PATHOLOGICAL INVESTIGATION OF BOVINE TUBERCULOSIS IN RANGPUR DISTRICT IN BANGLADESH

A THESIS

BY

DR. ENAMUL HAQUE

SEMESTER: JULY – DECEMBER/ 2012
REGISTRATION NO.: 1105024
SESSION: 2011-2012

MASTER OF SCIENCE (M. S.)

IN

PATHOLOGY

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Approved as to style and contents by

(Professor Dr. S. M. Harun-ur-Rashid)
Supervisor

(Dr. Md. Nazrul Islam)
Co- Supervisor

(Professor Dr. S. M. Harun-ur-Rashid)
Chairman
Examination committee
&
Chairman

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2012

Dedicated

To My

Beloved Parents

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ABSTRACT

Bovine tuberculosis (bTB) is one of the most economically important zoonotic diseases and tuberculin test has been widely used to detect its prevalence worldwide. This study was designed to investigate the pathological condition of bovine tuberculosis (BTB) using caudal fold tuberculin test in different dairy farms and free ranging cattle in Rangpur district during january - December, 2012. A total of 240 cattle (over the age of 6 months) were selected to have tuberculin test by bovine PPD (purified protein derivative) at caudal fold area in intradermal route regardless of age and sex. In the present study, 9 (3.75%) out of 240 cattle showed tuberculin positive reaction (distinct and visible swelling) and 7(2.92 %) cattle showed suspicious reaction (indistinct and invisible swelling). Out of 98 male cattle 3 (3.06%) and out of 142 female cattle 6 (4.22%) showed tuberculin positive reaction. The percentage of female cattle reactors were somewhat higher than the percentage of male cattle in CFT test and younger animals (7months- 3 years) were found more susceptible to tuberculosis than older animals. Grossly congested and consolidated lungs containing fairly distinctly demarcated caseous yellowish nodules throughout both parts of the lungs and divided macroscopically into haemorrhagic, fibrotic lesion and localized type. The spleen was enlarged but grossly nodular lesions were not seen. Histopathologically granulomatous nodular lesions with caseous necrotic mass surrounded by various reactive cells and fibrous connective tissues were found in lungs pursued by necropsy of selected tuberculin positive animal. The study dictates a positive correlation among the caudal fold tuberculin test, gross and histopathological study of the visceral organs.

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LIST OF ABBREVIATIONS AND SYMBOLS

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-aminoe tetra acetic acid
ELISA	-	Ezyme Linked Immunosorbent Assay
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
PM	-	Postmortem
PNCEBT	-	Plan of national control and eradication of bovine tuberculosis
PPD	-	Purified protein derivative
ROI	-	Republic of Ireland
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
rpm	-	Rotation per minute
sq	-	Square
TB	-	Tuberculosis
TU	-	Tuberculin unit
%	-	Percent
µg	-	Micro gram

CHAPTER I

INTRODUCTION

Tuberculosis (TB) is an important zoonotic disease caused by an intracellular acid-fast organism *Mycobacterium sp.* It has been recognized from 176 countries as one of the important bovine diseases causing great economic loss (Hines *et al.*, 1995; Martin *et al.*, 1994; Samad, 2008). *M. tuberculosis*, *M. bovis* and *M. avium* all the three species are capable of causing disease in humans although *M. tuberculosis* is by for the most common in humans with continues to be a major cause of morbidity and mortality throughout the world (Lima *et. al.*, 2005).

Tuberculosis (TB) is a contagious disease, which can affect most warm-blooded animals, including human being. Cattle, goats and pigs and the domestic livestock are most susceptible to infection, while horses are relatively resistant to infection. It is characterized by chronic debilitating, emaciation and progressive development of granulomatous lesions or tubercles in the lung tissue, lymph nodes, or other organs (Blood and Radostits, 1989; Thoen *et al.*, 2009). It is the most important zoonosis associated with enormous economic losses in the animal industries and severe hazards to human health worldwide, particularly in the developing world (WHO, 2002; Kiboss and Kibitok, 2003; Ayele *et al.*, 2004; WHO, 2009).

The devastating effects of the disease have varied widely with time and in different regions; but its occurrence has largely been influenced by man-made factors, such as urban crowding and poverty (Fätkenheuer *et al.*,1999). Early understanding of cross species infection or zoonotic nature of these agents began at the time that humans domesticated and lived closely with animals (Biet *et al.*, 2005). The causative agents have since spread to all groups in the human population and constitute major threats to human health globally (Tan *et al.*, 2003; Ayele *et al.*, 2004; Thoen *et al.*, 2009).

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).

Bovine tuberculosis in the most developed countriesis can be controlled by test-and-slaughter or test-and-segregation methods. Affected herds are re-tested periodically to eliminate cattle that may shed the organism; the tuberculin test is generally used. 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.

Tuberculosis is usually diagnosed in the field with the tuberculin skin test. A presumptive diagnosis can also be made by histopathological examination of acid-fast bacilli and isolation of *M. bovis* on selective culture media. The identity of the organism can be confirmed with biochemical tests and culture characteristics, or polymerase chain reaction (PCR) assays (Q-PCR) (Sawyer *et al.*, 2007). Other assays are typically used as ancillary tests to the tuberculin test. The lymphocyte proliferation and gamma-interferon assays are blood tests that measure cellular immunity. Enzyme-linked immunosorbent assays (ELISAs) measure antibody titers to *M. bovis*. The tuberculin test is the standard method of diagnosis in live cattle and cervids, and the prescribed test for international trade.

In the regions of Rangpur district pathological investigation of bovine tuberculosis was not performed. Under these circumstances, it is necessary to explore a study to detect the prevalence of and to investigate the pathological conditions of TB infection in animals. The rapid and accurate detection of *Mycobacterium sp.* is of paramount importance in the effective treatment, control and eradication of TB in man and animal.

Considering the above fact, the present study was designed to obtain the following objectives:

- To identify the prevalence of bovine TB using caudal fold tuberculin (CFT) tests in Rangpur district ;
- To compare the prevalence of bovine tuberculosis of different age and sex;
- Gross and histopathological study of necropsy specimens collected from typically TB affected animals.

CHAPTER II

REVIEW OF LITERATURE

The present study have been conducted to investigate bovine tuberculosis using caudal fold tuberculin (CFT) test highlighting different pathological explorations after a brief overview on etiology, prevalence; epidemiology, diagnosis and zoonotic threat. The present research work related to the presumptive study is very limited in Bangladesh. In this viewpoint, the research works relevant to the present study in different countries have been reviewed and presented in this chapter.

2.1. Tuberculosis

Tuberculosis (TB) is a disease of animals and man caused by pathogenic members of the genus Mycobacterium and characterized by progressive development of granulomatous lesions or tubercles in the lung tissue, lymph nodes, or other organs (Blood and Radostits,1989; Thoen *et al.*, 2009). It is the most important zoonosis associated with enormous economic losses in the animal industries and severe hazards to human health worldwide, particularly in the developing world (WHO, 2002; Kiboss and Kibitok, 2003; Ayele *et al.*, 2004; WHO, 2009). TB is the leading cause of human death due to a single infectious agent (O'Reilly and Daborn, 1995; Cosivi *et al.*, 1998; Larson, 2000a; Tan *et al.*, 2003; Thoen *et al.*, 2009) with over 9.27 million new cases and nearly 2 million deaths recorded every year (WHO, 2009; 2010).

In central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (Prodinger W.M., 2005). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis. In countries with tuberculosis eradication programmes, clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals before signs appear. Prior to the national

tuberculosis eradication campaigns, however, clinical signs associated with tuberculosis were commonly observed (Cousins D.V., 2001).

At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. Macroscopically, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be purulent. The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Histologically, lesions caused by *M. bovis* are often paucibacillary (having few organisms) and the absence of acid-fast organisms does not exclude tuberculosis in lymphadenitis of unknown aetiology.

Mycobacterium bovis has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterised. The incidence of pulmonary tuberculosis caused by *M. bovis* is higher in farm and slaughterhouse workers than in urban inhabitants. The transmission of *M. bovis* to humans via milk and its products is eliminated by the pasteurisation of milk. One of the results of bovine tuberculosis eradication programmes has been a reduction in disease and death caused by bovine tuberculosis in the human population.

Bovine tuberculosis in wildlife was first reported in 1929 in greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmi*) in South Africa and by the 1940s, the disease was found to be endemic in greater kudu. In 1982 in Uganda, a prevalence of 10% in African buffalo and 9% in warthog (*Phacochoerus aethiopicus*) was found, and in Zambia, *M. bovis* infection has been reported in Kafue lechwe (*Kobus leche kafuensis*) and in a single eland (*Traurotragus oryx*). An outbreak of tuberculosis in wild olive baboons (*Papio cynocephalus anubis*) was reported in Kenya. *Mycobacterium bovis* infection has also been diagnosed in African buffalo in the Kruger National Park in South Africa

(Bengis R.G., 1996), and more recently spill over to other species such as chacma baboon (*Papio ursinus*), lion (*Panthera leo*) and cheetah (*Acynonyx jubatus*) as well as greater kudu has occurred. The rigorous application of tuberculin testing and culling of reactor cattle has eliminated *M. bovis* infection from farmed bovine populations in some countries, but this strategy has not been universally successful. The risk that these reservoirs of infection constitute for domestic animals and humans is quite variable depending on the specific epidemiological situation for the species and the environment (Corner L.A.L., 2006); Morris R.S., Pfeieer D.U. & Jackson R.,1994). Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated. *Mycobacterium bovis* has been isolated from farmed and free-living cervidae.

2.2. Etiology of tuberculosis in human and cattle

TB in humans and animals is caused by the tubercle bacilli of the *Mycobacterium tuberculosis* complex (MTC) and other environmental non-tuberculous or atypical mycobacteria. The typical MTC agents: *Mycobacterium tuberculosis, M. bovis, M. africanum, M. microti* and *M. canetti* cause TB in an exceptionally wide range of domestic and wild mammalian hosts including humans (O'Reilly and Daborn, 1995; van Soolingen *et al.*, 1997; Kremer *et al.*, 1998; van Soolingen *et al.*, 1998; Harris and Barletta, 2001; Aranaz *et al.*, 2003; Biet *et al.*, 2005; Brudey *et al.*, 2006; Une and Mori, 2007; Thoen *et al.*, 2009).

