INTRODUCTION

Tuberculosis is a chronic bacterial disease in animals and humans characterized by the progressive development of specific granulomatous lesions of tubercles in affected tissues. It is most important zoonotic chronic disease with high prevalence among human, domestic and wild animals in developing countries (Caswell and Williams, 2008).

*Mycobacterium bovis*, the causative agent of bovine tuberculosis, also infects other domesticated and wild mammals. The disease affects all age groups of susceptible hosts and is accountable for more deaths throughout the world than any other bacterial disease ever today (Omer et al., 1995). Tuberculosis (TB) remains a global health threat, with 9 million new cases and up to 2 million deaths annually (Kauffmann, 2007).

Tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs. Within the host, *M. bovis* is known to persist within granulomas with, distinct lesions represented by a caseonecrotic core surrounded by epithelioid macrophages, T cells, B cells, Langhan’s type multinucleated giant cells, and fibroblasts (Ulrichs and Kaufmann, 2006). Survival of the host depends on the ability to limit mycobacterial proliferation through effective granuloma formation.

After death, infection is diagnosed by pathological and bacteriological techniques. Mycobacterial culture is time consuming and, like molecular methods, does not permit identification of other causes of *in vivo* positive reactors. Histopathology, on the other hand, offers the major advantage of producing results within three days. Furthermore, the technique has a high specificity because it can histomorphologically characterize lesions unrelated to mycobacterial agents (e.g. parasites, neoplasia). Ziehl Neelsen (ZN) staining has high specificity but low sensitivity.

Nitric oxide (NO), the product of the enzyme nitric oxide synthase (NOS), is a highly reactive nonpolar gas that leads to a number of biochemical reactions that ultimately result in inactivation of various
pathogens (Clancy and Abramson, 1995). Inducible nitric oxide synthase (iNOS) is important in the control of a number of intracellular pathogens, including mycobacteria, and is a marker of classic macrophage activation. NO has been found to be involved in granuloma formation of both infectious and noninfectious granulomas of humans and animals. However, NO has not been implicated in all types of granulomas; for example, evidence of NO is not detectable in foreign body granulomas and nonspecific granulomatous lymphadenitis (Facchetti et al., 1999). The primary product of iNOS is mycobactericidal and this is consistent with a role for iNOS in controlling tuberculosis. In mammalian cells, NO production is catalyzed by three distinct isoenzymes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Clancy and Abramson, 1995). In macrophages and other cells, iNOS is stimulated by endotoxin, cytokines, and other activators (Jungi et al., 1996). Once produced, NO reacts with superoxide to generate peroxynitrite and other reactive nitrogen intermediates.

Experimental evidence exists that NO plays a role in controlling infection with members of the \textit{M. tuberculosis} complex (Chan et al., 1992) and bovine macrophages have been shown to express iNOS \textit{in vitro} (Adler et al., 1995) and \textit{in vivo} (Fligger et al., 1999). In contrast to those in mice, bovine macrophages require additional signals from bacterial products (e.g., lipopolysaccharide, lipoarabinomannan) in addition to Interferon-\(\gamma\) (IFN-\(\gamma\)) for maximal NO production (Jungi et al., 1997). Moreover, \textit{in vitro}, it appears that levels of NO produced by bovine macrophages are insufficient to control intracellular replication of virulent \textit{M. bovis} (Carpenter et al., 1998).

It is envisaged that the immunohistochemical demonstration of iNOS in the tissue sections found positive for bacilli by acid fast staining will contribute in better understanding of the pathogenesis of tuberculosis in bovines.

Keeping the above facts in mind the study is proposed with the following objectives:

1. Pathomorphological study of the tuberculous lesions in bovine.
2. Immunohistochemical demonstration of inducible nitric oxide synthase (iNOS) in tubercular granuloma.
REVIEW OF LITERATURE

The present research work was conducted to study the pathomorphology of tuberculous lesions in bovine and to demonstrate inducible nitric oxide synthase (iNOS) in the tubercular granuloma immunohistochemically. The relevant literature is briefly reviewed.

**Bovine tuberculous granuloma**

Cancela and Marin (1993) used the avidin-biotin complex peroxidase (ABC-P) method to detect *M. bovis*, and the results were compared with those obtained by the Ziehl-Neelsen (ZN) technique. Lesions were examined from 18 cows and 24 goats with tuberculosis. All animals showed pulmonary lesions, which in the cattle were mainly minor (i.e. primary complex) but in the goats were sometimes minor and sometimes severe. Microscopically, typical granulomas were seen in the lungs and lymph nodes, with central necrosis and the cellular components of chronic inflammation, but mycobacteria were either seen in small numbers or were not detectable. The ABC-P technique was more sensitive than the ZN method, as shown by the number of positive animals detected, the intensity of staining, and the successful use of low magnification. Caprine lesions, although more severe than bovine lesions, appeared to contain fewer organisms.

Whipple *et al.* (1996) determined the distribution of lesions in *M. bovis* infected cattle. Tuberculosis was confirmed in 15 cattle with evidence of infection in the following tissues: medial retropharyngeal, parotid, tracheobronchial, mediastinal, caudal deep cervical and subiliac lymph nodes, palatine tonsil and lung. Gross and histologic lesions were present most frequently in lymph nodes of the thoracic region. *M. bovis* was isolated from three cattle that had no gross lesions of tuberculosis. Results of this study indicate that not all cattle infected with *M. bovis* have visible lesions of tuberculosis in sites that are routinely inspected.

Kumar *et al.* (1998) conducted studies on tuberculosis in crossbred dairy cattle. They recorded tuberculosis in 9.8% cattle with a higher occurrence in adult animals. The frequency of lung involvement was 98.6%.
Maity *et al.* (2000) studied pathology of lymph nodes in cattle. They found 13.25% caseated lymph nodes. Grossly and microscopically lesions were suggestive of tuberculosis. *Mycobacterium* *sp.* was only identified from these lymph nodes.

Milian-Suazo *et al.* (2000) in their study found 400 (16%) of 2,500 cattle carcasses had gross lesions typical of tuberculosis. Of the 400 infected cattle, 336 (84%) had lesions in ≥ 1 lymph node. Infection was confirmed in 87% of cattle with gross lesions by histologic examination, in 77% and 59% cattle, respectively by bacteriologic culture in two different laboratories.

Jeyakumar *et al.* (2004) reported tuberculosis meteritis in a heifer. Numerous caseated and calcified nodules were present in lung, lymph nodes, liver and kidney. Grossly, uterus was enlarged contained thick fluid from cervix to tip of both horns. There was diffuse caseation with calcification. Epithelioid cells were noticed. Acid fast bacilli could be demonstrated in the cytosol of epithelioid cells.

Katoch *et al.* (2004) reported genital tuberculosis in a Jersey crossbred cow. The histopathological examination of the tissues collected from various parts of the reproductive tract revealed typical granulomatous inflammation of the oviduct and uterine wall showing focal to diffused lesions consisting of central caseous necrosis followed by macrophages, epithelial cells and giant cell infiltration. The granulomas were enclosed in thin fibrous encapsulation with mild lymphocytic reaction.

Kumar and Swamy (2005) reported miliary tuberculosis in crossbred cows. Lungs contained large number of nodules of varying size containing cream coloured pus. Impression smear showed presence of acid fast and alcohol fast Gram positive bacilli, morphologically similar to *M. bovis*. Microscopically, the tubercle comprised caseous necrosed centre surrounded by macrophages, plasmacytes, lymphocytes and fibroblasts. Characteristic Langhan’s type giant cells were also seen.

Shitaye *et al.* (2006) determined prevalence of bovine tuberculosis by abattoir meat infection. The retrospective meat analysis
showed that 699 (0.052%) of 1336266 cattle were found positive. Firm, creamy, whitish nodular focal lesions were predominantly (63.9%) observed in the lung cavity. The typical tuberculous lesions, granulomas with calcification, and multinucleated giant cells of Langhan’s type were detected in two (4.2%) and one (6.7%) lung and mesenteric lymph node respectively. Microscopically, no mycobacteria were detected in all examined tissue samples stained by ZN staining technique.

Ameen et al. (2008) investigated bovine tuberculosis in 17676 cattle. The granulomatous lesions in lung, lymph nodes (mediastinal, bronchial retropharyngeal and mesenteric) liver, spleen and intestine were observed in 97 cattle out of 17676 and they were positive by ZN technique. The most affected organs were 49 lungs (48.51%) and 26 lymph nodes (25.74%).

