hemalatha *et al.*, 1995; Sattar and Mirza, 2009; Mohankumar *et al.*, 2010; Javed *et al.*, 2010
Molecular detection	Liebana *et al.*, 1995; Sreevatsan *et al.*, 2000; Kim *et al.*, 2001; Taylor *et al.*, 2001; Sreedevi *et al.*, 2004; Mishra *et al.*, 2005; Young *et al.*, 2005; Nassar *et al.*, 2007; Sawyer *et al.*, 2007

2.11.1. Tuberculin test

Tuberculinisation or intradermal tuberculin tests, have been used for more than 100 years, and are currently the method most widely used for diagnosing BTB in cattle (Monaghan *et al.*, 1994).

All animals over 3months of age should be tested and positive reactors disposed of according to local legislation. Suspicious reactors are retested at intervals appropriate to the test used (Radostits *et al.*, 2000). At the initial test, a careful clinical examination should be conducted on all animals to ensure that there are no advanced clinical cases

which will give negative reactions to the test. Doubtful cases and animals likely to have reduced sensitivity, particularly old cows and those that have calved within the previous 6 weeks, may be tested by one of the special sensitivity or serological tests.

The test, also known as single cervical intradermal tuberculin test (SITT) or caudal fold test (CFT) is based on an injection of a purified protein derivative (PPD) of *M. bovis* origin (bovPPD). When performed in parallel to the injection of PPD of *M. avium* PPD (avPPD), the test is known as the comparative cervical intradermal tuberculin test (CITT). Seventy-two hours after injection, the skin thickness is measured with calipers, as skin swelling is a measure of hypersensitivity to the antigens used (Brasil, 2006).

Cattle infected with *M. avium, M. tuberculosis, M. avium paratuberculosis*, *Nocardia farcinius,* or other mycobacteria could be reactive to bovine PPD, leading to false-positive results. As mycobacteria shares several antigens, cross reactions are common, reducing test specificity. Therefore, comparative intradermal tests are performed with the purpose of reducing the occurrence of such cross reactions; however, this approach does not completely eliminate nonspecific reactions (Collins *et al.,* 1994).

Though bovine PPD (made from *M. bovis*) is specific for BTB, however, reactivity to tuberculin made from both human and bovine bacilli (mammalian tuberculin) is similar and is usually greatest in animals sensitized specially to these bacilli (Francis *et al.,* 1978).

One thousand eighty seven animals were tested for tuberculosis by comparative intradermal reaction test. 202 cattle (18.58%) were found positive. The herd prevalence rate was 94.44% (34 of 36 herds tested) (Sidibe *et al.* 2003). Results showed that the positive individual comparative intradermal prevalence varied according to the age and breed of the animals, with higher incidence in adults over 10 years old (44.18%) and in imported cattle breeds and crossbreds (22.42%).

Mycobacterium bovis was isolated from 43 animals. Using all 7 herds, the sensitivities of the caudal fold test (CFT), the caudal fold and comparative cervical skin tests used in

series (CFTCCT) by using tuberculin; and gross necropsy were 93.02%, 88.37%, and 86.05%, respectively. The sensitivities of the 2 skin tests were slightly higher when 2 or more gross lesions were present, and the sensitivity of gross necropsy was significantly higher (P 5 0.049). The sensitivity of the CFT was found to be notably higher than most estimates in other studies (Bo Norby *et al.*, 2004).

2.11.2. Isolation and identification of organisms

2.11.2.1. Sample collection

The choice of appropriate clinical specimen is very important for isolation of *M. bovis* and *M. tuberculosis* from cattle. A total of 768 specimens (heparinized or EDTA containing blood (162), fine needle aspirates from prescapular lymph gland (PSLG,160), milk (154), pharyngeal swab (PhS, 98), rectal pinch (RP, 97) and faecal sample (97) from 161 cattle of organized cattle farms in north India suspected to be suffering from tuberculosis were analyzed (Wood *et al.,* 1992). After decontamination by modified Petroff's method isolation of *M.tuberculosis* complex was done on Lowenstein-Jensen medium (with and without pyruvate). The culture isolates were identified *as M. tuberculosis* and *M. bovis* on the basis of biochemical tests.

Some group observed the prevalence of the tuberculin skin test in 10 dairy farm areas in Addis Ababa; and detects the tuberculous lesions and causal agents from tissue samples of the respiratory tract and mesenteric lymph nodes of the slaughtered cattle (Adams *et al.,* 2001).

2.11.2.2. Suitable Media

A variety of different media for the cultivation of mycobacteria have been described but a few of them are in use today.

Therefore, media containing sodium pyruvate, in lieu of glycerol, are used for isolation of *M. bovis* (WHO, 1996). Furthermore, it is generally accepted that mycobacteria grow more rapidly in liquid medium (Salfinger and Pfyffer, 1994).

Those currently used can be characterized by three basic types (Kekkaku *et al.*, 1998); the first is egg-based media represented by Ogawa and Löwenstein-Jensen. The second type is

agar-based media; the most common one are Middlebrook 7H10 and 7H11. The third type is liquid media such as Middlebrook 7H9.

Two systems, the newly developed Mycobacteria Growth Indicator Tube (MGIT) and biphasic Septi-Chek AFB based on liquid media, proved to be significantly better than the egg-based solid media for the isolation of mycobacteria from clinical specimens (Rinsho *et al.*, 1998). The isolation of Mycobacterium tuberculosis by MGIT occurred 8 days previous to the isolation by the conventional Ogawa method. These results indicate that the MGIT system is efficient for the recovery of mycobacteria.

Growth of *M. bovis* may take up to 6-8 weeks (Wards *et al.*, 1995). On a suitable pyruvate-based solid medium, colonies are smooth and off-white. Although characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*, every isolate needs to be confirmed (OIE, 2008). The identification is made in two steps: the first is to obtain a primary culture of the bacillus, and the second is identification, based o physiological and biochemical characteristics (Thorel, 1994).

2.11.2.3. Identification

The quality of specimens collected and the proper transport of those specimens to the laboratory are critical to successful isolation of etiological agents. Most mycobacteria grow at a relatively slow rate. Therefore, the acid-fast smear plays an important role in the early diagnosis of mycobacterial infection. There are several methods of determining the acid-fast nature of an organism. In the carbolfuchsin procedures (Ziehl-Neelsen, Kinyoun), acid-fast organisms appear red, and in the fluorochrome procedures (auramine O, auramine-rhodamine, acridine orange), the acid-fast organisms fluoresce yellow to orange. Fluorochrome-stained slides may be directly restained with any of the carbolfuchsin staining procedures (Kekkaku *et al.*, 2003). This may be done to confirm a positive fluorochrome slide and to study organism morphology.

Although microscopic examination of smears is faster and cheaper than any other method, visualization of acid-fast bacilli (AFB) is not able to discriminate among members of the *Mycobacteriaceae* family, or between members of the genus

Mycobacterium and other organisms which share this acid-fast staining characteristic, including certain species of *Legionella, Nocardia, Rhodococcus, Tsulunnurella, Cryptosporidium,* and *Cyclospora* (Eisenstadt and Hall, 1995). Additionally, this method lacks sensitivity (Wards *et al.,* 1995) and can only reveal the presence of AFB when concentrations are exceeding 104 bacteria per milliliter (Rodriguez *et al.,* 2004).

2.11.3. Histopathology and Immunohistological examination

Histological examinations are practical and inexpensive, and useful to make decisions on grossly suspect carcasses (Wards *et al.,* 1995). Another advantage of histopathology is increased diagnostic sensitivity when it is performed in conjunction with culture (Liebana *et al.,* 2008). Fráguas *et al.* (2008) examined 97 tuberculin-reactive animals and tested the value of histological examination as a complementary tool. In that study, 64.9% of the samples had characteristic lesions, with concordance among macroscopic evaluation, histological examination, and microscopy. This high concordance could be a consequence of a correct carcass gross examination. Despite those advantages, the requirement for obtaining postmortem samples limits the diagnostic process (Lilenbaum *et al.,* 1999), and most lesions can be paucibacillary (Liebana *et al.,* 2008), leading to false-negative results.

The immunohistological examination is more sensitive than the traditional Ziehl-Neelsen technique. In addition to being a diagnostic tool, it also provides information regarding host immune responses (Pollock *et al.,* 2005). Immunological approaches include the use of cell markers (Bai *et al.*, 2004; Miranda *et al.*, 2007), cytokines (Bai *et al.*, 2004; Kiszewski, 2006), *Mycobacterium* cell-wall antigens (Purohit *et al.*, 2007), and adhesion molecule markers (Miranda *et al.*, 2007). Various anti-BCG antibodies for immunohistochemistry are commercially available, but Purohit *et al.* (2007) demonstrated that the use of anti-MTP-64, a specific antigen for *M. tuberculosis* complex, seems to be a more sensitive and specific marker.

2.11.4. Serological test

It is well established that cattle infected by *M. bovis* develop early immune responses, dominated by cell-mediated immunity (Fifis *et. al.,* 1994; Alito *et al.,* 2000; McNair *et al.,* 2001; Pollock *et al.,* 2005; Welsh *et al.,* 2005). Therefore, serology is less efficient to

identify cattle in the early stages of infection, when antibodies titers are low (Wood and Rothel, 1994).