Human TB is caused mainly by *M. tuberculosis* but *M. bovis* the etiological agent of bovine TB can also be responsible for the human disease, making *M. bovis* an important zoonotic species (Blood and Radostits, 1989; Cosivi *et al.*, 1998; Biet *et al.*, 2005; OIE, 2009) while *M. africanum* is problematic to humans in tropical Africa (Cosivi *et al.*, 1998). Human and other non-host specific infections caused by *M. avium, M. microti,* and *M. canettii* have also been documented (Falkinham

3rd 1996; van Soolingen *et al.*, 1997; Kremer *et al.*, 1998; van Soolingen *et al.*, 1998; Biet *et al.*, 2005).

2.3. Bovine tuberculosis

Bovine TB is a chronic disease of animals caused by infection with the slow-growing, obligate intracellular bacterium *Mycobacterium bovis* (Bourne and others, 2007; OIE, 2009). This highly-adapted and 'successful' pathogen has a world-wide distribution and in several countries bovine TB remains a major, costly infectious disease of cattle and other domesticated, feral and wild animal populations, including; badgers, possums, deer, goats, sheep, camelids etc (Pollock and Neill, 2002; Mathews and others, 2006; Carslake and others, 2011). Bovine TB is an OIE (World Organisation for Animal Health) listed (formerly List B) disease: "one that is considered to be of socioeconomic or public health importance within countries and of significance to the international trade of animals and animal products" (OIE, 2011, Cousins and Roberts, 2001). Bovine TB affects cattle health, impacts negatively on profitability and trade and can decimate years of genetic improvement towards desirable production traits (Boland and others, 2009). It also impacts negatively on the welfare of affected farming families (Farm Crisis Network, 2009). Although effectively controlled by herd testing, pasteurization, meat inspection, health surveillance and BCG vaccination, transmission to humans can occur and is still considered a public health risk (Moda and others, 1996, Smith and others, 2004, de la Rua-Domenech, 2005), although some more recent opinion considers this risk to be negligible (Torgerson and Torgerson, 2010). Hence, bovine TB control is currently more concerned with trade implications. Despite sustained and costly implementation of eradication programmes since the 1950s bovine TB has not been eradicated from either the UK or ROI. Indeed, there has been a sustained and largely unexplained increase over the last 20 years in parts of the UK (Gilbert and others, 2005). Consequently, bovine TB is the most complex and difficult multi-species endemic disease currently facing government, the veterinary profession and the farming industry in the UK and ROI (Reynolds, 2006, More and Good, 2006). Whilst it is important to view bovine TB as an infectious disease

which requires preventive as well as control measures, M. bovis infection in cattle now rarely appears to present as clinical disease. More commonly it appears as apparently healthy animals responding to an immunological test based on tuberculin, an entirely different scenario to that which existed when control programmes were first introduced (Collins, 2006). The problem of bovine TB in the UK is exceptionally complicated and the relationship between evidence, uncertainty and risk is difficult to communicate (Krebs, 2011). It is recognised as a very significant policy challenge and continues to be, almost inevitably, highly politicized (Spencer, 2011). Key to understanding bovine TB epidemiology is the relationship between infection and disease (TB) and the relationship between disease and transmission. Risk factors are known to influence both transmission and susceptibility.

2.4. Bovine TB – A Global View

Bovine TB is distributed throughout the world and has been reported on every continent (except Antarctica). Basically, where there are cattle there is, or has been, bovine TB (Smith and others, 2006b); with the notable exception of those countries that have eradicated bovine TB using a test-and-slaughter policy to remove infected cattle, such as Australia, some Caribbean islands (including Cuba) and parts of South America. In other countries, notably the USA, Canada, South Africa and most of the European Union (EU), with the exception of the UK and Republic of Ireland (ROI), bovine TB has been reduced to negligible levels, although sporadic, and sometimes persistent and damaging outbreaks associated with the import of infected cattle or the existence of wildlife reservoirs have been reported (Smith and others, 2006b; Reviriego Gordejo and Vermeersch, 2006). In New Zealand (NZ), the control of bovine TB in cattle and farmed deer has proven difficult, and a wildlife reservoir in brush-tailed possums has been implicated. Their control programme targets bovine TB in cattle and in local wildlife. Recent evidence indicates that NZ is now making significant progress towards eradication, although the epidemiology is different to that in the UK and ROI. The combination of cattle

surveillance and controlling bovine TB in local wildlife has also limited a persistent outbreak associated with white-tailed deer in Michigan (USA).

The suspected wildlife reservoir in Michigan, Canada and the Kruger National Park in South Africa is itself of economic/social value. Social issues and public approval could also be important factors in eliminating the disease, and has strong resonance for the UK, where the badger, a protected species, has been implicated as a reservoir host (Smith and others, 2006b).

2.5. Epidemiology

2.5.1. Host range of tubercle bacilli

The ability of MTC (*Mycobacterium tuberculosis* complex) bacteria to infect a wide variety of mammalian species has been attributed to their different routes of transmission from infected to susceptible hosts (Kaneene and Pfeiffer, 2006). The rate of transmission is determined by the infectiousness of the host expelling the pathogens, the concentration of the organisms and length of exposure; and immune status (or susceptibility) of the host at risk (Menzies and Neill, 2000; Tiruviluamala and Reichman, 2002). Similarly, the susceptibility levels vary with different host species, pathogenic mycobacteria, route of exposure, and infective dose and virulence of the tubercle bacilli strain (Biet *et al.*, 2005; Une and Mori, 2007; OIE, 2009; Thoen *et al.*, 2009). Susceptible non-specific hosts that become infected can serve as reservoir or "spillover" hosts with sporadic infections or persistent disease within affected animal populations if these maintenance hosts are present in the environment (Blunden and Smith, 1996; Gunn-Moore *et al.*, 1996; Gupta and Katoch, 2005; Cassidy, 2006; Corner, 2006; Ellis *et al.*, 2006; Monies *et al.*, 2006; Pollock *et al.*, 2006; Une and Mori, 2007; Shrikrishna *et al.*, 2009).

TB is not commonly reported in wild animals, except when they have been exposed to infected domestic animals or humans. Infected wild animals may be spillover cases where the disease is present but cannot be maintained in the wildlife

population. However, several wildlife species have been recognized as reservoirs of *M. bovis,* though cattle are the most probable natural reservoir hosts (Kaneene and Pfeiffer, 2006; Thoen *et al.,* 2009).

2.5.2. Source of infection

Infected cattle are important sources of infection for healthy cattle, but wildlife reservoirs of infection have also been reported in many regions (Wedlock *et al.,* 2002; Philips *et al.*, 2003; Kaneene and Pfeiffer, 2006; Thoen *et al.*, 2009); thereby complicating the epidemiological picture. Bovine TB is an FAO–OIE "List B" disease because of its important socio-economic and public health impact (Benkirane, 1998; OIE, 2008).

Bovine TB is also a serious zoonosis transmitted to humans through the consumption of contaminated raw or poorly cooked animal products (e.g.: fresh milk and meat), inhalation of aerosols from infected animals and contamination of breaks in the skin (Blood and Radostits, 1989; Ayele *et al.*, 2004; Kaneene and Pfeiffer, 2006; Wilsmore and Taylor, 2008; Cfsph, 2009).

2.5.3. Routes of transmission of the tubercle bacilli

There are several routes of entry and transmission of the tubercle bacilli but the respiratory and gastrointestinal tracts are the primary routes and less frequently incisions in the skin (Neill *et al.,* 1994; Goodchild and Clifton-Hadley, 2001; Philips *et al.*, 2003; Ayele *et al.,* 2004; Biet *et al.*, 2005; Kaneene and Pfeiffer, 2006; Wilsmore and Taylor, 2008).

Respiratory transmission through direct inhalation of contaminated aerosols is the most important route of infection in groups of susceptible hosts that remain in repeated close contact or in a confinement with infected individuals (Goodchild and Clifton-Hadley, 2001; Hussain *et al.*, 2003; Cassidy, 2006; Kaneene and Pfeiffer, 2006; Palmer and Waters, 2006).

The oral route is accomplished when feed or water contaminated with mucous, nasal secretion, saliva, discharging lesions, faeces and urine that contain the infective organism; and unpasteurised milk or raw meat from an infected animals are consumed by the healthy host (Moda *et al.*, 1996; Ayele *et al.*, 2004; Biet *et al.*,2005; Kaneene and Pfeiffer, 2006; Palmer and Waters, 2006). Also, the ingestion of *M. bovis* directly from cows to nursing calf and indirectly from contaminated pasture or farm tools to other animals (O'Reilly and Daborn, 1995; Cosivi *et al.*, 1998; Ayele *et al.*, 2004; Good, 2006; Goodchild and Clifton-Hadley, 2006; Delahay *et al.*, 2007) could be common in some regions.

Other modes of transmission though less common, include the transcutaneous mode of transmission of *M. bovis* from animals to humans who handle infected carcasses, with the spread of infection through cuts and abrasions; for example butcher□s wart in humans (Grange and Yates, 1994).

Congenital infections, genital transmission and vertical transmission have also been noted to occur when the reproductive organs are affected (Neill *et al.*, 1994; Cosivi *et al.*, 1998; Philips *et al.*, 2003; Figueiredo *et al.*, 2008), but these modes of transmission are rarely reported in regions with strict eradication programmes (Ayele *et al.*, 2004).

2.5.4. Incidence of infection

The incidence of the disease is higher in the developing nations because of the absence of any national control and eradication program, is also accelerating worldwide particularly in the Asian, African and Latin American countries (Bonsu *et al.*, 2001).

As stated earlier that bovine tuberculosis (bTB) has been significantly widely distributed throughout the world and it has been a cause for great economic loss in animal production. In developed countries, BTB in animals is a rarity with occasional severe occurrences in small groups of herds. In developing countries,

however, such as in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and (particularly in Africa 11%) enzootic occurrences of bTB have been reported (Cosivi *et al.,* 1998).

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 and 3.05% cattle in the district of Mymensingh (Samad and Rahman, 1986) and 27.5% breeding bulls (Islam *et al.,* 2007) showed positive reaction to the tuberculin test. 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.

2.5.5. Prevalence of bovine tuberculosis

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).