Basaraba et al. (2008) demonstrated that increased expression of host iron-binding proteins precedes iron accumulation and calcification of primary lung lesions in experimental tuberculosis in the guinea pig. The workers demonstrated that ferric iron accumulates both intra- and extra-cellularly in the primary lung lesions of guinea pigs infected with the H37Rv strain of M. tuberculosis. Iron accumulated within macrophages at the periphery of the primary granulomatous lesions while extra-cellular ferric iron was concentrated in areas of necrosis.

Liebana et al. (2008) examined 400 cattle for determining the pathology of naturally occurring bovine tuberculosis. Stage IV granulomas, alone or in combination with other stages, constituted 63% of lesions, while 16% of lesions were stage I/II granulomas. Caseous necrosis and calcification were common features of the granulomas encountered in natural M. bovis infections. Granulomas often covered large areas of histological sections and typically contained only small numbers of acid fast bacilli.

Russell et al. (2009) in their study observed that the foamy macrophage appears to be a key player in both sustaining persistent bacteria and contributing to the tissue pathology that leads to cavitation and release of infectious bacilli.
Vural and Alcigir (2010) studied pathomorphological and immunohistochemical findings of tuberculosis in cattle. Amongst the 86 TB cases, 78 (91%) were located in the lungs, pleura and regional lymph nodes, five (6%) were located in the liver and portal lymph nodes, and three (3%) were generalized. Pathomorphologically, 81 cases (94%) comprised miliary-nodular tubercles, and five cases (6%) were determined to be chronic organ tuberculosis. The lesions were localized prominently in caudal lobe (65%), caudal-cranial lobe (13%), cranial lobe (3%), and all lobes (19%). These were protrusive, hard, whitish yellow nodules ranging from 2-5 mm to 3 cm in size that displayed dry and calcified areas in cut sections. Immunohistochemically, *M. bovis* antigens were seen in the macrophages, necrotic areas, and the Langhan’s giant cells (96%) in the same tissues but generally the number and intensity of the positivity were much higher than ZN.

Sanchez *et al.* (2011) compared microscopical and immunological features of tuberculoid granulomata and cavity pulmonary tuberculosis in naturally infected goats. They characterized the immunological mechanisms that lead to liquefaction and cavity formation by comparing granulomata and cavity lesions. In cavity lesions there was a substantial population of neutrophils and a significant decrease in the number of CD4+ T cells, with concomitant increases in other T-cell populations. The enzyme iNOS was strongly expressed by macrophages in the cavity lesions.

Sharma *et al.* (2011) examined total number of 1804 cattle lungs of different age groups, sex and breeds. Out of these 768 representative samples of lungs showing gross lesions, were further examined histopathologically. Out of these 23 cases (2.99%) were positive for pulmonary tuberculosis. Grossly, lungs were found to be consolidated and studded with grayish white nodules, mostly distributed in diaphragmatic lobes. Microscopically, granulomatous lesions typical of tuberculosis were evident in all cases.

Besirovic *et al.* (2012) examined twenty-eight cows euthanized after being found positive by comparative tuberculin skin test. Eleven animals were subject to field necropsy, and lesions consistent with tuberculosis were observed on the lymph nodes of the thoracic cavity and lungs in all carcasses.
Histopathologic examination by hematoxylin and eosin staining confirmed the presence of specific granulomatous lesions, while ZN staining demonstrated the presence of very few acid fast bacteria. Mediastinal lymph nodes from seven necropsied animals were submitted for bacteriology. Acid fast bacteria from five out of the seven submitted samples were isolated. All isolates were identified as *M. caprae*.

Shettar (2012) conducted studies on lesions of tuberculosis. Out of fifteen cases twelve animals revealed pulmonary form of tuberculosis, one with generalized form and two animals showed no observable lesions. The frequency of caseative lesions in affected animals was 73.73% in bronchial lymph nodes, 66.67% in lungs, 40.0% in mediastinal lymph nodes. The remaining organs such as prescapular, retropharyngeal, mesenteric lymph nodes, intestine and liver showed lesions at 6.67%. In histopathological study, granulomatous inflammation was observed in all affected organs characterized by focal or multifocal areas of central caseation with or without calcification, surrounded by a zone of inflammatory cells consisting of lymphoid cells, epithelioid cells and Langhan's type of giant cells.

**Immunoreactivity of iNOS in granuloma**

Goldmann *et al.* (1996) investigated the expression of iNOS in rat pulmonary cryptococcosis. iNOS immunoreactivity was detected in macrophages, neutrophils, vascular endothelium, and respiratory epithelium. iNOS immunoreactivity was detected in a selective population of epithelioid macrophages within some granulomas but not others. iNOS- granulomas were identical to iNOS+ granulomas with respect to morphology and immunohistochemical profiles. iNOS expression coincided with granuloma formation and preceded a decrease in lung fungal burden, suggesting an anticryptococcal role for NO.

Facchetti *et al.* (1999) using western blotting and immunohistochemistry, investigated iNOS expression in human lymph nodes with nonspecific reactions and in tissues containing granulomas caused by *Mycobacteria, Toxoplasma, Cryptococcus neoformans, Leishmania, Bartonella*, non infectious granulomas (sarcoidosis, foreign body), and other
histiocytic reactions. Immunohistochemistry demonstrated that iNOS was selectively expressed by the epithelioid and multinucleated giant cells within the granulomas.

Fligger et al. (1999) determined expression of inducible nitric oxide synthase in spontaneous bovine bronchopneumonia. High levels of iNOS were expressed by cells (probably leukocytes) surrounding necrotic foci. Occasionally, iNOS was expressed by intra-alveolar macrophages in viable parenchyma, by leukocytes within the airways, and by some chondrocytes in the supporting cartilage of bronchi. iNOS expressing cells were largely restricted to the cellular zone surrounding necrotic areas.

Pando et al. (2001) showed expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT) during the evolution of experimental pulmonary tuberculosis. In this study, they used a well-characterized mouse model of pulmonary tuberculosis to examine the local kinetics of expression and cellular distribution of iNOS and NT at the cellular and subcellular level. The histopathological study showed two phases of the disease. The early phase was characterized by mononuclear inflammation and granuloma formation. During this phase, high percentage of activated macrophages was observed that were immunostained for iNOS and NT. Immuno-electronmicroscopy showed NT immunoreactivity in lysosomes and mycobacterial wall and cytoplasm. The concentration of iNOS mRNA and NO metabolites were also elevated. The late phase was characterized by progressive pneumonia with focal necrosis and a decrease of iNOS mRNA and NO metabolites. Macrophages became foamy cells with scarce iNOS immunostaining but strong NT immunoreactivity.

Choi et al. (2002) analyzed nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. Immunohistochemical and morphometric analyses revealed that, compared with control subjects, iNOS, eNOS, and nitrotyrosine, but not nNOS, were significantly elevated in the inflammatory zone of the tuberculous granulomas, and in the nongranulomatous pneumonitis zone. Tumor necrosis factor-α (TNF-α) was also significantly increased in tuberculous lungs and was principally localized to the necrotic, and to a lesser extent, the inflammatory
and fibrotic areas of the granulomas. The NOS isoforms, nitrotyrosine, and TNF-α were expressed by the epithelioid macrophages and giant cells within the granulomas and in alveolar macrophages and epithelial cells in pneumonitis areas.

Hostetter et al. (2005) recorded iNOS immunoreactivity in intestine of cattle suffering from Johne’s disease. The vast majority of granulomas from *Mycobacterium avium paratuberculosis*, animals lacked iNOS immunoreactivity (scores of 0–1). Variation in granuloma morphology did not significantly alter iNOS expression, when overlap of iNOS and *Mycobacterium avium paratuberculosis* occurred within granulomas. Overlap was more frequent in dilated crypts, where clusters of mycobacteria and iNOS-positive cells intermixed with moderate numbers of neutrophils and cell debris were present (crypt abscesses). In 19 cases, iNOS staining was identified in low frequency near the basal aspect of the crypts in the deep lamina propria. In the lymph node granuloma from the *M. bovis* infected animal there was multifocal iNOS immunoreactivity within the granulomas (score of 2–3), which was most near the center as well as areas bordering foci of necrosis and neutrophil infiltration.

Suarez et al. (2006) demonstrated by western blot a high expression of natural resistant associated macrophage protein (NRAMP1) in peripheral blood mononuclear cells (PBMCs), alveolar macrophages (obtained by bronchioalveolar lavage), and lymph node granulomas from eight Holstein-Freisian cattle with bovine TB. Immunohistochemistry revealed the abundant expression of NRAMP1 and iNOS in lymph node and lung granulomas. Immunoreactivity was abundant in the cytoplasm of many epithelioid macrophages and multinucleated giant cells of the Langhan’s type. A striking accumulation of nitrotyrosine (NT), an indicator of iNOS activity and local NO production, was observed in granuloma cells, particularly in multinucleated Langhan’s cells.