Serological tests including complement fixation, Flurescent antibody, direct bacterial agglutination, precipitin and hemagglutination tests have been developed but have little potential value for the routine diagnosis of tuberculosis (Radostits *et al.,* 2000).Early enzyme linked immunosorbent assay (ELISA) tests to detect mycobacterial antigens had limited value but an ELISA which examines antibody to define antigens of *M, bovis* before and after skin testing appear useful in detecting non-specific reactors.

The ELISA (indirect) technique was tested for the detection of *Mycobacterium bovis* antibodies in the diagnosis of bovine tuberculosis, using in parallel the intradermal tuberculin test. 7032 cattle from 13 establishments with different degrees of prevalence of the disease were analysed (Silva *et al.,* 2001). The results showed a great correlation between both techniques in the establishments with low prevalence of the disease and a percentage of positive animals to both systems in all the cases.

The indirect ELISA technique measures the binding of specific antibodies to an antigen (Lilenbaum *et al.,* 1999). In order to diagnose cattle infected by *M. bovis,* antigens usually employed are the PPD and single or associated purified antigens from *M. bovis* (Fifis *et al.,* 1994; Lilenbaum *et al.,* 1999; Lilenbaum *et al.,* 2001; Silva, 2001; Waters *et al.,* 2006).

Although serological assays cannot be considered first choice diagnostic methods, many researchers describe strategies for their use (Silva, 2001; de la Rua Domenech *et al.,* 2006; Lilenbaum *et al.,* 2001; Fonseca, 2006). Their recommendations are based on the existence of anergic animals (Silva, 2001; McNair *et al.,* 2006), as well as increased antibody titres in more advanced stages of the disease (Pollock and Neill, 2002, Welsh *et al.,* 2005). Using ELISA, Lilenbaum and Fonseca (2006) identified tuberculous cows in 18 herds involved on a tuberculosis control program, and subsequently confirmed infection by isolation of *M. bovis* from lung lesions. In those cases, ELISA was employed as a complementary diagnostic test and improved the control of tuberculosis by identifying anergic cows.

2.11.5. Haematological and Biochemical Parameters

The causative organism not only produces tubercles but also affects hemato-biochemical parameters as various diseases have an adverse effect on the haematological parameters (Sattar and Mirza, 2009).

A drop in red blood cell (RBC) count and haemoglobin (Hb) concentration (Chandrasekharan and Ramakhshnan, 1969; Samad and Rahman, 1986; Amin, 1989; Rao *et al.*, 1992; Kumar *et al.*, 1994), PCV (Kumar *et al.*, 1994) and neutrophils (Samad and Rahman, 1986) had been reported in positively reacting cattle. The increase in monocytes (in buffaloes) and lymphocytes (in cattle) correlated to some extent with earlier finding in bison, where these were slightly higher than control animals (Miller *et al.*, 1989). A significant decrease in leukocytes in buffaloes, while non-significant decrease in cattle, was another supportive finding to tuberculin results that had been previously reported and related with peracute to acute form of the disease (Lepper *et al.*, 1977). Similarly, increase in lymphocyte percentage and also total leukocyte count had been reported (Samad and Rahman, 1986). Kumar *et al.* (1994) also reported that depending on the stage and progress of the disease, leukocytosis, leucopoenia, lymphocytosis, lymphopenia, neutrophilia and monocytosis can be observed.

The haematological studies revealed a considerable decrease in RBC, while the decrease in ESR in a significantly ($P = 0.05$) greater number of buffaloes showed a positive or doubtful reaction to mammalian PPD. Total leukocyte count and lymphocyte percentages were higher in a considerably greater number of buffaloes. However, while changes in monocyte and eosinophil count were less significant, combined monocyte count in both positive and doubtful reactors was higher in a considerable number of buffaloes (Javed *et al.*, 2006).

In tuberculin test positive reactors there was an increase in erythrocyte sedimentation rate, mean corpuscular volume, eosinophil & monocyte number and decrease in total erythrocyte count & hemoglobin. There was also increase in mean hemoglobin concentration and decrease in Packed cell volume and Mean corpuscular hemoglobin concentration, which were not statistically significant ($p>0.05$) (Mohankumar *et al.*, 2010).

A recent study was conducted to clarify the usefulness of haematological parameters and changes in serum proteins along with tuberculin test in different animal species. In cattle, significantly lower RBC count, PCV, neutrophils and serum albumin, while lymphocyte percentage was significantly higher in positive reactor animals. It was concluded that tuberculosis caused decrease in erythrocyte parameters including RBC count, PCV and Hb concentration, while it caused an increase in monocytes, eosinophils and lymphocytes (Javed *et al.,* 2010).

In biochemical study, there was no significant difference in the total protein contents between the tuberculin reactors and non-reactors but significant difference in the level of albumin, globulin where positive reactors showed an increase in globulin content and decrease in albumin content (Moses *et al.,* 1975; Kumar *et al.,* 1994; Hemalatha *et al.,* 1995; Mohankumar *et al.,* 2010). However, an increased level of serum total protein was observed in the animals, which had generalized tuberculosis which was observed in cases with large caseated tubercular lesions (Grys, 1965).

2.11.6. Molecular detection

Molecular assays primarily used to analyze the epidemiology of bovine tuberculosis; various strains, including laboratory and clinical samples, have been successfully employed (Cousins *et al.,* 1993; Griffin and Dolan, 1995; Kamerbeek *et al.*, 1997; Zumárraga *et al.,* 1999; Rodriguez *et al.*, 2004; Parra *et al.*, 2008).

A multiplex PCR technique was developed for specific detection of *Mycobacterium bovis*, *M. tuberculosis*, *M. avium* and *M. avium* subsp. *paratuberculosis*, using clinical samples from field cattle (Kim *et al.,* 2001).

Mycobacterium bovis is a slowly growing microorganism, and confirmation of the diagnosis by conventional culture is a lengthy process. A simple, rapid method for the extraction of DNA from bovine tissue samples was developed and used in a PCR designed for the diagnosis of tuberculosis. Tissues from 81 cattle from tuberculosis-infected herds (group 1) and 19 cattle from tuberculosis-free herds (group 2) were tested in this PCR, and the results were compared with those of conventional culture (Liebana *et al.,* 1995). The PCR assay detected 71.4% of the culture-positive animals from group 1. Tissue from all animals in group 2 was negative in the PCR assay and by culture. The described method

could be used as a rapid screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of bovine tuberculosis. The PCR technique is much faster than culture and reduces the time for diagnosis from several months to 2 days. It also provides for the detection of *M. bovis* when rapidly growing *Mycobacterium* spp. are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable.

Biopsy samples (n = 20) were collected from the prescapular lymph nodes of single intradermal tuberculin test positive cattle in an organized dairy farm. *M. bovis* was isolated from 4 samples (Sreedevi *et al.,* 2004). The isolates were cultured and extraction of DNA was carried out. Primers were designed for IS6110 element specific to *M. tuberculosis* complex group of organisms. A 1360 base pair PCR product was observed with the DNA of *M. bovis*, *M. tuberculosis* and *M. bovis* BCG strains while *M. phlei*, *M. avium* and *M. paratuberculosis* were negative. The purified PCR products were then subjected to RE analysis. PvuII enzyme did not vary among the different mycobacterial cultures.

Molecular diagnostic systems, especially based on the real-time PCR technology (Q-PCR), which are directly applied to biological samples, have been recently developed in order to carry out human tuberculosis diagnosis (caused by *M. tuberculosis)* (Miller *et al.,* 2002). The development of procedures for *M. bovis* has been very limited and there has existed just one significant research made on bovine tissue samples (Taylor *et al.,* 2001).

The utility of the hupB gene (Rv2986c in *M. tuberculosis*, or Mb3010c in *M. bovis*) to differentiate *M. tuberculosis* and *M. bovis* was evaluated by a PCR-restriction fragment length polymorphism (RFLP) assay with 56 characterized bovine isolates. The degree of concordance between the PCR-RFLP assay and the microbiological characterization was 99.0% ($P < 0.001$). A nested PCR (N-PCR) assay was developed, replacing the PCR-RFLP assay for direct detection of *M. tuberculosis* and *M. bovis* in bovine samples. The N-PCR products of *M. tuberculosis* and *M. bovis* corresponded to 116 and 89 bp, respectively. The detection limit of mycobacterial DNA by N-PCR was 50 fg, equivalent to five tubercle bacilli. *M. tuberculosis* and/or *M. bovis* was detected in 55.5% (105/189) of the samples by N-PCR, compared to 9.4% (18/189) by culture (Mishra *et al.,* 2005).

A real-time PCR assay for the measurement of gamma interferon (IFN-gamma) mRNA in European badger (*Meles meles*) blood cultures was developed. The levels of IFN-gamma mRNA in blood cultures stimulated with either bovine or avian tuberculin or specific mycobacterial antigens were compared with those in a nonstimulated control blood culture as the basis for determining the tuberculosis (TB) status of live badgers (Sawyer *et al.*, 2007). The assay was validated by testing 247 animals for which there were matching data from postmortem examination and culture of tissues. Relative changes in the levels of IFN-gamma mRNA in response to bovine tuberculin and specific antigens were found to be greater among badgers with tissues positive for TB on culture. The test was at its most accurate (87% of test results were correct) by using blood cultures containing bovine tuberculin as the antigen and when the response to avian tuberculin was taken into account by subtracting the avian tuberculin response from the bovine tuberculin response. At a specificity of 90.7%, the test was 70.6% sensitive.