Javed *et al.,* (2009) conducted a study to find the prevalence of bovine tuberculosis (bTB) in buffaloes in relation to different factors such as age, weight, breed, sex, milk production, lactation length, parity and physiological status. The comparative intradermal test (CIDT) was used to identify reactor buffaloes for bTB. Overall prevalence of bTB was found to be 2.22% in positive reactors. The results showed that bTB was significantly high ($P < 0.05$) in buffaloes aged 5-8 years (5.04%). Non significantly high ($P > 0.05$) percentage of positive cases were found in buffaloes having weight higher than 500 kg (3.49%), having more than 6 parity (16.67%) , those in lactating stage (3.66%) or cross bred buffaloes (3.85%). Significantly high ($P < 0.05$) prevalence was recorded in buffaloes producing more than 15 litres (9.52%) of milk per day and having lactation length more than 8 months (11.11%). These results suggest that high incidence of BTB were found in old, heavy, high milk and parity producing and having long lactation length.

Inangolet *et al.,* (2008) determined the prevalence of bovine tuberculosis in the transhumant and agro-pastoral cattle herds in the border areas of Katakwi and Moroto districts in Uganda was carried out from July 2006 to January 2007 using

comparative intradermal tuberculin test containing bovine and avian PPDs. A total of 1470 animals, 612 (41.6%) males and 858 (58.4%) females, 883 (60%) young, 555 (37.8%) adult and 32 (2.2%) old animals were included. The study involved a cross-sectional multistage sampling technique with random selection of individual animals from a herd. The results revealed a 1.3% overall prevalence of bovine tuberculosis in cattle herds in the study area, with a marked variation between sub-counties. The highest recorded prevalence was 6.0% in Kapujan, while no cases were recorded in Ongogonja, Magoro and Katakwi sub-counties. Distinctly different patterns in the avian-bovine reactions were also found in different sub-counties. A multivariate logistic regression showed more positive reactions (OR=6.3; 95% CI (1.4–26.34) in females than males. BTB prevalence did not differ significantly between cattle maintained in pastoral and agro pastoral production systems. The study demonstrated a relatively low prevalence of bovine tuberculosis in local zebu cattle reared under traditional husbandry systems in Uganda, suggesting low infectiousness of the disease under such mode of production. The risk associated with the consumption of raw milk among the pastoral communities and that, the pooling of milk together from different animals is a common practice, warrants more investigation into the zoonotic transmission of tuberculosis within these communities.

Shitaye *et al.,* (2006) conducted postmortem surveillance for the detection of tuberculous lesions. The 10 year (1996-2005) retrospective analysis of the inspection of 2 455 289 slaughtered animals revealed that 707 (0.028%) were found with tuberculous lesions in parenchymatous organs and were obtained from 699 (0.052%) out of 1 336 266 cattle, 4 (0.001%) out of 534 436 sheep, 3 (0.001%) out of 573 767 goats and 1 (0.009%) out of 10 820 pigs. The tuberculous lesions found in cattle were significantly ($P<0.01$) higher than in other animals. The bovine tuberculin skin tests conducted in 10 areas (85 farms and 2098 cattle) in Addis Ababa revealed that positive reactions were obtained from 9 farm areas in 41 (48%) herds which included 392 (19%) animals. Tuberculous lesions were found in 34 (3.5%) out of 984 cattle during meat inspection surveillance. Histopathologically,

granulomatous inflammation was evident in 3 (8.8%) animals with tuberculous lesions. A highly sensitive PCR (IS6110) was positive in 4 out of 34 (11.8%) animals with tuberculous lesions and in 1 (2.9%) animal without lesions.

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).

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. 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%).

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%).

Kazwala *et al.*, 2001) conducted a study in the Southern Highlands of Tanzania to determine the prevalence of bovine tuberculosis and the risk factors associated with the occurrence of the disease in cattle of different categories and in different climatic zones. The overall prevalence of the disease was 13.2%, and 51% of the herds tested contained reactor cattle.

Coleman and Cooke, 2001) indicated that bovine tuberculosis (bTB) is the most important disease of livestock in New Zealand, and it puts at risk the nation's trade in dairy, beef and venison products

In Bangladesh so far the single intradermal (SID) skin test with purified protein derivative (PPD) has been used to detect the prevalence of bTB, and an overall 5.9% cattle in the district of Pabna (Pharo *et al.*, 1981), 3.05% cattle in the district of Mymensingh (Samad and Rahman, 1986) and 27.5% breeding bulls (Islam *et al.*, 2007) showed positive reaction to the tuberculin test.

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.

2.6. 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.

2.7. Clinical findings

Bovine TB in cattle is a chronic and *wasting* (weight loss) disease and other *non-specific* clinical signs include anorexia, drop in production (eg: drop in milk yield), chronic intermittent cough (may be productive), dyspnoea and enlarged regional lymph nodes in advanced cases which may rupture (Blood and Radostits, 1989; OIE, 2009).

2.8. Pathogenesis and Pathology of tuberculosis in cattle

The pathogenesis of TB, host immune response to tubercle bacilli, dissemination and combination of tuberculous lesions in the initial focus of infection and regional (i.e. draining) lymph nodes have been documented (Griffin and Buchan, 1994;

Neill *et al.*, 1994; Dannenberg , 2001; Neill *et al.*, 2001; Smith, 2003; Gupta and Katoch, 2005; Cassidy, 2006; de la Rua-Domenech,2006b; Palmer and Waters, 2006; Pollock *et al.*, 2006; Thoen and Barletta, 2006).

TB is characterized by progressive development of granulomatous lesions or tubercles in affected tissues / organs (Blood and Radostits, 1989; McAdams *et al.*, 1995; Cassidy, 2006; Liebana *et al.*, 2008). Tuberculous lesions have been reported to be distributed mostly in the respiratory tract and associated lymph nodes of naturally infected cattle (Francis, 1971; Collins and Grange, 1983; Blood and Radostits, 1989; McAdams *et al.*, 1995; Cassidy *et al.*, 1998; Cassidy, 2006; Palmer and Waters, 2006; Liebana *et al.*, 2008), particularly in portions of the lungs close to the pleural surface (Cassidy, 2006). Predominant findings of lesions in the retropharyngeal, submandibular and parotid lymph nodes also exist in a considerable proportion of animals, suggesting potential foci of excretion on the upper respiratory tract surface (Corner, 1994; Neill *et al.*, 1994).

Macroscopic lesions of tuberculosis in cattle are typically caseous, yellow and mineralized (Dungworth, 1993), and 95% of lesions are located in the lungs and lymph nodes of the head, thorax and abdomen (Corner, 1994). The lesions caused by *M. bovis* and *M. tuberculosis* in cattle are similar in appearance, but *M. tuberculosis* infection usually does not progress beyond the development of small granulomas in the pharyngeal, thoracic and mesenteric lymph nodes (Cousins, Huchzermeyer, Griffin, Brückner, Van Rensburg & Kriek, 2004).

Microscopic 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 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).

This initial infection, also termed primary TB may resolve spontaneously in most individuals (Thoen *et al.*, 2009), the healed lesions appearing on chest radiograph as calcified parenchymal nodules (Leung *et al.*, 1992; Thoen *et al.*, 2009). Haematogenous spread of the tubercle bacilli to other body structures may follow the pulmonary reactivation to produce the extrapulmonary form (McAdams *et al.*, 1995).

After inhalation of contaminated droplets by adult cattle, initially lodge in the respiratory tract would result in local inflammatory reaction followed by spread to regional lymph nodes in the thorax and haematogenous dissemination to the head and abdomen (Liebana *et al.*, 2008; Thoen *et al.*, 2009). A low-grade fever and symptoms of respiratory illness may also be present. In cattle signs usually become visible at the *advanced stage* of the disease (Corner, 1994; Shitaye et al., 2006) and mainly in adult or old animals (Oloya *et al.*, 2006). Some infected livestock are apparently in healthy condition showing no evidence of infection but lesions may be found during slaughter / meat inspection (Murray *et al.*, 1991; Shitaye *et al.*, 2006).

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

It is necessary to determine the minimum infective dose of *Mycobacterium bovis* to stimulate specific immune responses and generate pathology in cattle. There are 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.

2.10. Immune response against mycobacterial infections

There is an initial interaction between macrophages and mycobacteria after infection 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.

2.11. Economic importance in Dairy sector

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). TB is of great economic importance in the animal industries (wild and domestic) worldwide, especially in countries where little information is available on the incidence of *M. bovis* infection in humans (Cosivi *et al.*, 1998; Enarson, 2006; Pavlik, 2006a; Pavlik, 2006b; Thoen *et al.,* 2006; Zinsstag *et al.,* 2006; Defra, 2008).However, *M. bovis* has been estimated to account for less than 0.5% – 7.2% of human TB in most industrialised countries and 10% – 15% in most developing countries (Cosivi *et al.,* 1998; Ashford *et al.,* 2001; de la Rua-Domenech, 2006b; a). Also, approximately 85% of the cattle and 82% of the human populations of Africa live in areas where animal TB is either partly controlled or not controlled at all (Ayele *et al.,* 2004; Shitaye *et al.,* 2006).

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. TB in dairy cattle has been reported to cause a reduction of 17% milk production in Mexico (Anon, 1995) and 4% in a herd of USA (Hermandez and Baca, 1998). Thus the TB is of paramount importance to cattle producers and public health authorities because of its economic and zoonotic implications (Hermandez and Baca, 1998). Bovine tuberculosis is still common in less developed countries, and severe economic losses can occur from livestock deaths, chronic disease and trade restrictions. In some situations, this disease may also be a serious threat to endangered species.

2.12. Zoonotic threat

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 bacteria particularly *M. bovis* have since spread to all groups in the human and animal populations; and constitute major threats to human health as well as animal health and production (Tan *et al.*, 2003; Ayele *et al.*, 2004; Une and Mori, 2007; Thoen *et al.*, 2009). Reliable information is not generally available on the incidence of human TB due to *M. bovis* in developing countries since poor attention is given to the problem due to limited diagnostic facilities.