Palmer et al. (2007) observed the lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with *M. bovis*. Tissues from four calves each were examined at 15, 28, 42, 60, 90, 180, 270, and 370 days after inoculation. Granulomas in the
medial retropharyngeal lymph nodes were staged (I–IV) on the basis of cellular composition and the presence or absence of necrosis and peripheral fibrosis. Immunohistochemistry for inducible nitric oxide synthase (iNOS), was performed. Abundant iNOS immunoreactivity was associated with granulomas from day 15 through day 60 but was minimal from day 90 to the termination of the experiment.

Thacker et al. (2007) determined associations between cytokine gene expression and pathology in M. bovis infected cattle. After 15, 30, 60 and 85 days post-infection (dpi) peripheral blood mononuclear cells (PBMC) were isolated and stimulated. In addition, gene expression adjacent to gross lesion in the retropharyngeal lymph nodes (LN) was analyzed. Pathology was evaluated at necropsy. Expression of IFN-γ, TNF-α, iNOS and IL-4 by PBMC increased in response to infection, whereas, IL-10 expression decreased. Gene expression in PBMC and LN was compared between animals in the high and low pathology groups. Cells from animals in the high pathology group expressed more IFN-γ, TNF-α, iNOS and IL-4 than did animals in the low pathology group at early time points. IL-10 gene expression decreased.

Chiapello et al. (2008) demonstrated that glucuronoxylomannan (GXM) which is major component of Cryptococcus capsular polysaccharide, represents an essential virulence factor for this yeast. Cryptococcus neoformans infections in immunocompetent rats are associated with inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by macrophages. This study demonstrates in vitro and in vivo that GXM promotes iNOS expression with NO production in rat macrophages.

Beisiegel et al. (2009) performed studies on dual role of iNOS. They stated that combination of host susceptibility and virulence of M. tuberculosis determines dual role of nitric oxide in the protection and control of inflammation. Confrontation of limited host resistance with heightened bacterial virulence forms a most hazardous combination. They investigated extreme combinations, confronting inducible nitric oxide synthase deficient (iNOS_/_) and wild-type (WT) mice with two related M. tuberculosis strains that differed markedly in virulence, namely, the M. tuberculosis laboratory strains H37Rv and H37Ra. Deregulated chemokine signaling and excessive
neutrophil necrosis contributed to disproportionate neutrophil influx and exacerbated TB in iNOS/−/− mice infected with virulent *M. tuberculosis* (strain H37Rv), whereas resistant and susceptible mice controlled attenuated H37Ra equally well.

Moustafa *et al.* (2009) diagnosed TB peritonitis in human. TB granulomas were found in 9/16 cases (56%) and a diffuse granulomatous reaction was found in the remaining 7/16 cases (44%). Immunoreactivity to iNOS was intensely expressed in macrophage rich TB granuloma and in the diffuse granulomatous TB reaction. Most Langhan’s cells (multinucleated giant cells) showed strong reactivity of iNOS. An increased local expression of iNOS in granuloma associated macrophages of untreated patients was observed indicating excess NO production in the active stage of this form of tuberculosis.

Yang *et al.* (2009) evaluated the role of nitric oxide in mycobacterial infections. The workers reported that nitric oxide (NO) and inducible NO synthase (iNOS) are important in innate immune responses to various intracellular bacterial infections, including mycobacterial infections. It is generally recognized that reactive nitrogen intermediates play an effective role in host defense mechanisms against tuberculosis. In a murine model of tuberculosis, NO plays a crucial role in anti-mycobacterial activity.

Delgado *et al.* (2010) performed studies on expression of NRAMP1 and iNOS in *M. avium* subsp. *paratuberculosis* naturally infected cattle. Paratuberculosis (PTB) is a chronic disease caused by *M. avium* subsp. *paratuberculosis* (MAP). The changes in NRAMP1 and iNOS expression were surveyed by immunohistochemistry in tissue samples from MAP-infected cattle and healthy controls. A strong specific immunolabeling against both NRAMP1 and iNOS molecules, throughout granulomatous paratuberculosis (PTB) compatible lesions in ileum and ileocaecal lymph nodes from paratuberculous cattle compared with uninfected controls, suggesting a relationship between the expression of these molecules and the pathogenesis of PTB disease was observed. The workers postulated that natural resistance-associated macrophage protein 1 (NRAMP1) and the
inducible nitric oxide synthase (iNOS) molecules show nonspecific effects against several intracellular pathogens residing within macrophages.

Hermeyer et al. (2011) detected *Mycoplasma bovis* by *in-situ* hybridization and expression of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase (Mn-SOD) in the lungs of experimentally-infected calves. All infected calves had an increased number of cells expressing iNOS, NT and Mn-SOD in the inflamed lung tissue. These molecules were most strongly expressed by macrophages demarcating necrotic areas, by altered bronchiolar epithelial cells and by macrophages within obliterated bronchioles. Co-localization of *M. bovis* DNA, *M. bovis* antigen and macrophages expressing iNOS, NT and Mn-SOD was observed.
MATERIALS AND METHODS

1. Location and Place of Work

The proposed work was carried out in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Jabalpur and the Advanced Tuberculosis Diagnosis Laboratory, Centre for Wildlife Forensics and Health, Nanaji Deshmukh Veterinary Science University (N.D.V.S.U.), Jabalpur (M.P.).

Meteorological data and features of place

Jabalpur is situated at 2317˚ latitude and 79.57˚ E longitudes at 410.87 MSL (mean sea level) in the southern part of third agro-climatic zone, including Satpura Plateau and Kymore hills. It has tropical climate having average rainfall of 1241mm.

2. Materials

2.1 (a) Animals

Material for laboratory examination was collected from dead cattle irrespective of age sex or breed.

2.1 (b) Chemicals

The chemicals used in the histochemistry and immunohistochemistry procedures are enlisted below.

- Ziehl –Neelsen staining kit (K005-1KT, Hi-Media)
- Phenol (AS021, Hi-Media)
- Ferric Chloride (RM 1178, Hi-Media)
- Biebrich-Scarlet (RM 3204, Hi-Media)
- Phosphomolybdic Acid (RM 629, Hi-Media)
- Phosphotungstic Acid (RM 398, Hi-Media)
- Aniline Blue (RM901, Hi-Media)
- Orange G (O 0030, RANKEM)
- Sliver Nitrate (S0060, RANKEM)
• Sodium Thiosulphate (N:28005, RANKEM)
• Aluminium Sulfate (RM 282, Hi-Media)
• Nuclear Fast Red (RM 2354, Hi-Media)
• Potassium Ferrocyanide (RM602, Hi-Media)
• Oil Red O (TC256, Hi-Media)
• Propylene Glycol (P0618, RANKEM)
• Tris Base (H5131, PROMEGA CORPORATION)
• Serum albumin from bovine (A9647, SIGMA ALDRICH)
• Polyclonal antibody: Rabbit antimouse iNOS of Fisher Scientific having Product number PA3030A was used.
• ExtrAvidin Peroxidase kit for rabbit antibodies (Catlog No. EXTRA3, SIGMA ALDRICH): Contains biotinylated mouse anti-rabbit IgG and streptavidin-peroxidase.
• AEC staining kit (Catlog No. AEC101, SIGMA ALDRICH): contains acetate buffer, 3-amino-9 ethylcarbazole (AEC) and 3% hydrogen peroxide.
• Harris hematoxylin (SO34, Hi-Media)

2.1 (c) Equipment
Cryostat used for cryosections (LEICA, Model No.CM 1100)

3. Methods
3.1 Study period:
The study was conducted for a period of seven months from August 2012 to March 2013.

3.2 Collection of samples
Gross examination of over 250 bovine carcasses was done at slaughter houses and during postmortem examination. From these lungs and extrapolmonary tissue having nodular/ cystic appearance with a suspicion of tuberculosis were collected for further processing of histopathology and cryostat sections.
3.3 Direct smear examination

Impression smears (imprinting and scraping) were prepared from the lesions suspected of tuberculosis and stained by Ziehl-Neelsen as per the method described by Chauhan (2006). For this staining procedure ZN staining kit manufactured by Hi-media was used as described by their guidelines.

4. Pathological examination

External examination of the dead animals brought for postmortem and live animals prior to slaughter was done. All major visceral organs and regional lymph nodes were examined and incised. The gross lesions in different visceral organs and lymph nodes were noted. The frequency of lung lesions in different lobes was recorded.