A rapid and specific method, based on new polymerase chain reaction species-specific primers, which amplifies a 470-base pair fragment of the *M. bovis* genome, was evaluated. A total of 275 milk-producing cows were studied by intradermal tuberculin test (ITT) which gave 184 positive and 91 negative cases. From them, 50 animals were taken from a cattle ranch free of tuberculosis (Romero *et al.*, 1995). Three different samples were collected from each animal (blood, nasal mucus, and milk). Positive results were obtained from 26 animals by PCR (11.4%), 1 by bacteriological culturing (0.4%) and 1 by bacilloscopy (0.4%).

PCR primers specific for the *Mycobacterium tuberculosis* complex were used to detect the presence of *Mycobacterium bovis* BCG (Pasteur) in soil microcosms and *Mycobacterium bovis* in environmental samples taken from a farm in Ireland with a history of bovine tuberculosis. *M. bovis* genes were detected in soil at 4 and 21 months after possible contamination. Gene levels were found in the range of 1x103 to 3.6x103 gene copies g of soil-1, depending on the sampling area. Areas around badger sets had the highest levels of detectable genes and were shown to have the highest levels of gene persistence. *M. bovis*-specific 16S rRNA sequences was detected, providing evidence of the presence of viable

cells in Irish soils. Studies of DNA turnover in soil microcosms proved that dead cells of *M. bovis* BCG did not persist beyond 10 days (Young *et al.*, 2005).

In Brazil, Zanini *et al.* (2001), Leite *et al.* (2003), Figueiredo (2006), and Figueiredo *et al.* (2008a, 2008b), employed PCR techniques for identification of *M. bovis* from tissues, milk and colonies, demonstrating that those tests can be valuable to rapidly identify isolates with the minimum of 10CFU/mL limit of *M. bovis* in milk.

Mycobacterium was verified in animals from a Brazilian dairy herd, a total of 42 samples from 30 cows were submitted to culture and the isolated strains were analyzed by two polymerase chain reaction (PCR), the first specific for species belonging to the Mycobacterium complex (MTC) and the other for differentiating *M. tuberculosis* from *M. bovis* (Nassar *et al.*, 2007). Twenty seven samples (64.3%) from 18 animals (60%) were positive for mycobacteria by culture, including samples from 15 retrofaryngeal lymphnodes (55.5%), 9 prescapular lymphnodes (33.3%), 2 lungs (7.4%), and 1 liver (3.7%). All isolated colonies were confirmed by PCR to contain MTC organisms, and were identified as *M. bovis* by the same methodology.

2.11.7. Differentiation from *M. tuberculosis*

The aim of this work was the design and validation of a rapid and easy single tube multiplex-PCR (m-PCR) assay for the unequivocal differential detection of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Oligonucleotide primers were based on the uninterrupted 229-bp sequence in the *M. bovis* genome and a unique 12.7-kb insertion sequence from the *M. tuberculosis* genome, which is responsible for species-specific genomic polymorphism between these two closely related pathogens (Bakshi *et al.*, 2005). The m-PCR assay was optimized and validated using 22 *M. bovis* and 36 *M. tuberculosis* clinical strains isolated from diverse host species and 9 other non-tuberculous mycobacterial (NTM) strains. The designed primers invariably amplified a unique 168-bp (*M. bovis*-specific) and 337-bp (*M. tuberculosis*-specific) amplicon from *M. bovis* and *M. tuberculosis* strains, respectively. The accuracy of the assay, in terms of specificity, was 100%, as none of the NTM strains tested revealed any amplification product. As little as 20 pg of genomic DNA could be detected, justifying the sensitivity of the method. The m-

PCR assay is an extremely useful, simple, reliable and rapid method for routine differential identification of cultures of *M. bovis* and *M. tuberculosis*. This m-PCR may be a valuable diagnostic tool in areas of endemicity, where bovine and human tuberculosis coexist, and the distinction of *M. bovis* from *M. tuberculosis* is required for monitoring the spread of *M. bovis* to humans.

2.12. Control

Tuberculosis (TB), one of the most widespread infectious diseases, is the leading cause of death due to a single infectious agent among adults in the world. *Mycobacterium tuberculosis* is the most common cause of human TB, but an unknown proportion of cases are due to *M. bovis* (Cosivi *et al.,* 1998). In industrialized countries, due to animal TB control and elimination programs, together with milk pasteurization, have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans. In developing countries, however, animal TB is widely distributed, as control measures are not applied or are applied sporadically, and pasteurization is rarely practiced.

Human and livestock diseases can be difficult to control where infection persists in wildlife populations. In Britain, European badgers (Meles meles) are implicated in transmitting *Mycobacterium bovis*, the causative agent of bovine tuberculosis (TB), to cattle. Badger culling has therefore been a component of British TB control policy for many years. However, large-scale field trials have recently shown that badger culling has the capacity to cause both increases and decreases in cattle TB incidence. Here, we show that repeated badger culling in the same area is associated with increasing prevalence of *M. bovis* infection in badgers, especially where landscape features allow badgers from neighboring land to recognize culled areas. This impact on prevalence in badgers might reduce the beneficial effects of culling on cattle TB incidence, and could contribute to the detrimental effects that have been observed. Additionally, we show that suspension of cattle TB controls during a nationwide epidemic of foot and mouth disease, which substantially delayed removal of TB-affected cattle, was associated with a widespread increase in the prevalence of *M. bovis* infection in badgers (Woodroffe *et al.,* 2006). This pattern suggests that infection may be transmitted from cattle to badgers, as well as vice versa. Clearly, disease control measures aimed at either host species may have unintended

consequences for transmission, both within and between species. Our findings highlight the need for policymakers to consider multiple transmission routes when managing multihost pathogens.

Control polices for this disease rely on extensive annual testing and a test-and-slaughter policy (Flynn *et al.,* 2007).

2.13. TB vaccine

The current TB vaccine is a live vaccine derived from *Mycobacterium bovis* and attenuated by serial in vitro passaging. All vaccine substrains in use stem from one source, strain Bacille Calmette-Guérin. However, they differ in regions of genomic deletions, antigen expression levels, immunogenicity, and protective efficacy (Keller *et al.,* 2008).

BTB is increasing in incidence in the UK. Effective control strategies could involve vaccination; BCG, either alone or in prime-boost strategies, remains the most effective vaccine against bovine tuberculosis. However, BCG vaccination of cattle would require development of diagnostic tests able to accurately discriminate *Mycobacterium bovis*-infected from BCG-vaccinated animals (Sopp *et al.*, 2008). Herein, they demonstrated that the detection of secreted IFN-gamma following short term culture (4h) of whole blood with purified protein derived from *M. bovis* (PPD-B) allows such discrimination. This reflects, in part, the differential kinetics of IFN-gamma secretion in infected compared to vaccinated cattle. This is the first study to demonstrate that accurate, rapid distinction of BCG-vaccinated from *M. bovis*-infected cattle can be achieved in a short time period without the need for production of *M. bovis*-specific antigens, complex antigen mixtures or extensive laboratory procedures. They were also able to detect PPD-specific IFN-gamma release during short term culture of blood from a number of humans with active TB indicating that this test may have wider application and are potentially useful for the rapid diagnosis of disease in humans.

Current efforts are aimed at optimizing the protective efficacy of *Mycobacterium bovis* BCG by the use of vaccine combinations. The protection afforded by BCG alone is enhanced by vaccinating cattle with a combination of vaccines comprising BCG and a

protein tuberculosis vaccine, namely, culture filtrate proteins (CFPs) from *M. bovis* plus an adjuvant (Wedlock *et al.*, 2008).

2.14. Treatment of tuberculosis

Approximately one third of the world's population, including more than 11 million persons in the United States, is latently infected with *Mycobacterium tuberculosis*. Although most cases of tuberculosis in the United States occur in foreign-born persons from endemic countries, the prevalence is generally greater in economically disadvantaged populations and in persons with immunosuppressive conditions (Inge *et al.*, 2008).

Delays in detection and treatment allow for greater transmission of the infection. Compared with the traditional tuberculin skin test and acid-fast bacilli smear, newer interferon-gamma release assays and nucleic acid amplification assays lead to more rapid and specific detection of *M. tuberculosis* infection and active disease, respectively. Nine months of isoniazid therapy is the treatment of choice for most patients with latent tuberculosis infection. When active tuberculosis is identified, combination therapy with isoniazid, rifampin, pyrazinamide, and ethambutol should be promptly initiated for a two-month "intensive phase," and in most cases, followed by isoniazid and a rifamycin product for a four- to seven-month "continuation phase." Directly observed therapy should be used.