Indeed, zoonotic TB is neglected in most African countries and techniques for differentiating between organisms are not widely accessible (Cosivi *et al.*, 1998; Zinsstag *et al.*, 2006; Thoen and LoBue, 2007; Marcotty *et al.*, 2009). In countries where bovine TB is endemic and not controlled or partially controlled, human TB due to *M. bovis* may occur resulting from ingesting contaminated milk and milk products, other fresh animal products (ex: contaminated raw beef) and by inhaling cough spray from infected cattle (Collins and Grange, 1987; Moda *et al.*, 1996; Cosivi *et al.*, 1998; Etter *et al.*, 2006; Thoen *et al.*, 2006; Shitaye *et al.*, 2007). Also, human infection by *M. bovis* is clinically indistinguishable from that caused

by *M. tuberculosis* (Cosivi *et al.*, 1998; de la Rua-Domenech, 2006b; Thoen *et al.*, 2009) and can lead to pulmonary and extrapulmonary TB. Cervical lymphadenopathy, intestinal lesions, chronic skin TB (lupus vulgaris) and other extrapulmonary forms are common in human *M. bovis* infection (Cosivi *et al.*, 1998; Kazwala *et al.*, 2001a).

Zoonotic TB can occur as a result of occupational or accidental hazard among cattle farmers, handlers of fresh cattle products, veterinarians and migrants from countries where bovine TB is endemic (O'Reilly and Daborn, 1995; Ameni *et al.*, 2003; Ayele *et al.*, 2004; Etter *et al.*, 2006; Shitaye *et al.*, 2007; Regassa *et al.*, 2008). *M. bovis* infected cattle professionals may also be source of infection to cattle (Cosivi *et al.*, 1998; Ayele *et al.*, 2004) as well as other persons they come in contact with (Ocepek *et al.*, 2005; Evans *et al.*, 2007; Thoen *et al.*, 2009; Etchechoury *et al.*, 2010). Few studies have reported the isolation of *M. bovis* from humans suffering from pulmonary TB in parts of Africa such as Cameroon, Egypt, Nigeria, Democratic Republic of Congo and Tanzania (Cosivi *et al.*, 1998; Kazwala *et al.*, 2001a; Niobe-Eyangoh *et al.*, 2003; Zinsstag *et al.*, 2006). Also, an epidemiologic association between tuberculin skin positive cattle and human TB has been observed in Zambia (Cook *et al.*, 1996; Regassa *et al.*, 2008).

2.13. Diagnosis of tuberculosis

The diagnosis of tuberculosis in the living animal may be based on clinical findings, the tuberculin test (TB screening test), histopathological examination 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).

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

The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein

derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail.

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).

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). 72 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).

2.13.2. Isolation and identification of organisms

2.13.2. 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).

2.13.2.2. 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).

2.13.2. 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).

2.13.3. Demonstration of the tubercle bacilli

Presumptive findings based on histopathological techniques and microscopic demonstration of acid-fast bacilli have also been described (Chakravorty *et al.,* 2005; Johnson *et al.,* 2008). Direct smears from clinical samples (e.g. sputum) or suspected tissues typically lymph nodes are stained with the Ziehl - Neelsen stain, a fluorescent stain or immunoperoxidase techniques to demonstrate the acid-fast tubercle bacilli under the microscope (Strong and Kubica, 1985; WHO,1998a). The diagnosis is confirmed following growth and isolation of the *Mycobacterium* on

various selective media (e.g.: Lowentein-Jesen media, Middlebrook broth). Incubation of mycobacterial culture takes up to eight weeks for *M. tuberculosis* growth and twelve weeks for *M. bovis* (Strong and Kubica, 1985; WHO, 1998b; a; OIE, 2009). Further characterisation of the organism can be achieved by performing various biochemical tests (Nitrate reduction, Niacin and Catalase tests), culture characteristics (morphology of colony) and direct polymerase chain reaction (PCR) based genomic deletion typing for the presence or absence of various regions of difference (Frothingham, 1995; Brosch *et al.*, 2002; Parsons *et al.*, 2002; Smith *et al.*, 2006a; Warren *et al.*, 2006; Müller *et al.*, 2009a; Ameni *et al.*, 2010b). Detailed molecular typing or genetic fingerprinting techniques (e.g. Spoligotyping, Variable Number Tandem Repeat) may be used to further differentiate, characterise and geographically map different strains of *Mycobacterium* species (Frothingham and Meeker-O'Connell, 1998; van Soolingen *et al.*, 1998; Frothingham *et al.*, 1999; Sritharan and Sritharan, 2000; Watterson and Drobniewski, 2000; Drobniewski *et al.*, 2003; Chakravorty *et al.*, 2005).

2.13.4. 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.

2.13.5. Detection of cellular immunity

Various techniques to determine cellular immunity for the diagnosis of TB have been described. Tuberculin skin tests (TST) using purified protein derivatives (PPDs) of *M. tuberculosis, M. bovis* and *M. avium* are widely used to critically detect TB infection in humans and animals. However, blood tests that also measure cellular immunity such as the gamma-interferon and lymphocyte transformation assays have been used as ancillary tests to the TSTs for improved detection of the preclinical stages of TB in live subjects (Hoge *et al.* 1994; Gonza´lez-Llamazares *et al.*,1999; Ameni *et al.*, 2000; de Lourdes Garcia-Garcia *et al.*, 2000; Brock *et al.*, 2001; Ameni and Tibbo, 2002; Cousins and Florisson, 2005; de la Rua-Domenech *et al.*, 2006a; Coad *et al.*, 2008; Kim *et al.*, 2009). The lymphocyte proliferation test is uncommonly used in cattle, but may be useful in wildlife and zoo animals (Cfsph, 2009). Actually both tuberculin skin and gamma interferon (IFN-γ) tests have been used to maximise the efficacy of detecting latent TB and early stages of the disease in immunocompromised hosts (Ameni *et al.*, 2000; Kim *et al.*, 2009; Ameni *et al.*, 2010a).

The gamma-interferon (IFN-γ) test, an in vitro immunoassay based on the specific release of IFN-γ as the indicator of a response to the *M. bovis* antigen, was

described by Wood *et al.*, to enhance sensitivity, specificity, and to reduce handling events during tuberculin skin screening (Alicia et al., 2006). IFN-γ can be detected using an enzyme immunoassay and the sensitivity of the IFN-γ test was higher compared to the TST and the specificity of the IFN-γ assay was 90.6 to 98.6% (Alicia *et al.* 2006; de la Rua-Domenech *et al.*, 2006a). However, the performance and specificity of both tuberculin skin and IFN-γ tests can be affected by co-infecting agents, the most frequent being the presence of other mycobacterial infections such as paratuberculosis leading to dual infections of bovine tuberculosis and paratuberculosis (Alicia *et al.*, 2006). False positive reactors with the TST and/or the IFN-γ tests, most probably caused by cross-reactivity with *M. avium* subsp. *paratuberculosis*, have been reported (Biet *et al.*, 2005; Alicia *et al.*, 2006; de la Rua-Domenech *et al.*, 2006a). Though the TST are widely used for international field diagnosis of bovine TB in live animals (de la Rua-Domenech *et al.*, 2006a; de la Rua-Domenech *et al.*, 2006b), they may not be appropriate to eradicate bovine tuberculosis in herds with dual mycobacterial infections.

2.13.6. Detection of humoral immunity

Tests of humoral immunity employing the Enzyme-linked immunosorbent assays (ELISAs), immunochrommatographic (lateral flow) assay and other serologic based tests may complement tests of cellular immunity in anergic hosts. However, anti-TB antibodies titres are inconsistent and rise only in the late stages of infection while a limited cocktail of selected *Mycobacterium* antigens (e.g.: ESAT-6, MTSA-10, MPTS1, MPT63, MPB59, MPB64, MPB70, MPB83) are employed to detect circulating antibodies (Lyashchenko *et al.*, 1998; Pollock *et al.*, 2001; Banerjee *et al.*, 2003; Cousins and Florisson,2005; Lyashchenko *et al.*, 2007; Lyashchenko *et al.*, 2008).

Therefore the benefits of using multiple diagnostic tests to detect mycobacteria infected animals and humans cannot be overemphasized. It would be interesting to combine anti-bovine TB antibodies detection assays with tuberculin skin and or IFN-γ techniques for the accurate detection of *M. bovis* infected in animals.

2.13.7. 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.

2.14. Control / eradication of tuberculosis

Control and eradication programs for bovine TB, human TB and zoonotic TB of humans due to *M. bovis* are based on early accurate detection and removal of infected animals, chemotherapy of infected humans and vaccination of target populations to attenuate or prevent the manifestation of the disease (Citron, 1988; Abernethy *et al.,* 2006; Good ,2006; Goodchild and Clifton-Hadley, 2006; Pavlik, 2006a).

Finland was probably the first country to eradicate the disease in animals in 1899. Some countries followed the method of 'test and slaughter'. Such a plan is, however, not practicable in India, (Lall *et al.,* 1967). The test-and-slaughter policy is the basis for international bovine TB control and eradication programs using the TST to detect affected herds (and re-test) periodically and removing reacting cattle (Gilbert *et al.,* 2005; Abernethy *et al.*, 2006; Good, 2006) that may shed the infective organism.

In many industrialized countries there is "effective" compulsory reporting of *M. bovis* infection of all animals, quarantine of infected herds, tracing and re-testing of animals in contact with bovine tuberculin skin positive reactors, movement restrictions of cattle herds *not* yet tested for TB as well as controlled animal movement out of known TB infected herds and endemic areas (Citron, 1988; Gilbert *et al.,* 2005; Abernethy *et al.*, 2006; Good, 2006; Goodchild and Clifton-Hadley, 2006; Pavlik, 2006a; OIE, 2008; 2009). However, the test-and-segregation program, a modified form of the test-and-slaughter policy, may be more useful for developing countries, where the test-and-slaughter policy cannot be practicable for the whole cattle population (WHO, 1994b). Thus, interim measures to segregate infected herds and phased slaughter of reactors are done. In most countries with strict TB eradication programmes, the test-and-segregation strategy made up the early stages followed by the test-and-slaughter methods in the final stage (Cfsph, 2009) and infected slaughter / meat cases during inspection are traced back to the originating farms (Defra, 2008). Informed farm management decisions such as proper sanitation and disinfection are also important to reduce the spread of *Mycobacterium* within and between herds as well as the risks of exposure and transmission of bovine TB infection to humans (Wilsmore and Taylor, 2008).