4.1 Collection of tissue for histopathology

Sample of lymph nodes and pulmonary tissues showing enlargement, nodule formation and caseation were collected. The tissue samples were collected in 10% formalin and processed for histopathological examination (Gridley, 1960).

The samples were also collected in buffered formalin for demonstration of immunoreactivity of iNOS on paraffin embedded tissue sections.

Bovine lung tissues showing no gross changes (n=6) were also collected from slaughter house.

4.2 Processing of samples

The small tissue pieces (approximately 0.5x0.5cm) were dehydrated in three changes of acetone. These sections were then cleared in three changes of benzene. After that impregnation of wax was done as per the method described by Gridley (1960). The blocks were made after infiltration with the aid of L – molds.

4.3 Section cutting

The 5μm thick sections were cut by rotary type microtome. The sections were taken on clean glass slide with egg albumin as adhesive for
histopathology and gelatin as adhesive for immunohistochemistry.

4.3(i) Cryostat sections

The collected tissue slices were kept at -20 °C in deep freeze until processing. The tissues were thawed for about 5 minutes and were cut in 1 cm size and placed in the tissue holder using jung tissue freezing medium to bind tissue to specimen block. These were kept for about 10 minutes inside the cryostat machine LEICA CM 1100 model at -20°C for freezing. Sections of 6-8 μm thickness were taken on clean glass slide.

5. Staining of tissues

5.1. Routine staining :Hematoxylin-Eosin (HE)

Hematoxylin-Eosin (HE) staining was performed as per the method of Gridely (1960).

5.2. Special staining techniques

5.2(i) Demonstration of acid fast bacilli in tissue sections:

For demonstration of acid fast bacteria in tissue sections, Ziehl-Neelsen (ZN) and Kinyoun’s technique were followed as described by Chauhan (2006).

5.2(i) a. Ziehl-Neelsen (ZN)

Formalin preserved paraffin section was stained by Ziehl-Neelsen (ZN) as described by Chauhan (2006). For this, ZN staining kit manufactured by Hi-media was used.

Procedure

- The sections were deparaffinized and hydrated.
- Sections were stained in carbol fuchsin solution for 10-15 minutes along with heating.
- Washed in distilled water.
- Differentiated in acid alcohol till sections appeared pale pink.
- Counter stained in methylene blue solution for 30 seconds.
- Dehydrated, cleared and mounted in DPX.
With this staining the acid fast bacilli appeared singly or in clumps as bright red rods.

5.2(i)b. Kinyoun’s technique

Formalin preserved paraffin section was stained by Kinyoun’s technique as described by Chauhan (2006).

Procedure

- The sections were deparaffinized and hydrated.
- Sections were placed in Kinyoun’s carbol fuchsin solution and kept it in boiling water bath at 56°C for 1 hour.
- The slides were removed from water bath and kept at room temperature.
- Washed in running tap water.
- Differentiated in acid alcohol till sections appeared pale pink.
- Counter stained in methylene blue solution till colour of sections were pale blue.
- Dehydrated, cleared and mounted in DPX.

With this staining the acid fast bacilli appeared singly or in clumps as bright red rods.

5.2(ii) Demonstration of connective tissue

For demonstration of connective tissue (collagen) the tissue sections were stained by special staining method of Van-Gieson’s, Masson’s trichrome and Mallory Heidenhain stain.

5.2(ii) a. Van-Gieson’s stain

Formalin preserved paraffin sections were stained by Van-Gieson’s stain as described by Gridley (1960).

Procedure

- The sections were deparaffinized and hydrated.
- The sections were stained with Weigert’s hematoxylin solution for 10 minutes.
- Washed in distilled water.
- Counter stained in Van Gieson’s solution for 1 to 3 minutes.
- Dehydrated, cleared and mounted in DPX.
With this staining procedure the collagen stained deep red; muscles and cornified epithelium appeared yellow and nuclei were blue black.

5.2(ii) b. Masson's trichrome stain

Formalin preserved paraffin sections were stained by Masson's trichrome for collagen as described by Gridley (1960). The tissues were kept in Bouin's fluid for overnight. Procedure

- The sections were deparaffinized and hydrated.
- The sections were kept in Bouin's fluid for overnight at room temperature.
- Washed in running water until yellow color disappeared.
- Rinsed in distilled water.
- Stained with Weigert's hematoxylin solution for 10 minutes. Washed in running water for 10 minutes.
- Rinsed in distilled water.
- Kept in biebrich scarlet – acid fuchsin solution for 15 minutes.
- Rinsed in distilled water.
- Kept in phosphomolybdic acid- phosphotungstic acid solution for 10 to 15 minutes.
- Kept in aniline blue solution for 5–10 minutes.
- Washed in distilled water.
- Placed in 1% acetic water for 3 to 5 minutes.
- Dehydrated, cleared and mounted in DPX.

With this stain collagen, mucus appeared blue; cytoplasm, keratin, muscle, fibers, intercellular fibers red and nuclei black.

5.2(ii) c. Mallory Heidenhain stain

Mallory Heidenhain staining was done for collagen following the method of Humason (1962). Procedure

- The sections were deparaffinized and hydrated.
- The sections were kept in stain for 5 minutes.
- Washed in running tap water for 5 seconds.
- Dehydrated, cleared rapidly and mounted in DPX.
With this stain connective tissue appeared blue.

5.2(iii) Demonstration of calcium in tuberculous granuloma

Formalin preserved paraffin sections were stained by Von Kossa stain for demonstration of calcium following the method by Gridley (1960). Procedure

- The sections were deparaffinized and hydrated.
- The sections were kept in 5% silver nitrate solution for 60 minutes in direct sun light.
- Rinsed in distilled water.
- The sections were placed in 5% sodium thiosulphate for 5 minutes.
- Washed well in distilled water.
- Counter stained in nuclear fast red for 5 minutes.
- Dehydrated, cleared and mounted in DPX.

With this stain calcium salt appeared black.

5.2(iv) Demonstration of iron in tuberculous granuloma

Formalin preserved paraffin section was stained by modified Mallory reaction for demonstration of iron following the method of Gridley (1960). Procedure

- The sections were deparaffinized and hydrated.
- The slides were immersed in equal parts of 5% hydrochloric acid solution and 5% potassium ferrocyanide solution for 10 minutes.
- Washed thoroughly in distilled water.
- Counter stained in nuclear fast red for 5 minutes.
- Rinsed well in distilled water.
- Dehydrated, cleared and mounted in DPX.

With this staining iron appeared bright blue and nuclei red.
5.2(v) Demonstration of fat in granuloma

Oil Red O for Frozen Section

Cryostat frozen sections were stained by Oil red O for demonstration of fat following the method of Gridley (1960).

Procedure

- Frozen sections were cut and collected in distilled water.
- Sections were placed in absolute propylene glycol for 2 minutes.
- Sections were immersed in oil red O in propylene glycol for 30 minutes.
- Differentiated in 85% propylene glycol for about 1 minute. Agitated to prevent folds in tissue.
- Washed in two changes of distilled water.
- Counter stained in Harris’s hematoxylin for few seconds.
- Washed in two changes of distilled water.
- Mounted in glycerin jelly.

With this staining the fat appeared red and nuclei were blue.

6. Immunohistochemical demonstration of inducible nitric oxide synthase (iNOS) in tubercular granuloma

Immunoreactivity of inducible nitric oxide synthase (iNOS) was detected in paraffin embedded and cryostat sections. Sections were stained with the avidin biotin complex peroxidase method as described by Hostetter et al. (2005) with suitable modifications.

Solutions

0.3% hydrogen peroxide in methanol

Tris-EDTA Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>1.21g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.37g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

Add 0.5ml of tween 20.

Rat sera

5% Serum albumin from bovine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
</tr>
</tbody>
</table>
Polyclonal antibody: Rabbit antimouse iNOS
ExtrAvidin Peroxidase kit for rabbit antibodies
AEC staining kit:
Deionized water- 4 ml.
Acetate buffer- two drops
3-amino-9 aethylcarbazole (AEC) - one drop
3% hydrogen peroxide- one drop
Counterstain: Harris hematoxylin

Procedure for paraffin sections

- The sections were deparaffinized and hydrated.
- Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes.
- Antigen retrieval was performed by microwaving the slide in Tris- EDTA buffer for 5 minutes.
- The sections were incubated with mixture of equal volumes of rat sera and 5% bovine serum albumin for 20 minutes at room temperature.
- Thereafter, the sections were incubated with polyclonal antibody of 1:200 dilution for overnight at 4°C.
- The sections were incubated with biotinylated mouse anti-rabbit IgG of 1:400 dilution for 30 minutes and then with streptavidin- peroxidase reagent of 1:200 dilution for 15 minutes at room temperature.
- The sections were incubated with 3-amino-9 aethylcarbazole (AEC) reagent for 5 minutes at room temperature.
- Counter stained with Harris hematoxylin for 30 seconds.
- Following every step, sections were washed with phosphate buffered saline (PBS) of pH 7.4 (not done after incubation with mixture of normal rat sera and 5% bovine serum albumin).