CHAPTER III

MATERIALS AND METHODS

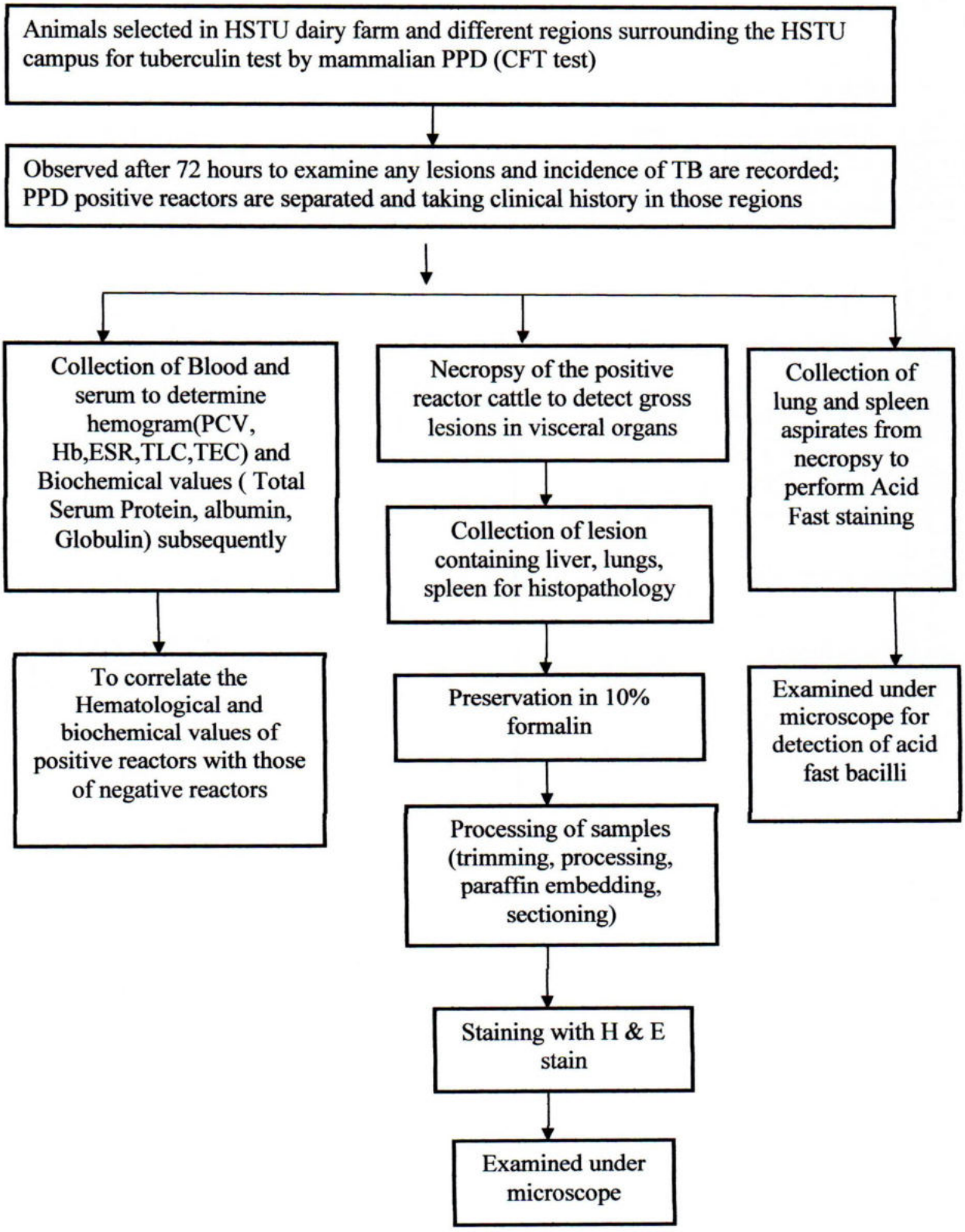
3.1. Experimental animals, areas, duration and experimental protocol

This study was designed to determine the prevalence of bovine tuberculosis in the HSTU dairy farm and free ranging cattle in village area surrounding the HSTU campus. The experimental sites were the HSTU dairy farm, Kornai village, Ranigonj and Rampur area. The experiment was performed in the Department of Pathology and Parasitology, Faculty of Veterinary and Animal Sciences, Hajee Mohammed Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. The experiment was conducted on selected cattle of HSTU dairy farm and free ranging cattle in Kornai, Ranigonj and Rampur area. These cattle were positive to tuberculin test. The duration of the study was March – December, 2011.

The major works of the present study

- Tuberculin test in selected farm and free ranging cattle;
- Hematological(Hemogram) study of tuberculin positive reactors;
- Biochemical (serum Protein) study of tuberculin positive reactors;
- Necropsy examination of visceral organs to detect lesions of tuberculosis in tuberculin positive animals;
- Acid-fast staining of lungs and spleen aspirates from necropsy;
- Histopathological examination of liver, spleen, lung of tuberculin positive cattle.

Experimental Protocol



3.2. The tuberculin test (the prescribed test for international trade)

Test materials

- Mammalian Tuberculin PPD (5ml vial)
- 1 ml syringe and needle

Test Procedure

- A total of 150 (above the age of 6 months) in Kornai village, Ranigonj, Rampur area and dairy farm of HSTU were selected to tuberculin test by mammalian PPD (Purified Protein Derivative) @ dose of 0.1ml (0.1 ml containing 10 TU) through intradermal route injection in caudal fold (CFT) area.
- Then after 72 hours, the injected areas were examined to observe any swelling, indurations, discoloration or any changes.

3.3. Hematological Examination

Blood sample was collected from the 5 tuberculin positive and 5 negative cattle with anticoagulant. Hematological examinations of tuberculin positive and negative reactors were accomplished to differentiate the values of packed cell volumes (PCV), Erythrocyte sedimentation rate (ESR), Hemoglobin (Hb), Total leukocyte count (TLC), Total Erythrocyte count (TEC).

The examinations were conducted in Physiology laboratory of HSTU and Life Diagnostic centre in Dinajpur and then the data of the tests were subjected to analysis using by MSTATC software.

Equipment and appliances for Hematological examination

- Collected blood
- EDTA(anticoagulant)
- Coplin jar
- Hemocytometer

- Distilled water
- Clean Slides
- Cover slips
- Microscope
- Hayem's solution
- Absolute methanol
- 1% HCl
- WBC pipette
- RBC pipette
- Sahli pipette
- Hemoglobinometer comparator
- Hemoglobinometer tube
- Special loading pipette
- Wintrobe hematocrit tube
- Centrifuging machine

3.3.1. Total leukocyte count (TLC)

Procedure

- The hemocytometer and cover slip was washed with distilled water and was dried. Then it was cleaned finally with acetone soaked tissue paper and dry tissue paper.
- Then the microscope was cleaned and was set the counting chamber under microscope of 10 power objective and bring the 1 square of WBC counting chambers (i.e. Left upper corner) into focus.
- Then unclotted blood was taken in white pipettes upto 0.5marks.
- The WBC pipette was filled with the diluting fluid (1% HCl) upto 11 marks and shaked for 2-3 mins by moving it with right hand in the way of 8 knot motion for mixing the blood and diluting fluid.
- 1-2 drops of mixture was discarded from the pipette and a small drop of it was placed on one side of the chamber and the cover slip was touched to spread the mixture on the counting chamber.
- All the 4 square chambers of WBC from the 4 corners were counted and were recorded.

Calculation

Depth counting chamber 0.1mm = 1/10
Dilution made in the pipette = 1/20
No. of counted square = 4

Multiplying factor = $\frac{1/1}{1/10 \times 1/20 \times 4/1}$ $=10 \times 5 = 50$

Now if we multiply the total no. of counted cells in 4 squares chambers by 50, we will get the total no. of WBC in thousand/cumm or /µl (-x10^3/µl).

3.3.2. Total RBC count

Procedure

- The hemocytometer and cover slip was washed with distilled water and air dried. Then it was cleaned finally with acetone soaked tissue paper and dry tissue paper.
- The counting chamber was placed under a microscope at 10X objective and brought the 1 corner of RBC counting chambers (i.e. left upper corner) into focus.
- The blood (unclotted) was taken in red pipettes upto 0.5marks after careful mixing the vial containing blood with EDTA.
- The RBC pipette was filled in with the diluting fluid, Hayem's solution upto 101 marks and was shaking for 2-3 mins by 8 knot motion.
- 1-2 drops of mixture was discarded from the pipette and a small drop of it was placed on one side of the chamber covered with a cover slip and allowed to spread the mixture between counting chamber and cover slip.
- All 5 of 25 chambers of RBC (i.e. 4 corners and 1 middle) was counted and recorded.

Calculation

Depth of counting chamber 0.1mm =1/10

Dilution made in the pipette = 1/200

No. of counted square = 5/25 = 1/5

Multiplying factor = $\frac{1/1}{1/10 \times 1/200 \times 1/5}$ $= 10 \times 200 \times 5 = 10{,}000$

Now if we multiply the total no. of counted cells in 5 chambers RBC by 10,000, we will get the total no. of RBC in million/cumm or /µl (-x10^6/µl).

3.3.3. Hemoglobin determination

Acid hematin method

- 1% HCl (approx, 1/10 normal) was taken upto 2 or 10 mark of the hemoglobinometer tube.
- The blood with EDTA was drawned into a sahli's pipette upto it's etched mark. The tip of the pipette was cleaned with cotton and merged upto the bottom of the hemoglobinometer and discharged the blood into the acid of the tube.
- The blood and HCl in the tube was shaked with glass stirrer and kept it stand for half an hour to form acid hematin.
- 1% HCl was added drop by drop to the mixture of the tube and stirred slowly with the small glass stirrer until it matches with the standard potassium dichromate colour standard in the hemoglobinometer comparator.
- Then the tube was removed from comparator and the reading of Hb in gm/100ml or percent was made.

3.3.4. Erythrocyte sedimentation rate (ESR) determination

Wintrobe method

- The special loading pipette was filled in by anticoagulated blood.
- The tip of the loading pipette was inserted upto the bottom of the wintrobe hematocrit tube and express blood by giving a uniform pressure on the bulb of the special loading pipette and the pipette was removed after filling the tube upto 0 or 10 marks.
- It was kept standing in a rack in vertical position for 1/2 hours and read the ESR result in mm/time.