The occurrence of *M. bovis* in wildlife reservoir hosts complicates eradication efforts. Culling to reduce population density can decrease animal TB transmission but the situation must be assessed carefully to avoid unanticipated effects such as the economic benefit and increase scattering members of the infected species

(Donnelly *et al.*, 2007; Smith *et al.*, 2007; Cfsph, 2009). The development of TB vaccines for wildlife reservoirs (Hughes *et al.*, 1996; Ayele *et al.*, 2004) and use in situations where the test-and-slaughter policy is totally impracticable (WHO, 1994b) is also being considered as an alternative. Also, human TB due to *M. bovis* is rare in countries where raw and poorly cooked meat are not consumed; and pasteurization of milk and milk products are components of bovine TB eradication programs (WHO, 1994b; Ayele *et al.*, 2004).

2.15. Vaccines used for tuberculosis

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).

2.16. 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 design

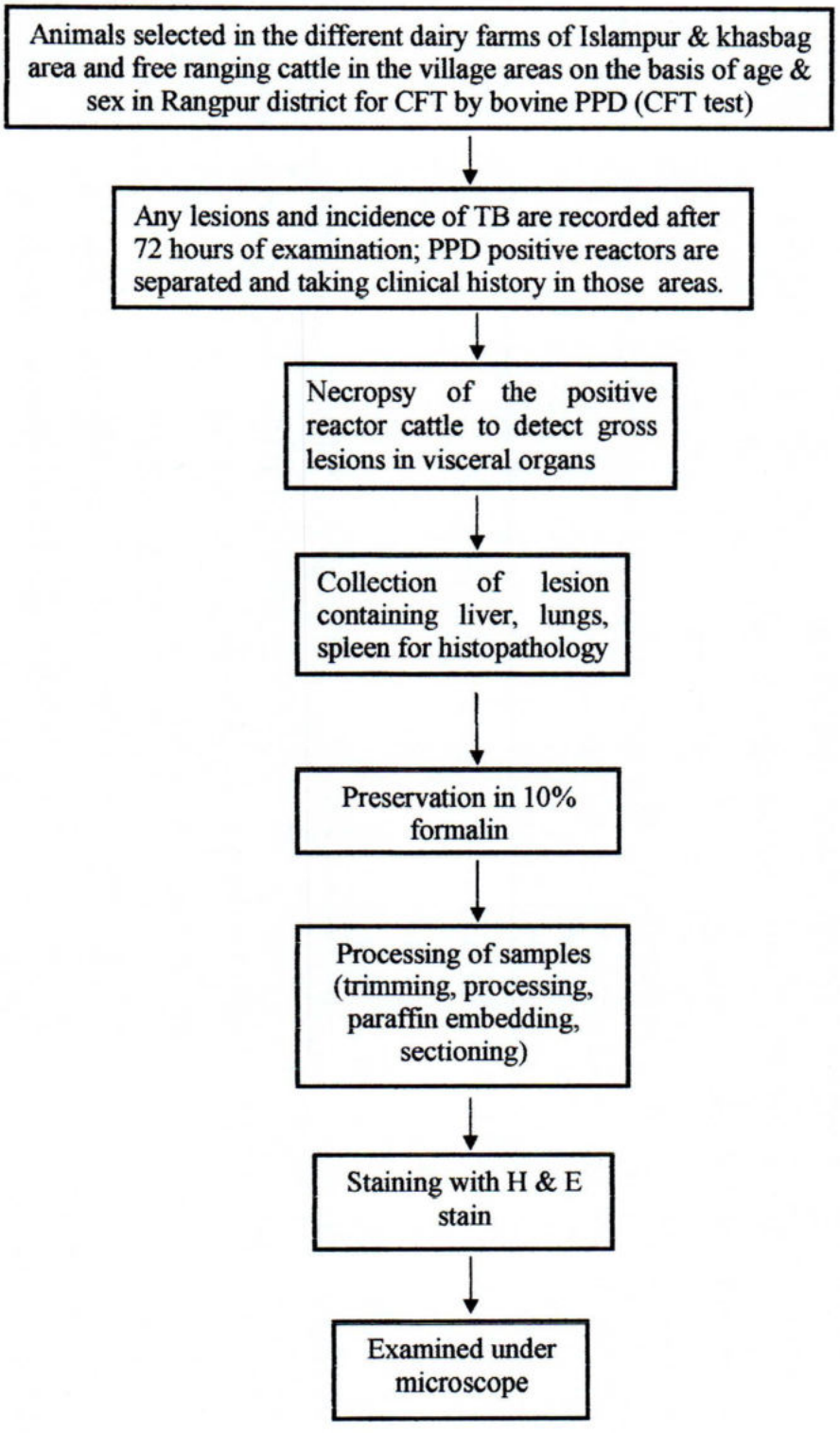
This study was represent the prevalence of bovine tuberculosis in cattle in Rangpur District. The experimental areas were the dairy farm of Islampur and khasbag village of sadar upazilla and free ranging cattle in Mahigong, Sampur, Najirer hat, Deodoba and Rampura area in Rangpur district. The experiment 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.

In this study a total of 240 cattle of the selected dairy farms and free ranging cattle of different regions were subjected to caudal fold tuberculin (CFT) test using bovine purified protein derivative (bPPD). Little amount of these cattle were positive to tuberculin test. The duration of the study was January – December, 2012.

The present study was performed the following major works

- Caudal fold tuberculin test in selected farm and free ranging cattle;
- To compare the prevalence of bovine tuberculosis of different age and sex;
- Necropsy examination of visceral organs to detect lesions of tuberculosis in tuberculin positive animals;
- Histopathological examination of lung of tuberculin positive cattle.

Experimental design



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

Materials required

- Bovine purified protein derivative (bPPD)
- Disposable syringe & needle(1 ml)
- Masks
- Ice carrier with ice pack
- Gumboot and Apron

Plate 1. Bovine PPD box

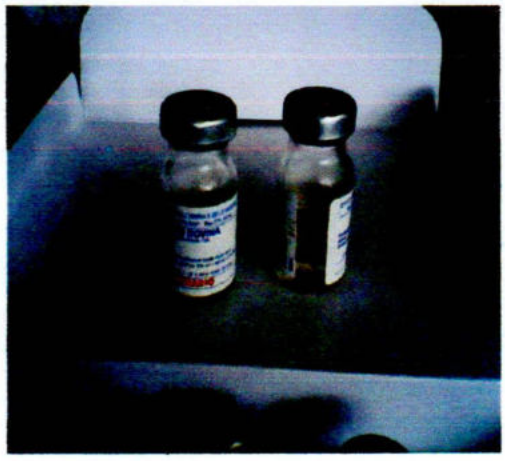


Plate 2. Bovine PPD vials

Plate 3. Tuberculin syringe

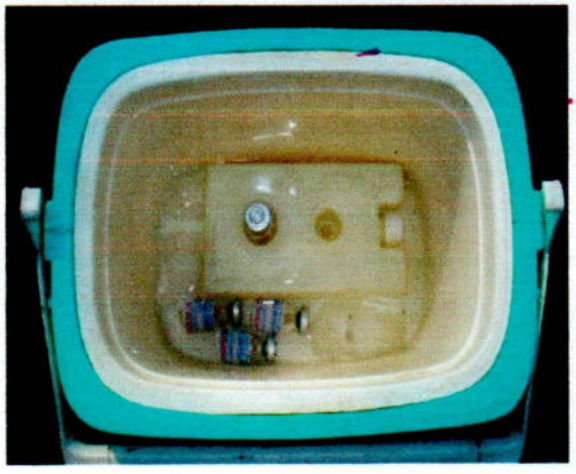


Plate 4. PPD vials with ice pack

3.2.1 Selection and grouping of animals

To determine the prevalence and risk factors associated with bovine tuberculosis infection in 240 cattle from different dairy farm and free ranging areas in Rangpur district were selected using CFT test. Of the 240 cattle, 98 were male 142 were female, among these cattle, are grouped into three subgroups: as group-1 are within 7 months-3 years, group-2 are within 4-6 years and group-3 are among over 6 years of age. Date of birth, age, sex and other managemental information were recorded in a questionnaire.

3.2.2 Selection of tuberculin and other necessary materials and instruments

For the CFT testing, bPPD was used in this study. The bPPDs was obtained from a licensed laboratory in Italy (*Instituto Zooprofilattico sperimentale dell'Umbria e delle Marche, Perugia).*

All the tuberculin vials were kept in a refrigerator for maintaining the potency and quality as for instruction of the manufacturer. For the purpose of cleaning and disinfection of the inoculation site, sterilized cotton and 70% ethyl alcohol were employed. For inoculation, 1 ml tuberculin syringe of 100 graduations, fitted with a short hypodermic needle was used.

3.2.3 Procedure of Caudal Fold Tuberculin (CFT) Test

This is the primary screening test to detect animals potentially infected with bovine TB. The test grossly measures the immune response to *Mycobacterium bovis*, the causative agent of bTB. According to the test intradermal injection of 0.1 ml bPPD was given with a hypodermic syringe in the skin of the caudal fold (the fold of skin at the base of the tail). If the animal has been exposed to mycobacteria, the immune system responds with inflammatory cells at the injection site to cause swelling and/or discoloration of the skin. After 72 hours the injection site is inspected and palpated to evaluate for a response. Marked edematous swelling, reddening at the

injection site classifies the animal as a responder. If no response is noted, the animal is classified as CFT test-negative.

The results were interpreted according to OIE standards (OIE, 2004):

a. If the reaction is ≥ 4.0 mm greater, then the test is considered reactor.

b. If the reaction is between 3.0 and < 4.0 mm, then the test is considered suspicious.

c. If the reaction is < 3.0 mm, then the test is considered negative.

Table 1: Schedule and procedure of inoculations

Test	Reagent	Site	Route	Dose	Time of observation post inoculation
CFT test	**bPPD**	**Caudal fold**	**i/d**	**0.1 ml**	**72 hours**

bPPD= Bovine purified protein derivative

3.2.4 Reading of the results of inoculations

In CFT test the reading was taken 72 hours after the inoculation. The positive tuberculin reaction was evident from an inflammation of sensitive nature at the point of inoculation. The swelling was either soft and edematous or somewhat hard in nature. The swelling was felt and estimated by palpation at the site of inoculation, while the animal expressed the sign of pain. Reading was taken after 72 hours, the injected areas were examined to observed any swelling, induration, discoloration or any changes.

3.3. Necropsy findings of suspected cattle

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

Equipment and appliances for necropsy

- Collected sample from suspected animals (cattle)
- Bone cutting saw
- Scissors (3)
- Forceps (4)
- Scalpel and blade
- Chisel
- 10% formalin
- Gloves
- Musk

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.