Procedure for cryosections

- Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes.
- The sections were incubated with mixture of equal volumes of rat sera and 5% bovine serum albumin for 20 minutes at room temperature.
• The sections were incubated with polyclonal antibody of 1:500 dilution for overnight at 4°C.
• The sections were incubated with biotinylated mouse anti-rabbit IgG of 1:400 dilution for 30 minutes and then with streptavidin- peroxidase reagent of 1:200 dilution for 15 minutes at room temperature.
• The sections were incubated with 3-amino-9 ethylcarbazole (AEC) reagent for 5 min at room temperature.
• Counter stained with Harris hematoxylin for 30 seconds.
• Following every step, sections were washed with phosphate buffered saline (PBS) of pH 7.4 (not done after incubation with mixture of normal rat sera and 5% bovine serum albumin).

  With this staining procedure the immunoreactivity of iNOS appeared as brown, amorphous material.

**Statistical analysis**

  Only percentage of tuberculous lungs with lesion distribution was calculated. The rest of the procedures and observations did not require any statistical analysis.
RESULTS

In the present study, for collection of tubercular lesions the post mortem examination was conducted according to the guidelines for the meat inspection. All major viscera and regional lymph nodes were examined and incised. The location and characteristic of each lesion were recorded.

The lung and lymph node samples were collected after examination of 252 bovine carcasses at Slaughter House, Municipal Corporation, Livestock Farm Adhartal, College of Veterinary Science and Animal Husbandry and Organized Dairy Farms in and around Jabalpur. Out of these 24 cases (9.52%) showed nodular lesions with casseating mass suggestive of tuberculosis. Of these 24 cases, 22 were of necropsy and only two were from post-mortem examination of slaughtered animals. The lung involvement was primarily observed in all the 24 cases (100%). Samples were taken from the lesions together with the normal adjacent tissue.

Demographic profile of animals with tuberculous lesions

The demographic profile of the 24 animals exhibiting tuberculous lesions is as shown in table 1. The mean age of the tuberculous animals was determined as five years and maximum number of cases was seen in Sahiwal breed (Fig. 1). Only one case was observed in cross bred male animal and two in non descript male. All the remaining cases were observed in female animals. Eleven cases were in lactating females and in two cases the animals were pregnant. The carcasses of only twelve animals exhibited emaciation or cachexia (Plate 1) whereas in the remaining carcasses the external examination revealed a normal healthy animal (Plate 2) with no features of chronic illness (muscle loss).
# Table 1: Demographic profile of animals exhibiting tuberculous lesions

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex/ lactation status</th>
<th>External appearance of carcass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sahiwal</td>
<td>4</td>
<td>Female, non lactating</td>
<td>Emaciated, dehydrated, rough hair coat</td>
</tr>
<tr>
<td>2.</td>
<td>Sahiwal</td>
<td>5</td>
<td>Female, non lactating</td>
<td>Emaciated, dehydrated, rough hair coat</td>
</tr>
<tr>
<td>3.</td>
<td>Cross bred Jersey</td>
<td>3.5</td>
<td>Female, lactating</td>
<td>Body condition fair, rough hair coat</td>
</tr>
<tr>
<td>4.</td>
<td>Non Descript</td>
<td>5</td>
<td>Female, non lactating</td>
<td>Emaciated, rough hair coat</td>
</tr>
<tr>
<td>5.</td>
<td>Cross bred HF</td>
<td>5</td>
<td>Female, lactating</td>
<td>Body condition good, smooth and shiny hair coat</td>
</tr>
<tr>
<td>6.</td>
<td>Cross bred HF</td>
<td>2.5</td>
<td>Female, non lactating</td>
<td>Emaciated with prominent rib cage</td>
</tr>
<tr>
<td>7.</td>
<td>Non Descript</td>
<td>4</td>
<td>Female, non lactating</td>
<td>Body condition fair, dehydrated carcass, matted hair coat</td>
</tr>
<tr>
<td>8.</td>
<td>Cross bred HF</td>
<td>4</td>
<td>Female, lactating</td>
<td>Body condition fair, smooth hair coat</td>
</tr>
<tr>
<td>9.</td>
<td>Cross bred Jersey</td>
<td>3</td>
<td>Male</td>
<td>Emaciated, dehydrated carcass</td>
</tr>
<tr>
<td>10.</td>
<td>Sahiwal</td>
<td>5</td>
<td>Female, non lactating</td>
<td>Body condition fair, dehydrated, rough hair coat</td>
</tr>
<tr>
<td>11.</td>
<td>Sahiwal</td>
<td>6</td>
<td>Female, lactating</td>
<td>Body condition normal, smooth hair coat</td>
</tr>
<tr>
<td>12.</td>
<td>Sahiwal</td>
<td>5</td>
<td>Female, lactating</td>
<td>Body condition normal, smooth and shiny hair coat</td>
</tr>
<tr>
<td>13.</td>
<td>Sahiwal</td>
<td>5</td>
<td>Female, lactating</td>
<td>Body condition normal, smooth and shiny hair coat</td>
</tr>
<tr>
<td>14.</td>
<td>Cross bred Jersey</td>
<td>4</td>
<td>Female, non lactating</td>
<td>Emaciated dehydrated carcass, matted hair coat</td>
</tr>
<tr>
<td>15.</td>
<td>Cross bred Jersey</td>
<td>4.5</td>
<td>Female, lactating</td>
<td>Body condition fair, smooth hair coat</td>
</tr>
<tr>
<td>16.</td>
<td>Sahiwal</td>
<td>6</td>
<td>Female, non lactating</td>
<td>Body condition normal, smooth and shiny hair coat</td>
</tr>
</tbody>
</table>
17. Non Descript 7 Male, castrated draught Dehydrated, extreme emaciation, rough hair coat

18. Non Descript 6 Male, castrated, draught Dehydrated, emaciated, rough hair coat

19. Sahiwal 7 Female, lactating Emaciated, rough hair coat

20. Cross bred Jersey 8 Female, lactating Emaciated, rough hair coat

21. Sahiwal 8 Female, lactating Cachexia, poor body condition, dehydrated, rough hair coat

22. Cross bred Jersey 4 Female, non lactating Body condition poor, emaciation, rough hair coat

23. Cross bred Jersey 5 Female, pregnant with 4 months approximately foetus, Body condition good, smooth and shiny hair coat

24. Sahiwal 4 Female, pregnant with approximately 3 months foetus Body condition good, Smooth hair coat

1. **Macroscopic examination**

   The animals were subjected to detailed post mortem examination. Each lung lobe was examined separately and cross-sectioned at 0.5 to 1.0 cm intervals. Lung lobes, left cranial, left caudal, right cranial, right caudal/middle, and accessory were examined.

1.1 **Scoring of lung lesions**

   The observations were subjected to the following scoring system for gross tubercular lesions as per the method described by Palmer et al. (2007) with suitable modifications (Fig. 2).

   No visible lesions: 0
   No external gross lesions, but lesions seen upon slicing: 2
   Gross lesions of <5-10 mm in diameter: 3
Gross lesions of <10-20 mm in diameter: 4
Distinct gross lesion of <20-30 mm in diameter: 5
Coalescing gross lesions: 6

**Table 2: Scoring of gross lesions**

<table>
<thead>
<tr>
<th>Gross observations</th>
<th>Number of animals</th>
<th>Scoring of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>No visible lesions</td>
<td>228</td>
<td>0</td>
</tr>
<tr>
<td>No external lesions but lesions seen on slicing</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Gross lesions &lt;5-10 mm in diameter</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Distinct gross lesions of &lt;10-20 mm in diameter</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Distinct gross lesions of &lt;20-30 mm in diameter</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>Coalescing gross lesions</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>Total score of lesions</td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>

**1.2 Location of lesions**

The lesions were prominently observed involving all the lobes (16/24, 66.66%), caudal lobe (6/24, 25%) (Plate 3) and caudal cranial lobes (2/24, 8.33%) (Fig. 3).

In 20 cases lesions suggestive for tuberculosis were located in the lungs and regional lymph nodes. In two cases tubercles were also observed in the pleura and the ribs (Plate 4). Only in two animals generalised lesions with involvement of liver, spleen and mesentery along with lungs and regional lymph nodes were noticed.