3.3.5. Packed cell volume (PCV)

Wintrobe method

- Two hematocrit tubes were filled in its 0 or 10 marks by blood with EDTA with a special loading pipette in the same way as desccibed in ESR determination.
- It was centrifuged at 3000 rpm for 30 mins and read the PCV result in percent.

3.4. Biochemical Examination

Blood collected from 5 tuberculin positive and 5 negative cattle without anticoagulant and serum was collected. Biochemical examinations of tuberculin positive and negative reactors were performed to differentiate the values of total serum protein (g/dl), serum albumin (g/dl) and serum globulin (g/dl).

The examinations were conducted in Life Diagnostic centre in Dinajpur and then the data of the tests were subjected to analysis using by MSTATC software.

Equipment and appliances for Biochemical Examination

- Blood sample
- Centrifuge machine
- Digital Automated Biochemical Analyzer (ERBA MANNHEIM GERMAN)
- Serum Protein reagent (ERBA MANNHEIM GERMAN)
- Albumin reagent (ERBA MANNHEIM GERMAN)

Procedure for determination of total serum protein, albumin and globulin

- Blood collected from tuberculin positive and negative animals
- Centrifugation of blood @ 3000 rpm for 10 minutes
- Serum collected from blood
- Setting up Digital Automated Biochemical Analyzer for total serum protein and albumin
- Serum mixed with total protein and albumin reagents in separate test tubes

- Then the mixed solution aspirated into the digital Biochemical analyzer
- Waited for 5 minutes
- Then Total serum protein and albumin were printed out in g/dl.
- Globulin was determined by the calculation:
  Globulin = Total serum protein-Albumin

3.5. Necropsy findings of suspected cattle

The necropsy was done on the selected cattle (showing typical PPD reaction) taken from Kornai village, Dinajpur. At necropsy, gross tissue changes were observed and recorded carefully by systemic dissection. The samples were also collected in 10% neutral buffered formalin for the histopathological study.

Equipment and appliances for necropsy

- Sample animals (cattle)
- Scissors (3)
- Forceps (4)
- Gloves
- Musk
- Bone cutting saw
- Scalpel
- Chisel
- 10% neutral buffered formalin

Procedure

At first the position of the animal was observed

↓

The animal was placed in dorsoventral position

↓

The external changes were observed

↓

An incision was made through mid-ventral line or through linea alba from chin to the anus

↓

The animal was skinning in such way that the longisimus muscle was exposed

↓

The pectoral and serratus muscle were cut such way so that the legs were fallen on the ground

↓

The medial thigh muscle was cut in the groin region of both legs such a way that coxo-femoral articulation was exposed and posterior leg was fallen on the ground

↓

The sternum was held in appropriate position

↓

The incision was made to the medio-lateral side of the rami

↓

The mandibular muscle was severed and the tongue was pulled out by holding from buccal cavity

↓

Assistant held the tongue and cut of the hyoid bone by knife or bone cutter

↓

The tongue, pharynx, larynx was pulled out from buccal cavity and cutting of the dorsal attachment of the tongue, trachea, esophagus when reach up to cariniform cartilage

↓

An incision was made transversely on the xyphoid cartilage on the anterior abdomen

↓

An incision was made through the costo-condral junction to both side of sternum from posterior to anterior up to the cuneiform cartilage

↓

The sternal attachment was severed

↓

The first 3-4 ribs were broken down in any sides to get enough space to enter into the thoracic cavity

↓

Entered the thoracic and examined the pleura.

↓

The heart and lungs were severed, the tongue was holding with esophagus reaching up to diaphragm

↓

The diaphragm was examined if there were any abnormalities and then diaphragm was cutting in tendinous and muscular part

↓

The tongue and esophagus was held, the dorsal attachment and abdominal cavity was cut.

↓

The visceral organs like liver, spleen, and lungs were examined.

3.6. Histopathological examination

Formalin-fixed samples of the liver, spleen and lungs from the sacrificed cattle collected by necropsy were processed for paraffin embedding, sectioned and stained with haematoxylin and eosin according to standard method (Luna, 1968) for histopathological study. Details of tissue processing, sectioning and staining are given below.

3.6.1. Equipment and appliances

- Sample (Liver, lungs, spleen)
- 10% neutral buffered formalin
- Chloroform
- Paraffin
- Alcohol
- Tape Water
- Xylene
- Hematoxylin and Eosin Stain
- Distilled water
- Clean Slides
- Cover slips

- Mounting media (dpx)
- Microscope

3.6.2. Processing of tissues and sectioning

- The tissues were properly trimmed to obtain a good cross section of the tissue.
- The tissues were washed under running tap water for overnight to remove the fixative.
- The tissues were dehydrated in ascending grades of alcohol using 50%, 70%, 80%, 90% alcohol, and three changes in absolute alcohol, for 1hr in each.
- The tissues were cleared in two changes in chloroform, 1.5hr in each.
- The tissues were embedded in molten paraffin wax at 56^0C for two changes, 1.5hr in each.
- Paraffin blocks containing tissue pieces were made using templates and molten paraffin.
- The tissues were sectioned with a microtome at 5mm thickness, which were allowed to spread on warm water bath (42^0C) containing small amount of gelatin and taken on oil and grease -free glass slides. The slides were air dried and kept in cool place until staining.

3.6.3. Hematoxylin and Eosin Staining Procedure

Preparation of Harris' hematoxylin solution

Hematoxylin crystals	5.0g
Alcohol (100%)	50.0 ml
Ammonium or potassium alum	100 g
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 g

Hemoatoxylin was dissolved in alcohol and alum in water by heat. The two solutions were thoroughly mixed and boiled as rapidly as possible. After removing from heat, mercuric oxide was added to the solution slowly. The solution was reheated to a simmer until it became dark purple, and then the vessel was removed from heat and immediately plunged into a basin of cold water until it became cool. 2-4ml glacial acetic acid was added per 100 ml of solution to increase the precision of the nuclear stain. Before use, the prepared solution was filtered.

Preparation of eosin solution

1% stock alcoholic eosin

Eosin Y, water soluble	1 g
Distilled water	20 ml
95% alcohol	80 ml

Eosin was dissolved in water and then 80 ml of 95% alcohol was added.

Working eosin solution

Eosin stock solution	1part
Alcohol, 80%	3 parts

0.5ml of glacial acetic acid was added to 100 ml of working eosin solution just before use.

Staining protocol

- Deparaffinization of the sectioned tissues was done by 3 changes in xylene (3 mins in each),
- Rehydration of the sectioned tissues was done through descending grades of alcohol (3 changes in absolute alcohol, 3 mins in each; 95% alcohol for 2 mins; 80% alcohol for 2 mins; 70% alcohol for 2 mins) and distilled water for 5 mins,
- The tissues were stained with Harris' hematoxylin for 10 mins,
- The sections were washed in running tap water for 10 mins,
- Then the staining was differentiated in acid alcohol (1part HCl and 99 parts 70% alcohol), 2-4 dips,
- The tissue sections were then washed in tap water for 5 mins and dipped in ammonia water (2-4 times) until sections became bright blue,
- The sections were stained with eosin for 1 min and then differentiated and dehydrated in alcohol (95% alcohol, 3 changes, 2-4 dips in each; absolute alcohol 3 changes, 2-3 mins in each),
- The stained sections were then cleaned by 3 changes in xylene, 5 mins in each and finally the sections were mounted with cover slip using DPX,

- Then the images of the stained section were taken by digital camera (Sony 14.2 Mega pixel).

3.7. Acid-fast staining (classic Ziehl–Neelsen stain) of lungs and spleen aspiration smear after necropsy

Equipment and appliances

- Alcohol
- Distilled water
- Running water
- Carbol fuchsin solution
- Methylene blue solution
- Clean Slides
- Cover slips
- Microscope

Preparation of Carbol Fuchsin Solution

Phenol crystal (melted)	25ml
Alcohol, 100%	50ml
Basic fuchsin	5gm
Distilled water	500ml

Preparation of Acid Alcohol Solution

Alcohol,70%	500ml
HCl conc.	5ml

Preparation of Methylene Blue Solution (stock)

Methylene blue	1.4gm
Alcohol, 95%	100ml

Preparation of Methylene Blue Solution (working)

Methylene blue(stock)	10ml
Distilled water	90ml

Staining procedure

- At first the aspirates taken from lungs and spleen by syringe and needle and smeared it in a clean glass slide and then air dried
- Stained the smear with carbol fuchsin solution for 30 minutes
- Washed well in running water
- Decolourized with acid alcohol solution until section are pale pink
- Washed thoroughly in running water for 8 minutes
- Counter stained by dipping a slide at a time in working methylene blue solution
- Washed with tap water, then rinsed in distilled water
- Then the slide was air dried and observed in oil immersion objective (100X)
- Then the images of the stained slides were taken by digital camera (Sony 14.2 Mega pixel).

Result

Acid fast bacilli	bright red rod
Other tissue element	pale blue

CHAPTER IV

RESULTS

4.1 Tuberculin test

Single intradermal hypersensitivity test with mammalian PPD was done to investigate the prevalence of bovine tuberculosis in the selected cattle (over 6 months) in the HSTU campus and surrounding regions of HSTU campus. After 72 hours, distinct visible swelling (8-10 mm) and indurations were considered to be positive reaction and indistinct indurations and swelling were considered to be doubtful reaction.