3.4. 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.

3.4.1. Equipment and appliances

- Sample (lungs)
- 10% formalin
- Chloroform
- Paraffin
- Alcohol
- Tape Water
- Xylene
- Hematoxylin and Eosin Stain
- Distilled water
- Clean Slides
- Cover slips
- Mounting media (dpx)
- Microscope

3.4.2. 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%, 95% 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.

3.4.3. 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

Hematoxylin 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).

CHAPTER IV

RESULTS

4.1 Caudal fold tuberculin test:

During January to December 2012, 240 cattle (over 6 months) were randomly selected from different dairy farms of Islampur and khasbag area of the sadar upazilla and free ranging cattle in village area at Rangpur District. These randomly selected 240 cattle were tested by using caudal fold tuberculin test with bPPD to investigate the prevalence of bovine tuberculosis. After 72 hours, distinct visible swelling and indurations were considered to be positive reaction and indistinct indurations were considered to be suspicious reaction.

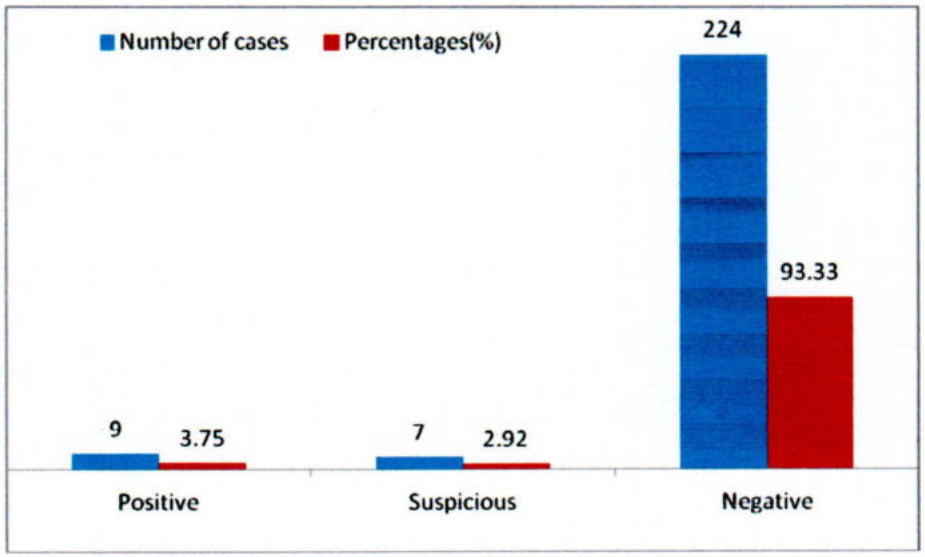
Table 2. Presence of BTB in the different regions at Rangpur District detected by caudal fold tuberculin test.

Name of place	**Animal tested**	**Positive reaction**	**Percentage of positive animals**	**Suspicious reaction**	**Percentage of suspicious animals**
Islampur dairy farm	35	0	3.75%	0	2.92%
Khasbag dairy farm	48	2		2	
Deodoba	47	2		1	
Najirer-hat	32	2		1	
Rampura	27	1		1	
Sampur	22	2		2	
Mahigong	29	0		0	
Total	240	9	-	7	-

Out of 240 animals, 9 animals with 3.75% reacted to CFT test with bovine PPD which showed marked swelling of the inoculation site at the caudal fold(Table.3.) and 7 animals with 2.92% showed suspicious reaction with the test which showed only slight swelling at the site of inoculation and the remaining 224 (93.33%) did not react or showed no swelling at the site of inoculation.

Table 3. Results of CFT test using bPPD

Total number of animals tested	Nature of reaction	Number of cases	Percentage (%)
240	Positive	9	3.75%
	Suspicious	7	2.92%
	Negative	224	93.33%



Graph-1. Graphical representations of the result of CFT test using bPPDs.

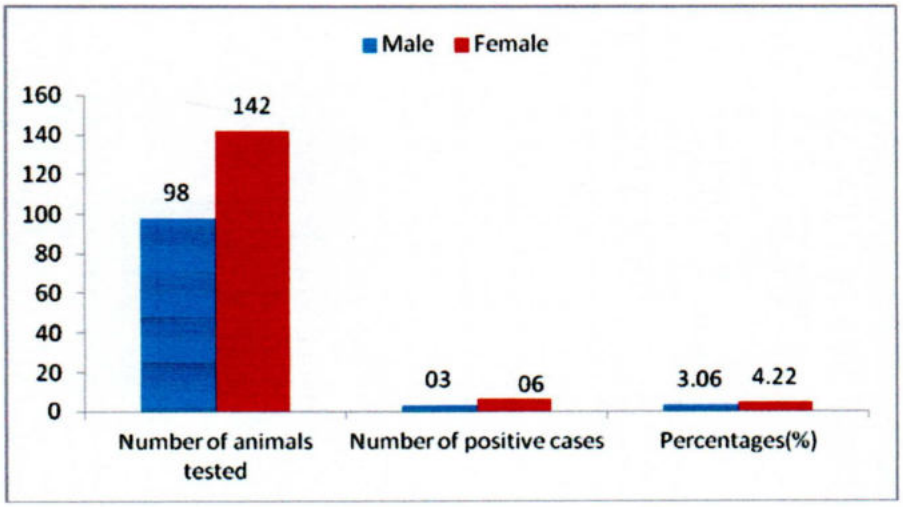
4.1.1 Results of CFT test on the basis of sex

4.1.1.1 Reactor animals based on sex

Of the 240 animals which were subjected to CFT test, the number of male reactor was 03 (3.06%) while the numbers of female reactors were 06 (4.22%).

Table 4. Reactors of CFT test with bPPD on the basis of sex.

Sex	Number of animals tested	Nos. of positive cases	Percentage (%)
Male	98	03	3.06
Female	142	06	4.22



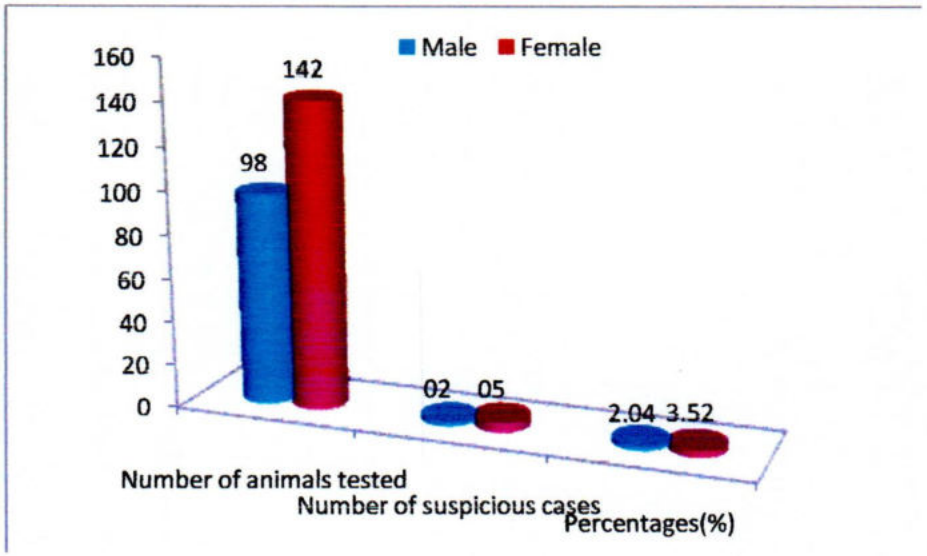
Graph- 2. Graphical representations of reactors of CFT test with bPPD on the basis of sex.

4.1.1.2 Suspicious cattle based on sex

Of the 240 animals which were subjected for CFT test, the number of suspicious male reactors was 02(2.04%) while the numbers of suspicious female reactors were 05(3.52%) in same test.

Table 5. Suspicious cases of CFT test with bPPD on the basis of sex.

Sex	Number of animals tested	Number of suspicious cases	Percentage (%)
Male	98	02	2.04
Female	142	05	3.52



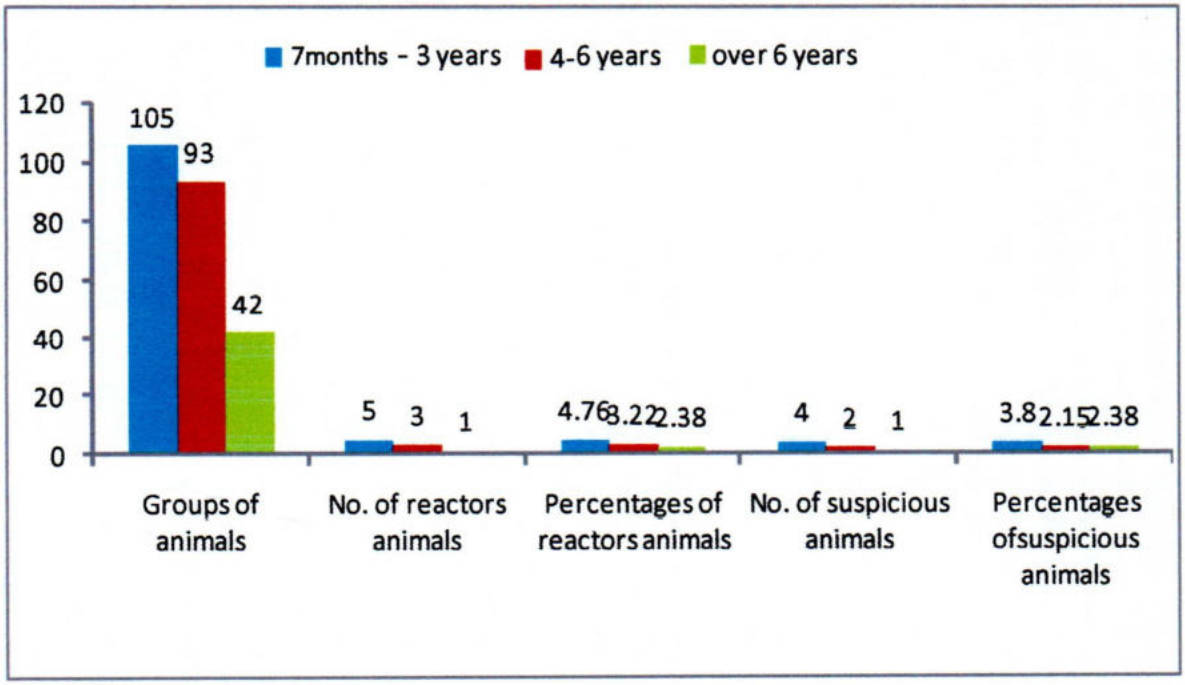
Graph- 3. Graphical presentation of suspicious reactors of CFT test with bovine PPD on the basis of sex

4.1.2. Results of CFT test on the basis of age

Among the 240 animals of different age those were subjected to initial CFT test, highest percentage of 4.73 positive reactor and also 3.80% suspicious animals were observed in the age group between 7months-3 years, 3.22 percentages of positive reactor and also 2.15% suspicious animals were found in the age group between 04-06 years, 2.38 percentages of positive reactor and also 2.38% suspicious animals were found in the age over 6 years.