Pathomorphologically, 20 cases (83.33%) comprised miliary-nodular tubercles (Plate 5) and only four cases (16.67%) were determined to be chronic organ tuberculosis (Plate 6).
1.3 Pathomorphology of gross lesions

1.3 (i) Miliary tuberculosis

Gross lesions of 18 cases were classified as military nodular and in maximum cases involved all the lobes of lungs. Several small sized nodules in lungs of 2-6 mm in diameter were observed. They were scattered in all the seven lobes of lung. These nodules were small, whitish yellow in colour and protruded from the organ. There was caseation and mineralization on cutting (Plate 7).

Conglomerate tubercles located particularly in the cranioventral lobes and lymph nodes were observed in four cases of miliary nodular tuberculosis. The upper surface of the tubercles was formed due to mergence of small nodules with irregular boundaries (Plate 8). The cut sections displayed a lobular appearance with mineralisation.

1.3(ii) Chronic organ tuberculosis

Gross lesions in four cases were classified as chronic organ tuberculosis. Of these in two cases, the carcass were emaciated and in poor body condition. The lungs revealed nodules which were fibrotic, hard, yellowish in colour. They were 2 mm to 2-3 cm in size. They were embedded in the organ. Nodules showed caseous mass in the centre on cutting (Plate 9). The gritty sensation was also felt.

In the remaining two cases of chronic organ tuberculosis the carcass were fair in body condition. Surface of lungs had nodules which were hard, protrusive and yellow in colour. They were of different size about 1-2 mm to 3 cm. Most of the nodules were present in cranial lobe (Plate 10). Some nodules were also found in middle and caudal lobes of lungs. Nodules showed caseous mass in the centre on cutting. The gritty sensation was also felt.

In addition to this tubercles of varying size localized in a narrow area in the form of a chain were observed scattered in the mucosa of the trachea in only one case of chronic organ tuberculosis.
1.3(iii) Lesions in lymph nodes

Tubercles were determined in the bronchial and mediastinal lymph nodes in all cases. The cut section of the enlarged lymph nodes had caseous calcified areas particularly in the cortex (Plate 11).

1.3(iv) Generalised tuberculosis

In the two cases the carcasses were fair in body condition. On PM examination lungs, pleura, peritoneum showed numerous small sized nodules. These nodules were hard, yellow in colour and embedded in organs. They were of same size about 1-2 mm in diameter. Nodules showed caseous mass in the centre on cutting. Little gritty sensation was felt. The regional lymph nodes were enlarged and showed caseation and calcification (Plate 12). The peritoneum was studded with these small sized nodules. Spleen had nodules of 2 cm in size (Plate 13) while liver had small sized numerous nodules.

2. Direct smear examination

Impression smear was prepared from the lesions of 24 bovine lung tissues suspected for tuberculosis and stained by Ziehl-Neelsen. All slides were examined carefully, scanning the entire area of each section at 100X magnification. They were considered positive when one or more acid-fast bacilli were detected in at least one section of the sample.

From the 24 cases examined only 12 cases showed acid fast bacilli in the impression smear of lung tissue whereas in the remaining 12 cases acid fast bacilli were not observed. Impression smears were also prepared from the mediastinal lymph nodes of all these 24 cases and from enlarged prescapular lymph nodes observed only in twenty animals. The number of acid-fast bacilli was high in all cases. They were mainly observed free in the caseous debris (Plate 14), within macrophage cytoplasm and also in Langhan’s type multinucleated giant cells (Plate 15).

Acid fast bacilli were also observed in the mediastinal lymph nodes impression smears of all the 12 cases in which acid fast bacilli were observed in lung tissue also. The bacilli were frequently observed in the cytoplasm of macrophages (Plate 16). In the impression smears prepared
from the 10 cases of enlarged prescapular lymph nodes acid fast bacilli were observed in the cytoplasm of macrophages (Plate 17).

3. Histopathology: Paraffin sections

3.1 Staging of granuloma in hematoxylin and eosin stained sections:

Microscopic sections of 5-6 µm thickness were prepared from different areas of the affected lung and lymph nodes of all the 24 cases and stained with haematoxylin and eosin. Several microscopic sections were prepared from different areas of lung lesions from a single case.

The histopathologic findings were evaluated microscopically and classified as positive, inconclusive or negative (Fig. 4). The positive cases comprised a classic granuloma as a characteristic lesion of tuberculosis composed of a central caseous necrosis with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan’s giant cells and were observed in 18 cases. Inconclusive lesions characterized by irregular unencapsulated clusters of epithelioid macrophages but not Langhan’s type multinucleated giant cells and necrosis, consistent with an initial stage granuloma was observed in four cases. Negative features not consistent with tubercular granuloma including significant eosinophilic infiltrates and lymphoid hyperplasia were seen in two cases.

The eighteen positive cases of the pulmonary granulomas were further staged from I to IV. The division of granulomas was based on accumulation of epithelioid macrophages, lymphocytes, and presence of multinucleated giant cells, connective tissue rim, central necrosis and mineralization. All four types of granulomas were found in miliary nodular tuberculosis.

Variable number of all four stages granulomas was seen in the same lung tissue. An overlapping of lesions with all the four stages present in the same lung tissue was observed in all cases. Thus, classification was done on the basis of frequency of the lesions observed.

Stage I or initial granulomas were characterized by accumulation of epithelioid macrophages with low number of lymphocytes and neutrophils (Plate18). Multinucleated giant cells were infrequent but necrosis
was absent. Lesions of eight cases which revealed very few and small nodules on gross examination mainly comprised stage I or initial granuloma on microscopic examination. Stage I granulomas were seen in proximity to granulomas of more advanced stages characteristic of satellite granuloma.

Stage II (solid) granulomas were characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes were present as well as multinucleated giant cells. Necrosis when present was minimal (Plate 19). Four cases of miliary nodular gross lesions mainly comprised of stage II granuloma on the basis of microscopic observations.

Stage III (necrotic) granulomas were characterized by complete fibrous encapsulation. Necrotic cores were surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes (Plate 20). Interestingly, both the cases of generalised tuberculosis with extra pulmonary tubercles had predominant microscopic features of Stage III granuloma in the pulmonary tissue.

Stage IV (necrotic and mineralized) granulomas were characterized by a thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contained foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surrounded necrotic areas, and there were often moderate-to-marked infiltrates of lymphocytes (Plate 21 and 22). All the four cases showing gross features of chronic organ tuberculosis demonstrated microscopic features of stage IV granuloma.

Microscopic evaluation of all affected lymph nodes revealed caseous necrosis in the center with varying degrees of calcification. A layer of inflammatory cells, consisting of lymphocytes, macrophages, epithelioid cells, and Langhan’s giant cells, surrounded this necrotic area (Plate 23). These inflammatory cells were surrounded by an extensive layer of fibrous connective tissue.

The single case of spleen and liver nodules were also processed for microscopical examination. The lesion comprised focal areas of
central caseation surrounded by a zone of inflammatory cells consisting of lymphoid cells, epithelioid cells and Langhan's type of giant cells (Plate 24).

3.2 Special staining of tissues

For better understanding of the pathomorphology of tubercular lesions the sections prepared from different parts of the granuloma were also stained with special stains for demonstration of acid fast bacilli, connective tissue, calcium, iron and fat.

3.2(i) Demonstration of acid fast bacilli

Two procedures were followed for demonstration of acid fast bacilli in the tissue sections of granulomas.

3.2(i) a. Ziehl-Neelsen (ZN) stained tissues

Ziehl-Neelsen stained sections revealed the presence of clumps of acid-fast bacilli around the necrotic centre, in the cytoplasm of macrophage and Langhan’s type giant cells. On ZN stained tissues the acid fast bacilli stained bright red were observed in lungs for 10 cases which showed acid fast bacilli in direct smears also (Plate 25). In sections of lymph nodes from six animals clumps of acid fast bacilli were observed. Acid fast granulomas were present intra-cellulary in the macrophages in the stage I and II granuloma (Plate 26). In the stage IV granuloma the acid fast bacilli were frequently present extracellulary in necrotic tissue and were infrequent in the macrophages or multinucleated giant cells.

3.2(i) b. Kinyoun’s technique

Formalin preserved paraffin section were also stained by Kinyoun’s technique. In only three cases the acid fast bacilli could be seen in the microscopic section of lung tissue as red stained rods (Plate 27).

3.2(ii) Demonstration of connective tissues

Extensive fibrosis was observed in the stage III and IV granulomas as evident from thick bands of red collagen around the granulomatous regions in Van-Gieson’s stained sections (Plate 28,29) and blue band in Masson’s trichrome stained tissues (Plate 30,31) and Mallory
Heidenhain (Plate 32). In stage I and II granulomas the fibrosis was mild and a thin band of collagen fibres was visible.