Table 2. Prevalence of BTB in the regions surrounding the HSTU campus detected by single intradermal tuberculin test

Name of place	Animal tested	Positive reaction	Percentage of positive animals	Doubtful reaction	Percentage of doubtful animals
HSTU dairy farm	10	0	3.33%	0	4.00%
Kornai village	98	4		3	
Ranigonj area	25	1		2	
Rampur area	17	0		1	
Total	150	5		6	

4.2. Hematological examination

Blood sample was collected from the 5 tuberculin positive and 5 negative cattle with anticoagulant. The collected unclotted blood was subjected for routine hematological examination (TEC, TLC, ESR, PCV%, Hb). Then the data of the test were subjected to analysis using by MSTATC software.

Table 3: Comparison of mean Hematological parameters ((TEC, TLC, ESR, PCV%, Hb) in tuberculin positive and negative reactor cattle

Parameters studied	Negative reactor (n=5) (Mean ± standard deviation)	Positive reactor (n=5) (Mean ± standard deviation)	Level of significance
TEC (-x10^6/µl)	10.20 ± 0.4472	9.80 ± 0.2828	*
TLC (-x10^3/µl)	7.800 ± 0.6519	8.000 ± 0.4123	*
Hb determination (gm %)	12.48 ± 0.6044	10.14 ± 0.9556	*
ESR (mm fall per ½ hour)	0.550 ± 0.0707	0.400 ± 0.1118	NS
PCV (%)	30.60 ± 1.1958	26.20 ± 1.8908	*

Values are expressed as mean ± standard error of means. NS: Statistically insignificant (P > .05). * Statistically significant (P≤ .05).

4.3 Biochemical examination

Blood collected from 5 tuberculin positive and 5 negative cattle without anticoagulant. Serum total proteins (g/dl), Serum albumin (g/dl), Serum globulins (g/dl) were detected by Digital Automated Biochemical Analyzer (ERBA MANNHEIM GERMAN) in Life Diagnostic centre, Dinajpur. The results were printed out as g/dl. Then the data were statistically analyzed by MSTATC software.

Table 4: Comparison of mean Biochemical parameters (serum proteins) in tuberculin positive and negative reactor cattle

Parameters studied	**Negative reactor (n=5) (Mean ± standard deviation)**	**Positive reactor (n=5) (Mean ± standard deviation)**	**Level of significance**
Serum total protein(TP) (g/dl)	6.770 ± 0.1715	6.116 ± 0.2712	*
Serum albumin (g/dl)	2.566 ± 0.2747	1.802 ± 0.4344	NS
Serum globulins (g/dl)	4.036 ± 0.1867	4.396 ± 0.2952	NS

Values are expressed as mean ± standard error of means. NS: Statistically insignificant ($P > .05$). * Statistically significant ($P \leq .05$).

4.4. Clinical findings of the TB hypersensitivity test

Fig. 1: Picture of cattle showed positive reaction to tuberculin. The cattle contained mild cough, emaciated body in respect to its age.

Fig. 2: Picture of caudal fold swelling after 72 hours followed by intradermal injection of mammalian tuberculin PPD (white arrow).

4.5. Necropsy findings of suspected cattle

Necropsy findings included different lesions in different regions of lungs entailing congested lesions with tiny nodules of different sizes and shapes especially in front portion of lungs. Nodular lesions were found in liver as well. The spleen was enlarged, but no visible nodules were found.

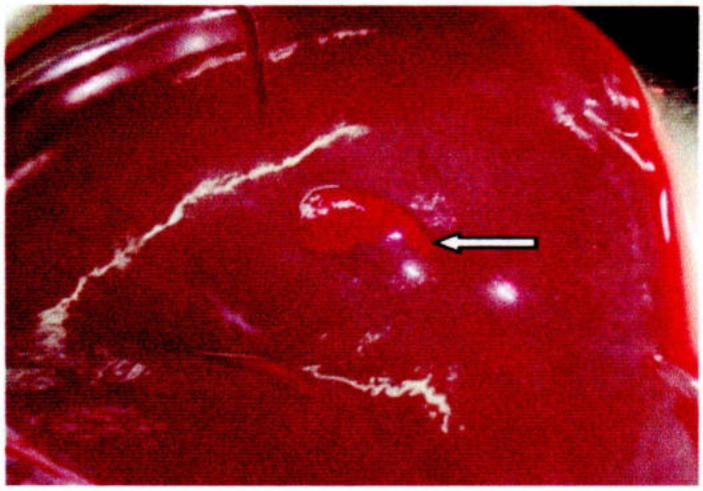


Fig. 3: Picture of liver collected from tuberculin positive cattle at Kornai village. Grossly nodular lesions were found in liver (white arrow).

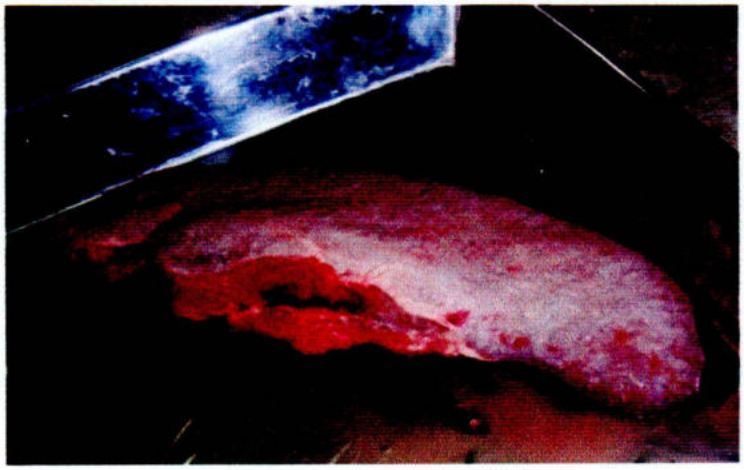


Fig. 4: Spleen collected from a tuberculin +ve cattle at Kornai village. The spleen was enlarged. Grossly nodular lesions were not seen.

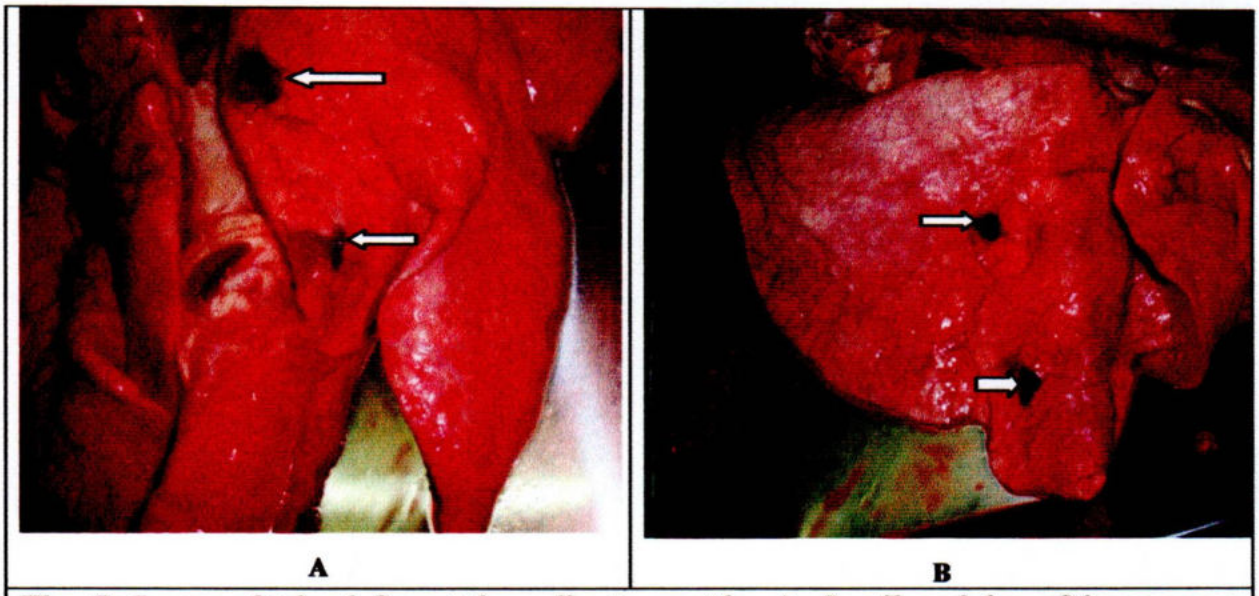


Fig. 5: Lungs obtained from tuberculin +ve cattle; A. Small nodules of lungs were found, B. Congested, necrosed and consolidated band regions were found (white arrows).