Table 6. Results of CFT test on the basis of age

Groups of animals	Age of the animals	Number of animals tested	Number of reactors & percentages (%)	Number of suspicious cases & percentages (%)
First group	7months-3 years (young)	105	5(4.76)	4(3.80)
Second group	04-06 years (adult)	93	3(3.22)	2(2.15)
Third group	Over 6 years (Old)	42	1(2.38)	1(2.38)



Graph- 4. Graphical representation of the results of CFT test on the basis of age

4.2. Clinical findings of the TB affected animals

Fig. 1: The animal is clinically affected with bovine TB. The cattle contained mild cough, anorexia, emaciated body condition in respect to its age.

Fig.2. Caudal fold inoculation of bPPD

Fig. 3: Picture of caudal fold swelling after 72 hours of inoculation of bovine PPD which indicate that the animal was affected with TB(arrow).

4.3. Necropsy findings of suspected animals

The necropsy examination included different lesions in different regions of lungs entailing enlarged, congested and consolidated lungs containing a large and fairly distinctly demarcated caseous nodules on the dorsal surface (Fig. 4). There are

presence of nodule within a lung (Fig.5) and haemorrhagic, necrosed and consolidated band regions were found on the ventral surface of the lungs and fibrotic lesions were found at the border of lung (Fig.7,8). The spleen was enlarged but grossly nodular lesions were not seen. (Fig.9).

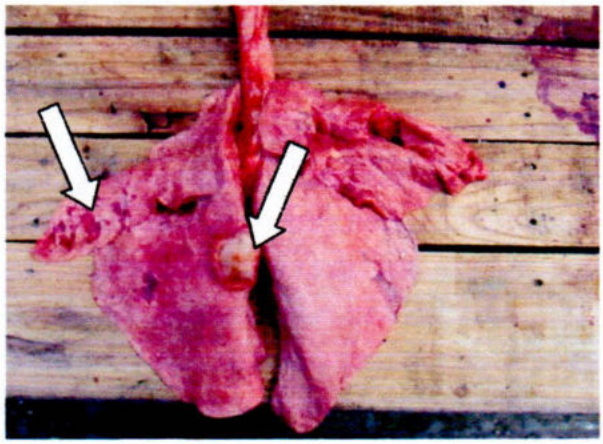


Fig.4. A picture of Lungs obtained from tuberculin +ve sample. The lung were congested and contains a large nodules(arrows).

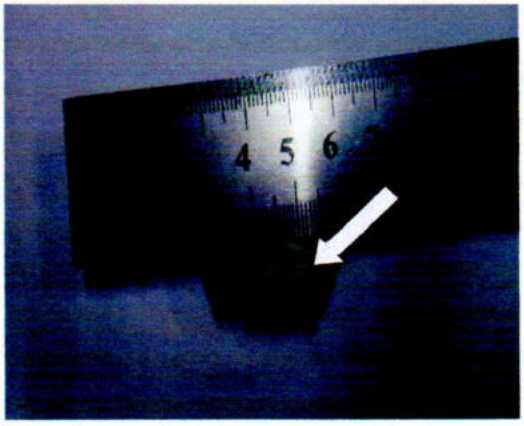


Fig. 5. Presence of nodules within a lungs obtained from a tuberculin +ve cow(arrow).

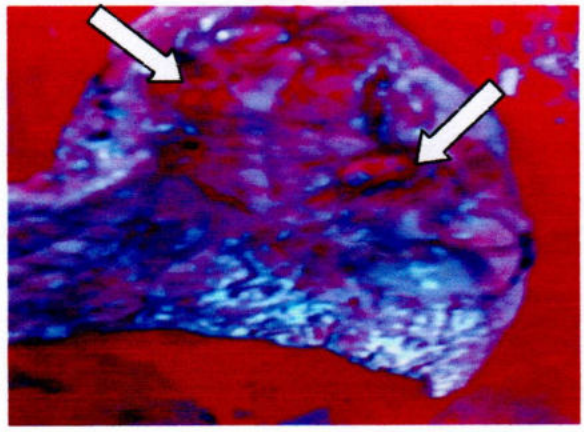


Fig.6: Picture of lungs collected from tuberculin positive cattle. Presence of nodules within lung parenchyma(arrows).

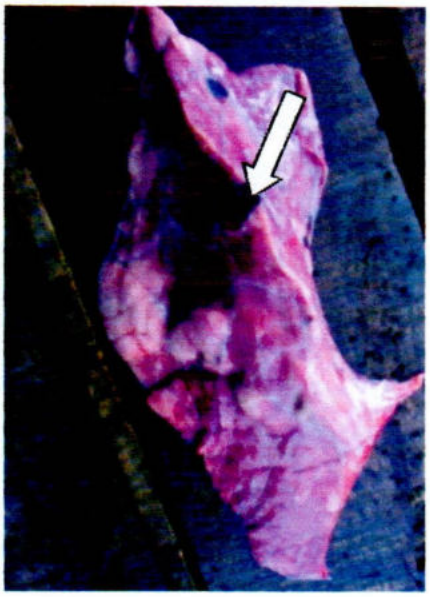


Fig. 7: Picture of lungs collected from tuberculin positive cattle. Haemorrhagic, necrosed and consolidated band regions were found on the ventral surface of the lungs(arrow).

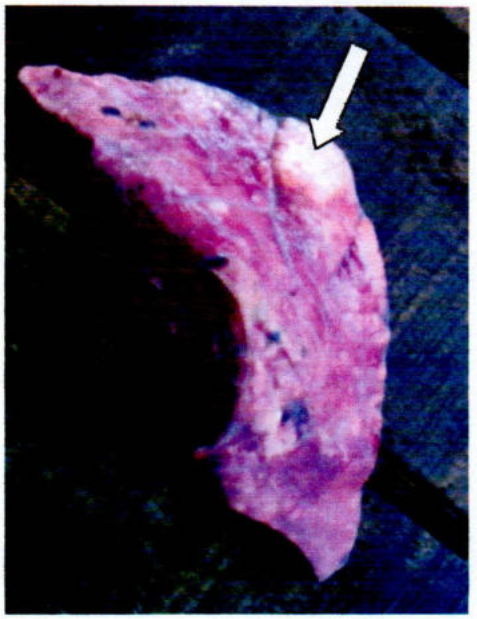


Fig. 8: A picture of lungs collected from a tuberculin +ve cattle. Fibrotic lesions were found at the border of lung(arrow).

Fig. 9. A picture of spleen obtained from a tuberculin +ve cow. The spleen was enlarged. Grossly nodular lesions were not found.

4.4. Histopathological examination

A granulomatous nodule were seen in the lungs of tuberculin positive cow. There was caseous necrotic center, which is surrounded by thick fibrous connective tissue capsule (Fig. 10). There were presence of necrogranulomas with central caseous necrosis infiltrated by neutrophils (Fig. 11). Alveoli of lungs of tuberculin positive cattle were field with exudate (Fig. 13). Heavily infiltration of reactive cells were seen in the lungs of tuberculin positive cattle (Fig. 14) and also proliferation of fibrous connective tissue were seen in the lung parenchyma along with fewer reactive cells (Fig. 15).

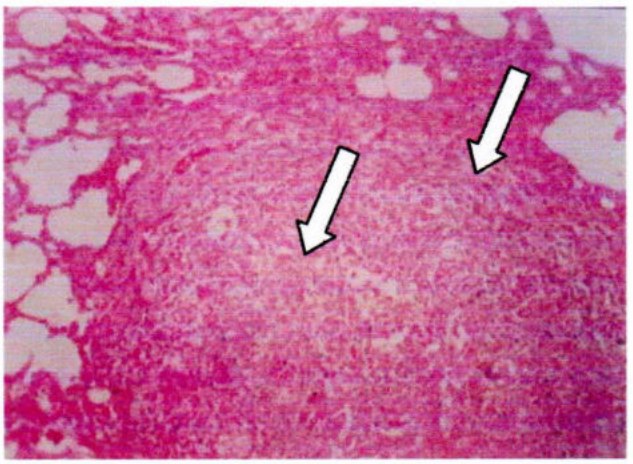


Fig. 10. A granulomatous nodule was seen in the lungs of tuberculin positive cow, stained with H&E. There was caseous necrotic center, which is surrounded by thick fibrous connective tissue capsule(arrows).

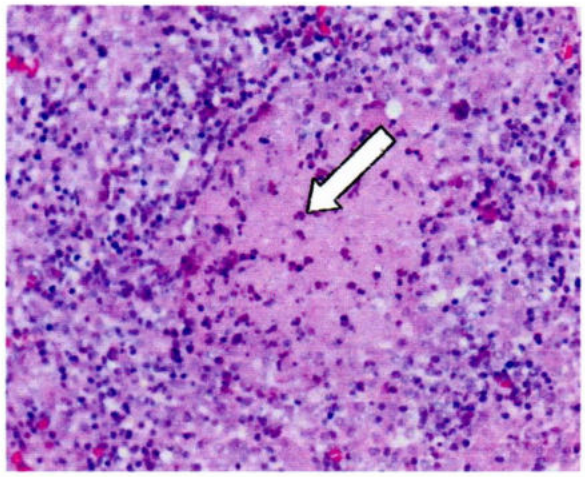


Fig. 11. Presence of necrogranulomas with central caseous necrosis infiltrated by neutrophils (arrow).