3.2(iii) Demonstration of calcium

Calcium was demonstrated in the tuberculous lesions by staining the paraffin embedded sections with Von Kossa stain. The mineralised tissue appeared black in colour. Maximum calcification was observed in the stage IV granulomas (Plate 33) followed by stage III (Plate 34). In stage I and II granulomas calcification was not very prominent.

3.2(iv) Demonstration of iron

Iron was demonstrated in the tuberculous lesions of paraffin sections stained by Modified Mallory method. It was observed that iron accumulated within macrophages at the periphery of the primary granulomatous lesions while extra cellular ferric iron was concentrated in areas of necrosis (Plate 35). Maximal iron accumulation was observed in stage IV granulomas (Plate 36).

4. Histopathology : Cryosections

4.1 Demonstration of fat

Fat was demonstrated in the tuberculous lesions in cryosections stained with Oil Red O. Maximum fat, stained red, indicating foamy macrophages was observed in stage IV granuloma followed by stage III (Plate 37, 38).

The pathomorphology of the tuberculous granuloma as assessed visually in sections stained by routine (HE) and special stains (ZN, Kinyoun’s, Van Gieson’s, Masson’s trichrome, Mallory heidenhain, Von Kossa, Modified Mallory, Oil Red O) is depicted in table 3. Maximum calcification, fibrosis, fat and iron were observed in stage III and IV granulomas. The acid fast bacilli were uniformly observed in increased number in the areas of caseous necrosis.
Table 3: Visual microscopic assessment of granuloma

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Stage of granuloma</th>
<th>Calcification</th>
<th>Fibrosis</th>
<th>Fat</th>
<th>Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I</td>
<td>±</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>II</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3.</td>
<td>III</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>4.</td>
<td>IV</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

5. Demonstration of inducible nitric oxide synthase (iNOS)

Immunoreactivity of inducible nitric oxide synthase (iNOS) was detected on paraffin embedded and cryo sections. Sections were stained with the avidin biotin complex peroxidase method. Immunoreactivity was observed more distinctly in the cryosections (Plate 39) although iNOS could also be demonstrated in the paraffin embedded sections. The cryosections were found better for demonstration of iNOS with less number of non specific reactions.

Immunoreactivity for iNOS was present in all the stages of granulomas and was visible in the macrophages and multinucleated giant cells (Plate 40). iNOS was observed in the area adjacent to the caseous necrotic centre (Plate 41). Immunoreactivity for iNOS was observed only in cells associated with the granuloma and was absent in the surrounding tissue.

Maximum iNOS expression was observed in the stage I granulomas and the immunoreactivity decreased in the later stages of III and IV granulomas (Plate 42). iNOS was also expressed in the affected lymph nodes within the cells.
DISCUSSION

In recent years, a more integrated view of tuberculosis pathogenesis has prevailed; the granuloma is not only recognized as a tissue reaction to limit bacillary growth and sequester infection but also as part of the successful life cycle of *Mycobacterium* sp., thus representing the dynamic combat zone between both, the pathogen and host defense elements. The pathogen *Mycobacterium* sp. has evolved to cause infection in many, but active disease in few individuals. The WHO estimates that 1/3rd of the planet's population harbors this bacterium, yet only 2-23% will develop disease during their lifespan. Intriguingly, there are no biomarkers for disease progression because, as far as has been ascertained to date, the systemic immune response is comparable in those individuals developing disease versus those with effective containment. Progression to active disease is determined locally, at the level of the infection site, the granuloma. Therefore, appreciating the interplay between the pathogen and the localized tissue response is critical to understanding the progression of infection to active disease, and ultimately, transmission. Thus, the first objective of the research was the pathomorphological study of the tuberculous lesions in bovine.

A tentative diagnosis of bovine tuberculosis can be made following the macroscopic detection at necropsy of typical lesion. The sensitivity of gross post mortem examination is affected by the method employed and the anatomical sites examined. Vural and Alcigir (2010) have stated that the diagnosis of TB in cattle can be made when typical tubercles are detected in carcasses at postmortem examination and this may be a more rapid and reliable diagnosis of bovine TB. In the present study, collection of tubercular lesions was done after the detailed post mortem examination of 252 bovine carcasses. The location and characteristic of each lesion were recorded. Out of these 24 (9.52%) cases showed nodular lesions with caseating mass suggestive of tuberculosis, moreover, lung involvement was seen in 100% cases.

Earlier workers have reported a variable prevalence of bovine tuberculosis from different geographical locations. Kumar *et al.* (1998) in their
study recorded tuberculosis in 9.80% cattle with 98.6% lung involvement. Milian-Suazo et al. (2000) identified tuberculosis in 400 (16%) of 2,500 cattle carcasses which had gross lesions typical of TB. Shitaye et al. (2006) determined 0.052% prevalence of bovine tuberculosis by abattoir meat infection with 63.9% lung involvement. Ameni et al. (2007) reported a 13.5% prevalence of bovine TB whereas Sharma et al. (2011) found prevalence of 5.38% of tuberculosis in cattle. However, it is opined that TB prevalence might be under estimated in tuberculous cattle because of undetected lesions in early infections or because small lesions might be missed as a result of poor postmortem techniques or meat inspectors being discountenanced under pressure from butchers.

Thus, the prevalence of bovine tuberculosis varies with the geographical location and the managerial conditions. In accordance with our findings the lung involvement was maximal in earlier studies also indicating a high incidence of pulmonary bovine tuberculosis.

One of the main individual risk factors identified by numerous studies in both developed and developing countries is the age of animals. The duration of exposure increases with age; older animals are more likely to have been exposed than younger ones, as shown by several cross-sectional studies carried out. The mean age of the tuberculous animals in our study was determined as five years. Kazwala et al. (2001) observed that older cattle were more affected by the disease than yearlings and calves. Similarly, Demelash et al. (2009) found that the incidence of bovine tuberculosis was higher in old and young animals than middle age group. The findings are contradictory to our findings as the incidence was observed more in the middle age group and our in accordance with the observations of Ameni et al. (2007) who observed more tuberculosis in animals between five and nine years of age. Animals might get infected at a young age, but only express the disease clinically when they are adults. Mycobacteria have the ability to remain in a latent state for a long period before reactivation at an older age. Nevertheless, till date scientists have not proved that a true dormant state exists in cattle.
In our study, maximal cases were seen in female animals. Gender has only been mentioned as a risk factor in few studies and opinions diverge regarding its influence on the susceptibility to a *M. bovis* infection. Kazwala *et al.* (2001) reported significant differences between male and female with a higher incidence of bovine tuberculosis in male population whereas Inangolet *et al.* (2008) found significantly more females positive to the skin test than males. However, it is opined that inference of sex predisposition cannot be drawn from our study as maximal postmortem were of female animals. Gender-linked factors are probably related to management practices or behavioral habits; males and females are managed differently, both in developed and developing countries.

In the present study eleven cases were in lactating females and in two cases the animals were pregnant. Kazwala *et al.* (2001) reported no significant difference in bovine TB cases between pregnant and non pregnant cattle but observed that significantly more (14.6%) lactating cattle reacted in the tuberculin testing than did non-lactating cows. In the present study the population size was too low to draw any inferences regarding the predisposition of TB for pregnant and lactating animals.

In our study both the cases of tuberculosis were in castrated bulls. Previously, Kazwala *et al.* (2001) also observed that the castrated bulls, often used for draught power, were more frequently affected than the entire bulls, mainly used for breeding.

Maximal cases in the present study were observed in Sahiwal breed. Earlier, scientists have emphasized a greater prevalence in exotic than local breed. However, all the conclusions were based on the reactivity in skin tests. It is questionable whether a variability of the reaction to the skin test exists depending on the cattle breed. If established, this variability would imply that diagnostic tests should be suitably designed and applied according to the animal breed to be tested. The difference of susceptibility between breeds is likely to be related to differences in the breed population in an area and the management practices as imported dairy animals are generally kept under intensive conditions. However, the number of cattle in present study whether
indigenous or cross bred was too few to make relevant comparisons on breed susceptibility.

In our study, the body condition of affected animals ranged from poor (emaciated) to good. Earlier, tuberculosis has always been associated with chronicity and debilitation. However similar to our observations, Firdessa et al. (2012) recorded a poor correlation of the body condition with the tuberculous lesions in animals and observed that the majority of these animals were highly diseased with severe pathology and little or no clinical signs typical for tuberculosis were observed. Thus, if bovine TB gets into a herd where no diagnostic measure is in place then it can be difficult to detect infected animals on a visual basis solely and to prevent further transmission.