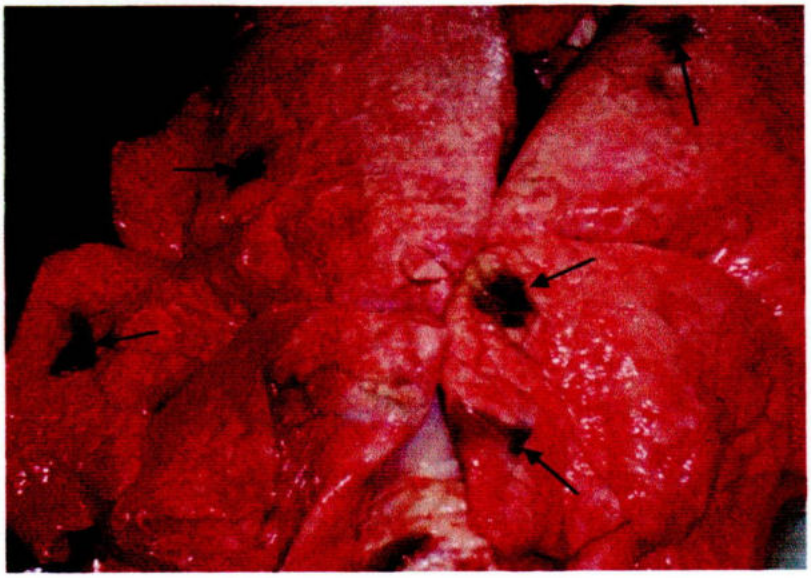


Fig. 6: Band and localized type of lesions distributed throughout the lungs obtained from tuberculin +ve cattle (black arrows).

4.6. Acid-fast staining (classic Ziehl–Neelsen stain) of aspirate smears of lung, spleen

Aspirate smears were taken from the lungs and spleen. Then acid fast staining was performed in Nova pathology, Dinajpur.

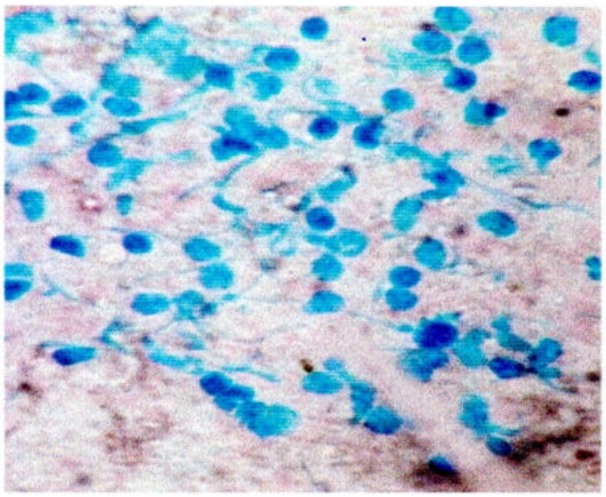


Fig. 7: Acid fast staining of aspirate smear taken from spleen. Only clumps of spleen contents were seen. Acid fast bacilli were not detected (100X).

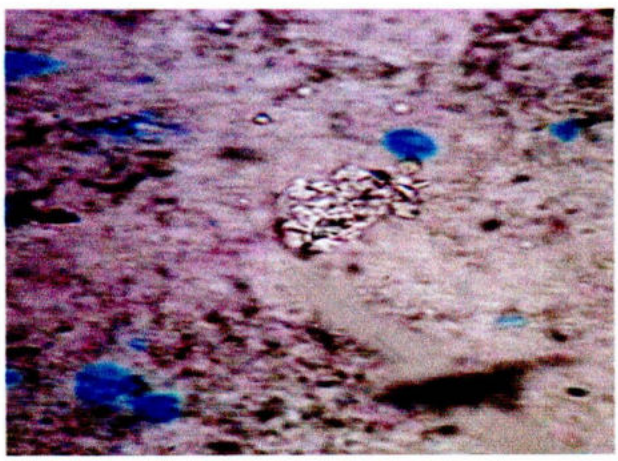


Fig. 8: Acid fast staining of Lungs aspirates smear. Acid fast bacilli were not detected under microscope (100X).

4.7. Histopathological examination

Severe congestion and accumulation of reactive cells and fibrin in the lung parenchyma was seen. Purple colour granular mass with mononuclear cellular infiltration, proliferation of fibrous connective tissues were seen in lungs. No granulomatous lesions were found in liver.

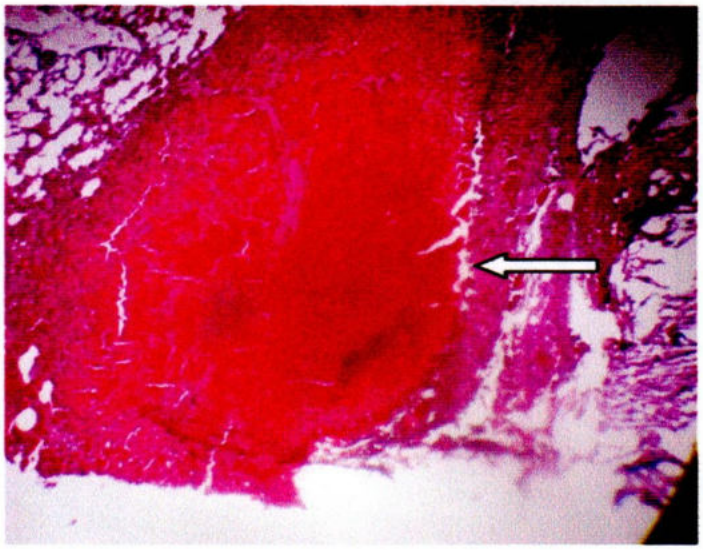


Fig. 9: A granulomatous lesion seen in the lungs of tuberculin positive cattle. There was a large purple coloured granular mass surrounded by thick fibrous connective tissue capsule (white arrow) (4X).

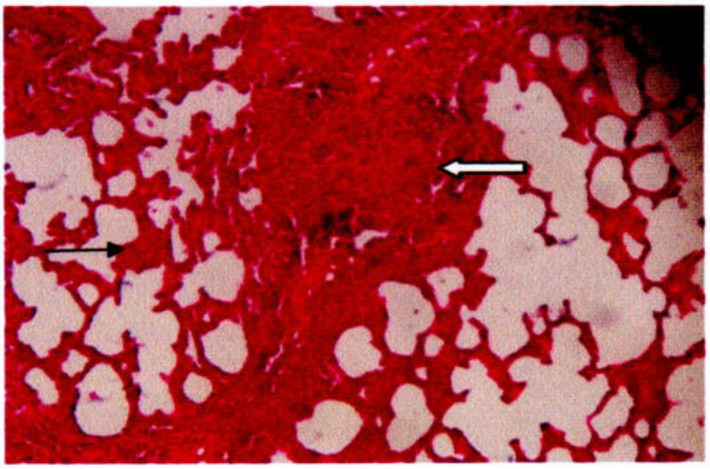


Fig. 10: Purple coloured granular necrotic center (white arrow) surrounded by huge reactive cells with thickened, disrupted interalveolar space (black arrow) was seen in the lungs of tuberculin positive cattle though there was no distinct encapsulation (10X).

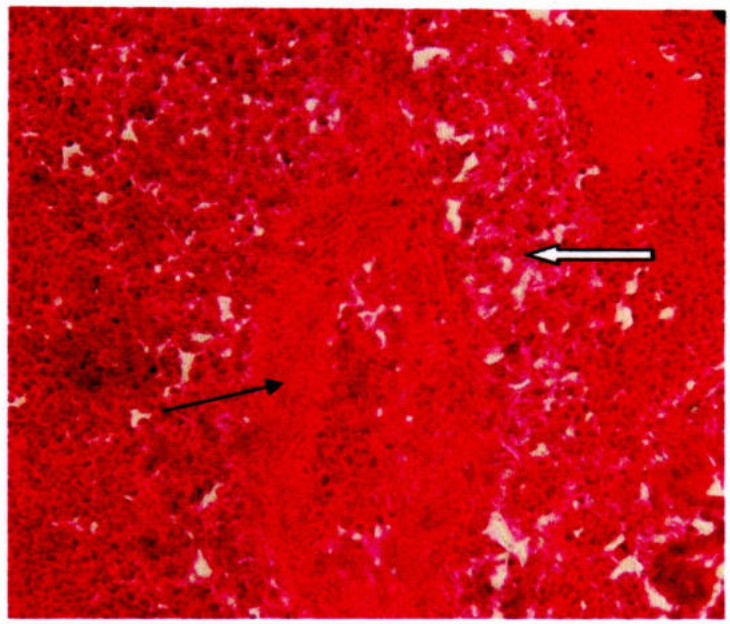


Fig. 11: A granulomatous nodule seen in the lungs of tuberculin positive cattle. There was a tubercular necrotic center with thick fibrous connective tissues (black arrow) surrounded by huge reactive cells infiltration (white arrow) (40X).

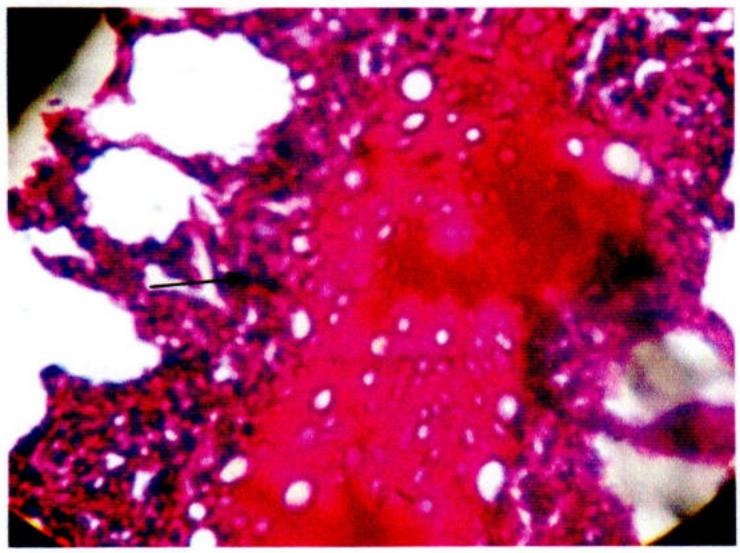


Fig. 12: Lungs of tuberculin positive cattle showed purple proteinaceous mass surrounded by mononuclear cells infiltration (black arrow) (40 X).