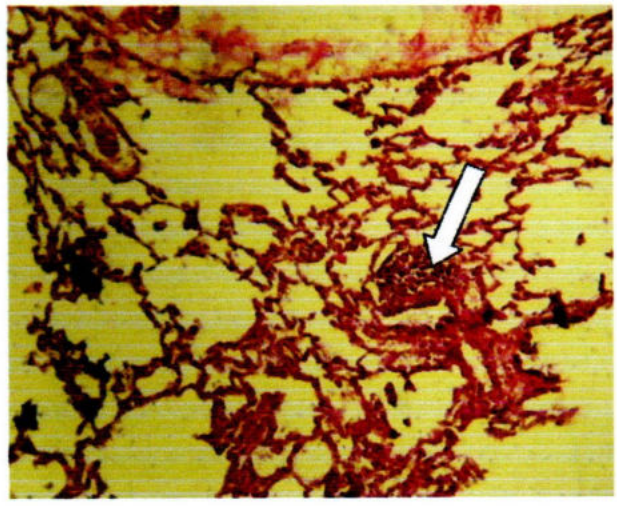


Fig. 12: Accumulation of reactive cells seen in the lungs of tuberculin positive cattle (arrow).

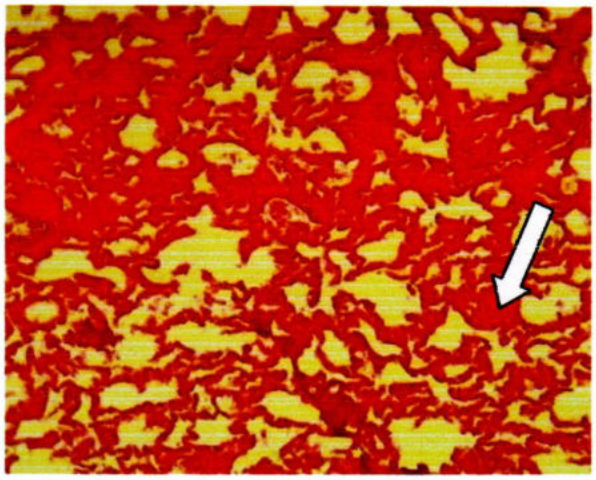


Fig. 13: Alveoli of lungs of tuberculin positive cattle were field with exudates (arrow).

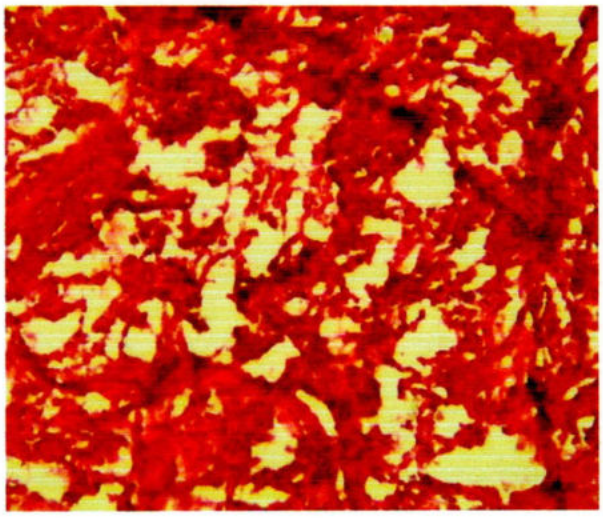


Fig. 14: Heavily infiltration of reactive cells seen in the lungs of tuberculin positive cattle.

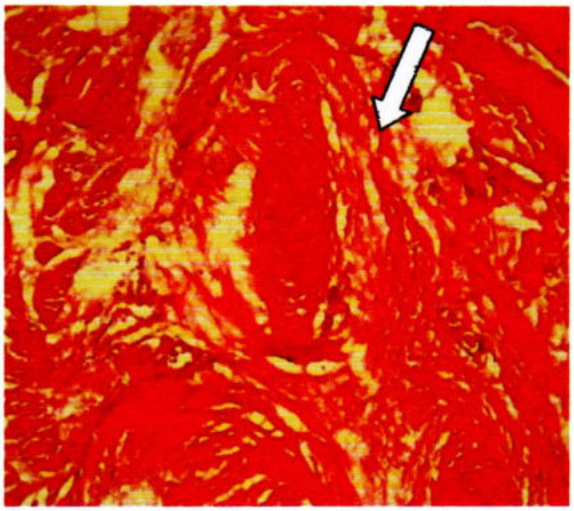


Fig. 15: Proliferation of fibrous connective tissue was seen in the lung parenchyma along with fewer reactive cells (arrow).

CHAPTER V

DISCUSSION

5.1. Caudal fold 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, 9 (3.75%) out of 240 cattle showed distinct tuberculin positive reaction and 7 (2.92%) out of 240 cattle showed suspicious reaction (Table 2) by caudal fold tuberculin test in case of free ranging cattle considering the age over 6 months regardless of age and sex. 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% suspicious reaction. 3.05% cattle in the district of Mymensingh (Samad and Rahman, 1986) and 27.5% breeding bulls (Islam *et al.*, 2007) showed positive reaction to the tuberculin test. 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.

Sex and age related prevalence

Prevalence of bovine tuberculosis is influenced by many factors such as geographical situation of a country, and it's temperature, hygienic status of humans and animals and enforced regulatory laws in Public health and Veterinary Public Health sectors that also affect the prevalence percentage. It has previously been reported that the animals older than 10 years were within high risk of infection (Tschopp *et. al.*, 2009). Similarly, Cleaveland *et. al.*, (2007) and Philips *et al.*, (2002) suggested that older animals are more susceptible to tuberculosis.

Inangolet *et al.*, (2008); also determined the prevalence of bovine tuberculosis in the transhumant and agro-pastoral cattle herds in the border areas of Katakwi and Moroto districts in Uganda was carried out from July 2006 to January 2007 using comparative intradermal tuberculin test containing bovine and avian PPDs. A total of 1470 animals, 612 (41.6%) males and 858 (58.4%) females, 883 (60%) young, 555 (37.8%) adult and 32 (2.2%) old animals were included. In this study, the percentage of female reactors was higher than the male and the youngers are more susceptable than adult.

From this study, it was found that a number of 240 animals which were subjected to CFT test, the number of male positive reactors were 03 (3.06%) while the numbers of female reactors were 06 (4.22%) and also the number of suspicious male reactors was 02(2.04%) while the numbers of suspicious female reactors were 05(3.52%) which indicated that positive reactor of female cattle were greater than the male cattle reactors which was somewhat similar according to Inangolet *et al.*, (2008). Although the exact cause of higher prevalence of female reactors can not be explained but it can be hypothesized that some hormonal influences may be associated with this phenomenon. Lioyd (1983) reported that higher level of prolactin and progesterone hormones make the female individual more susceptable to any infection. Moreover stresses of production such as pregnancy and lactation make the female animals more susceptable than male animals.

In case of age related prevalence, the present study was indicate that out of 240 animals of different ages, the highest 4.73 percentage of positive reactor and also 3.80% suspicious animals were observed in the age group between 7months-3 years, 3.22 percentages of positive reactor and also 2.15% suspicious animals were found in the age group between 04-06 years, 2.38 percentages of positive reactor and also 2.38% suspicious animals were found in the age over 6 years which was somewhat similar to Inangolet *et al.*, (2008). The younger groups of animals are mostly reacted to PPD, these may be due to the fact that the population size of younger are larger than the older or the disease is transmitted through the milk from affected cows to younger. It is also evident that younger have less develop immune system than the

older. Further studies are needed to rule out the genetics, epigenetic/environmental and Mycobacterium interplay of the different age and sex group of animals.

5.2. Clinical findings of the TB affected animals

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

5.3. Gross examination suspected cattle

This study represents different lesions in different regions of lungs entailing enlarged, congested and consolidated lungs containing a large and fairly distinctly demarcated caseous yellowish nodules on the dorsal surface of lungs (Fig. 4) in the tuberculin positive cattle. There were found haemorrhagic, necrosed and consolidated band regions on the ventral surface of the lungs and fibrotic lesions at the border of lung. The lesions were distributed throughout both parts of the lungs and divided macroscopically into haemorrhagic, fibrotic lesion and localized type (Fig. 7,8). The spleen was found enlarged but grossly nodular lesions were not seen (Fig. 9) during necropsy of the suspected animals.

(Blood and Radostits ,1989; McAdams *et al.,* 1995; Cassidy, 2006; Liebana *et al.*, 2008; Thoen *et al.*, 2009) found that the lesions or tubercles in affected tissues / organs were characterized by progressive development of granulomatous nodules reported to be distributed mostly in the respiratory tract and associated lymph nodes of naturally infected cattle and macroscopic lesions of tuberculosis in cattle are typically caseous, yellow (Dungworth, 1993) which are somewhat similar to this present study but the nodular lesions were not developed in other organs . This observation suggests the early stages of infection of the animal and primary site of lesions development.

This is in accordance with (Goodchild and Clifton-Hadley, 2001; Palmer and Waters, 2006) who state that respiratory transmission through direct inhalation of contaminated aerosols is the most important route of infection in groups of susceptible hosts that remain in repeated close contact or in a confinement with infected individuals which indicate that the mode of transmission of the infection could more likely be attributed to respiratory route.

5.4. Histopathological examination

A granulomatous nodular lesions were seen in the lungs of tuberculin positive cow. There was caseous necrotic center which is surrounded by thick fibrous connective tissue capsule(Fig. 10). There were presence of necrogranulomas with central caseous necrosis infiltrated by neutrophils (Fig. 11). Alveoli of lungs of tuberculin positive cattle were field with exudate (Fig. 13). Heavily infiltration of reactive cells were seen in the lungs of tuberculin positive cattle (Fig. 14) and also proliferation of fibrous connective tissue were seen in the lung parenchyma along with fewer reactive cells (Fig. 15). But the liver and spleen showed no granulomatous lesions though enlarged spleen (Fig. 9) were found macroscopically which might likely be due to chronic tissue level changes of visceral organs whereby lungs was followed by other organs.

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 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 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, spleen and other organs subsequently.

CHAPTER VI

CONCLUSION

The present study was conducted mainly to explore a pathological investigation of bovine TB based on caudal fold tuberculin test and to identify the prevalence of BTB and to compare the prevalence of bovine tuberculosis of different animals on the basis of age and sex. Distinct gross and histopathological lesions were found followed by necropsy of the tuberculin positive reactor cattle. The presence of bovine tuberculosis may have a zoonotic threat as occasionally human beings are infected with tuberculosis in these regions. 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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