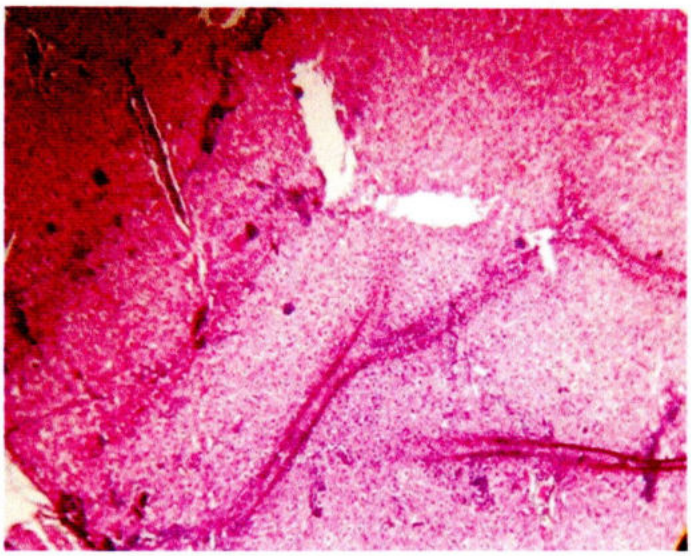


Fig. 13: No granulomatous nodules were seen in the liver of tuberculin positive cattle (4 X).

CHAPTER V

DISCUSSION

5.1. Tuberculin test

Bangladesh is an endemic area for tuberculosis infection, since a large number of people and animals are exposed to this infection.

In the present study, 5 (3.33%) out of 150 cattle showed distinct tuberculin positive reaction and 6 (4.00%) out of 150 cattle showed doubtful reaction (Table 2) by single intradermal tuberculin test in case of free ranging cattle considering the age over 6 months regardless of sex, age, breed. Pharo *et al.,* (1981) detected overall 5.9% incidence of BTB at Pabna (Bangladesh) milk shed areas under the cattle development project by intradermal tuberculin test of which 3.4% showed positive reaction and 2.6% doubtful reaction. Singh *et al.* (2004) also observed higher prevalence rate (9.09%) of bovine tubetculosis in India by single intradermal tuberculin test.

The prevalence of bovine tuberculoisis in the present study was somewhat similar to Pharo *et al.* (1981) and lower than Singh *et al.* (2004) which indicate that the prevalence of bovine tuberculosis did not reduce throughout the country might be due to no comprehensive control and culling strategy of TB infected animals.

5.2. Hematological examination

The results of hematological evaluation of tuberculin test positive reactors (n=5), and non-reactors (n=5) have been presented in Table 3.

In tuberculin test positive reactors there was an increase in total Leukocyte count and decrease significantly in total erythrocyte count ($p<0.05$), hemoglobin and packed cell volume ($P=0.05$) when compared to nonreactors. There was also decrease in erythrocyte sedimentation rate in tuberculin test positive reactors which was statistically insignificant ($p>0.05$) and was contrast to the findings of Mohankumar *et al.* (2010) whereby significant increase of ESR was observed. Erythrocyte sedimentation rate was not considered as a specific diagnostic value in

tuberculous animals unlike in human beings, where it is used frequently to evaluate the degree of the infection (Rao, 1992).

In the present investigation, significant increase in the total Leukocyte count in positive reactors was observed ($p<0.05$) indicating bacterial infection. Mohankumar *et al.* (2010) recorded slightly higher values of Total leukocyte count (TLC) that was statistically insignificant (P 0.05) which can be considered to be subtle contradictory to the present study regarding the statistical significance.

A drop in red blood cell (RBC) count and haemoglobin (Hb) concentration (Chandrasekharan and Ramakhshnan, 1969; Samad and Rahman, 1986; Amin, 1989; Rao *et al.*, 1992; Kumar *et al.*, 1994), PCV (Kumar *et al.*, 1994) were observed in the tuberculin positive reacting cattle as well as in animals with tubercular lesion. The same agreement went for the present study. This decrease in PCV, Hb and TEC values could be ascribed to poor health condition and chronicity of the disease and any sort of bone marrow atrophy or disorders.

5.3. Biochemical examination

The changes in the various biochemical constituents of serum in tuberculin positive test reactors and non-reactors have been summarized in Table 4. The results indicated that there was a decrease in the total serum protein contents in the positive reactors compared to non-reactors which was significant ($P=0.05$).Positive reactors showed an increase in globulin content and decrease in albumin content though those were statistically insignificant (($p>0.05$).

There was no significant difference in the total serum protein contents between the tuberculin positive reactors and non-reactors (Mohankumar *et al.,* 2010) but significant difference in the level of albumin, globulin where positive reactors showed an increase in globulin content and decrease in albumin content (Moses *et al.,* 1975; Kumar *et al.,* 1994; Hemalatha *et al.,* 1995; Mohankumar *et al.,* 2010).

However, an increased level of serum total protein was observed in the animals, which had generalized tuberculosis which was observed in cases with large caseated tubercular lesions

(Grys, 1965) which was different from this present study. This variation in the protein spectrum was probably due to moderate and severe forms of pulmonary and generalized infection.

5.4. Clinical findings of the TB hypersensitivity test

The cattle showing positive hypersensitivity test were observed to have poor body condition, emaciated body and mild cough (Fig. no 1 and 2) which had been selected for necropsy to investigate gross and histopathological abnormalities.

5.5. Gross pathology

In this present study, grossly tuberculous nodular lesions were found in the lungs (Fig. 5 A.) with congested, necrosed and consolidated band lesions in anterior portions of lungs (Fig. 5 B.) in the tuberculin positive cattle. The lesions were distributed throughout both parts of the lungs especially proximal portion of lungs and divided macroscopically into band and localized type (Fig. 6). The spleen was found enlarged but grossly no nodular lesions were found (Fig. 4). In liver, grossly slight nodule like raised portions were found (Fig. 3) during necropsy of the suspected animal.

Pfeffer *et al.* (1979) found that small lesions in the anterior lobes of the lungs of young animal were divided macroscopically into band and localized types. Localized lesions were subdivided into red, red-grey, grey-red and grey categories which are somewhat similar to this present study. Proximal portions of lungs infections underpin that the mode of transmission of the infection could more likely be attributed to respiratory route.

5.6. Histopathological examination

Granulomatous lesion with large purple coloured granular mass surrounded by thick fibrous connective tissue capsule (Fig. 9), Purple coloured granular necrotic center surrounded by huge reactive cells with thickened and disrupted interalveolar space (Fig. 10), tubercular necrotic center with thick fibrous connective tissues surrounded by mononuclear and various reactive cellular infiltrations (Fig. 11), purple proteinaceous mass surrounded by mononuclear cells infiltration with thickened and condensed alveolar wall indicating emphysematous and pneumonic lesions (Fig. 12) were seen in lungs of tuberculin positive cattle. But the liver showed no granulomatous lesions though small nodular lesions were found macroscopically (Fig. 13) which might likely be due to chronic tissue level changes of visceral organs whereby lungs was followed by liver.

Neill *et al.* (1994) depicted the lesions of bovine tuberculosis which were typically characterized by the presence of tubercles with central caseation and calcification. In the early stages of infection, these lesions were not encapsulated, but were surrounded by condensed alveolar tissue. Initially, there was presence of epitheloid and giant cells at the center of the tubercle, and, as the disease progress, they are surrounded by lymphocytes, plasma cells and monocytes, developing a peripheral fibroplasia and central caseous necrosis which was homogenous to this present study except calcification. This observation suggests the early stages of infection of the animal and primary site of lesions development in the visceral organs is lungs followed by liver and other organs subsequently.

5.7. Acid fast staining (classic Ziehl–Neelsen stain)

Acid fast bacilli were not found in spleen and lung aspirate smears by classic Ziehl–Neelsen stain (Fig. 7 and 8). It could be due to low load of organisms and early stage of infection which was supported by Wards *et al.* (1995) and Rodriguez *et al.* (2004) as it was depicted that the presence of acid fast bacilli can be revealed only when concentrations are exceeding 104 bacteria per milliliter.

CHAPTER VI

CONCLUSION

The present study was conducted mainly to explore a pathological investigation of bovine TB based on tuberculin hypersensitivity test and determination of prevalence of BTB and to see the correlation of biochemical and haematogical values between tuberculin positive reactors and non-reactors. Distinct gross and histopathological lesions were found followed by necropsy of the tuberculin positive reactor cattle and variation of hemogram and biochemical values between tuberculin positive reactors and non-reactors were also observed even though acid fast bacilli were not found in direct aspirate samples of the visceral organs. The prevalence of bovine tuberculosis may have a zoonotic impact as occasionally human beings are infected with tuberculosis in these regions. It can be deduced from the present study that acid fast staining cannot be considered as the standard diagnostic tool even if gross and histopathological study, hematological and biochemical study followed by tuberculin test showed positive correlation. However, further studies are needed to be explored to determine a quick diagnostic protocol to detect the strain of the organisms leading to a comprehensive control strategy to contain the disease since it has disastrous effect upon animal and man.

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