In the present study in 20 cases lesions suggestive for tuberculosis were located in the lungs and regional lymph nodes. In two cases tubercles were also observed in the pleura. Only in two animals generalized lesion with involvement of liver, spleen and mesentery along with lungs and regional lymph nodes were noticed. In agreement to our findings Shettar (2012) in his abattoir study observed that out of fifteen cases 12 animals revealed pulmonary form of tuberculosis, one with generalized form and two animals showed no observable lesions. The involvement of regional lymph nodes in primary pulmonary tuberculosis has been earlier reported by many workers (Whipple et al., 1996; Milian-Suazo et al., 2000; Kumar and Swamy, 2005; Shitaye et al., 2006; Ameen et al., 2008 and Besirović et al., 2012). Thus, it can be inferred that pulmonary form of tuberculosis is most prevalent in bovines with a high incidence of regional lymph node involvement.

Vural and Alcigir (2010) have stated that M. bovis infection have a greater distribution in lungs, thoracic lymph nodes and pleura and these predilection sites have been interpreted as evidence of respiratory route of infection. Observations obtained from naturally M. bovis infected cattle submitted to low intensive farming conditions have demonstrated that the majority of lesions were present in mesenteric lymph nodes (Ameen et al., 2008). In contrast, naturally M. bovis infected bovines exposed to intensive husbandry systems display augmented frequency of bovine TB lesions in the respiratory tract (Liebana et al., 2008) Thus, it is possible that intensive
husbandry systems favor *M. bovis* dissemination among dairy herds as a result of increased animal contact. Moreover, the high frequency of lesions observed in the respiratory tract suggests that the major route of *M. bovis* transmission was most likely aerogenous.

However, Prasad *et al.* (2005) have established that *M. bovis* predominates in extra pulmonary forms of tuberculosis and attributed this to infection by *M. bovis* occurring via the oral route. Contradictory to this, the present finding of increased prevalence of pulmonary tuberculosis raises the question of reverse zoonosis and infection in dairy animals being caused due to the *Mycobacterium* sp. via human transmission. More work establishing the species of mycobacteria causing bovine tuberculosis in the area is required for conclusive epidemiology.

**Lung lobe involvement**

The lesions in our study were prominently observed involving all the lobes (16/24, 66.66%), caudal lobe (6/24, 25%) and caudal cranial lobes (2/24, 8.33%). The predilection for lesion development in caudal lung lobes has been previously reported in cattle, where 90% of pulmonary lesions are found in the diaphragmatic lobes, approximately 50% of which are located in the distal one third. Palmer *et al.* (2007) in their experiments on tuberculosis determined that 52% of pulmonary lesions were present in the left caudal and right caudal/middle lung lobes combined with the right cranial lobe being the third most common lung lobe with 20% involvement. Liebana *et al.* (2008) reported greater frequency of pulmonary TB like lesions in the caudal lobes. Vural and Alcigir (2010) in their study observed that the tubercular lesions were localized prominently in caudal lobe (65%), caudal-cranial lobe (13%), cranial lobe (3%), and all lobes (19%).

Palmer *et al.* (2007) hypothesized that such a lesion distribution may be due to the fact that the majority of lung parenchyma is located within the right and left caudal lobes. Alternatively, regional lesion distribution within the lung could also be due to factors such as a difference in oxygen tension. A higher ventilation/perfusion ratio exists in cranio-ventral regions of the bovine lung compared with the caudo-dorsal region, creating a relative hypoxia in the
caudo-dorsal region. Such hypoxia may result in suboptimal macrophage function. Interestingly, the distribution of pulmonary lesions in tuberculous cattle is dissimilar to the typical cranio-ventral distribution of many bacterial pneumonia of cattle where, like tuberculosis, aerosol exposure is presumed. Regional lesion distribution has also been noted in human tuberculosis, where there is a predilection for granuloma development in the apical lung lobes. Due to the advanced tuberculous lesion in our present study the lesions were present in all the lobes followed by the caudal lobes.

Gross lesions of 18 cases were classified as military nodular and in maximum cases involved all the lobes of lungs. Several small sized nodules in lungs of 2-6 mm in diameter were scattered in all lung lobes. These nodules were small, whitish yellow in colour and protruded from the organ. There was caseation and mineralization on cutting. Similar gross appearance of miliary nodular tubercles were reported by Headley (2002), Jeyakumar et al. (2004), Kumar and Swamy (2005), Shitaye et al. (2006) and Vural and Alcigir (2010). In the present study gross lesions in four cases were also classified as chronic organ tuberculosis with larger sized fibrotic, hard, yellowish nodules. The variability in lesion appearance is probably a reflection of the progression of infection as determined by the host cell mediated immune response and ranges from early small granulomatous lesions to chronic encapsulated necrogranulomas and proliferative nodules. The tubercles (granulomas) or tumor-like masses form as a result of the body’s defence mechanisms to localize, or wall off, the invasion of the bacteria. It is commonly accepted that *M. bovis* primarily infects macrophages, where they are able to survive, replicate and disseminate into different anatomical sites (Volkman et al., 2004). Progression of mycobacterial disease and survival of the host are thought to depend on their ability to limit mycobacterial growth by an effective granulomatous response. In the case of experimental *M. bovis* infection in cattle, different stages of granuloma development have been observed to be associated with disease progression (Palmer et al., 2007) pointing out a dynamic process of the tuberculous granuloma structure.
Acid fast staining of impression smears and tissue sections

Direct impression smears were prepared from the lesions and paraffin sections of 24 lung tissues suspected for tuberculosis and stained by Ziehl-Neelsen. From the 24 cases examined only 12 cases showed acid fast bacilli in the impression smear of lung tissue whereas in 12 cases acid fast bacilli were not observed. However, in the positive smears the number of acid-fast bacilli was high and sometimes seen as clumps.

Ziehl-Neelsen stained sections revealed the presence of clumps of acid-fast bacilli around the necrotic centre, in the cytoplasm of macrophages and Langhan’s type giant cells in only ten sections. They were frequently observed free in the caseous debris. Similarly, Cancela and Marin (1993) and Virieux et al. (2006) found less sensitivity of acid fast staining technique and advocated the use of immunohistochemical technique for detecting positive cattle under field conditions. Varello et al. (2008) observed the relative sensitivity and specificity of ZN staining were 33.9% and 100% respectively.

An explanation for ZN low sensitivity may be a low survival rate of mycobacteria in the environment of the central caseation or loss of bacterial structure owing to immune responses operating in granulomatous inflammation in mycobacteriosis. For this reason, the identification of acid-fast bacilli using the ZN method needs to be reconsidered for its application in eradication schemes with other diagnostic methods such as auramine O /rhodamine staining or immunohistochemistry.

Hooja et al. (2011) in their study observed that fluorescent microscopy has higher sensitivity and comparable specificity which is further enhanced by concentration. Now with the advent of newer inexpensive Light Emitting Diode (LED) based fluorescent microscopes (FM), which are easier to use, fluorescent microscopy can be widely used even in peripheral laboratories where culture facilities are not available.

However, we agree with the opinion of Thoen et al. (1995) that lesions determined to be tuberculous upon examination of HE stained section with no acid fast bacilli may be regarded as suggestive of tuberculosis.
Histopathological diagnosis of tuberculosis

The histopathologic findings were evaluated microscopically and classified as positive, inconclusive or negative. The positive case comprised a classic granuloma as a characteristic lesion of tuberculosis composed of a central caseous necrosis with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan’s giant cells and was observed in 18 cases. Inconclusive lesions characterized by irregular unencapsulated clusters of epithelioid macrophages but not Langhan’s type multinucleated giant cells and necrosis, consistent with an initial stage granuloma was observed in four cases. Negative features not consistent with tubercular granuloma including significant eosinophilic infiltrates and lymphoid hyperplasia were seen in two cases only.

In previous studies, histologic examination was mainly applied in addition to acid fast staining to maximize the identification of *M. bovis* infected cattle (Headley, 2002; Jeyakumar et al., 2004; Kumar and Swamy, 2005 and Shitaye et al., 2006) and for the evaluation of lesions evolution in experimental studies of pathogenesis (Palmer et al., 2007). A study on the accuracy of histopathologic techniques was carried out on limited number of samples from cattle with classical histology features of tuberculosis by Virieux et al. (2006). The accuracy of histopathologic techniques on large number of samples are available only in cervids. Varello et al. (2008) claimed that histopathology demonstrated high sensitivity (93.4%) and specificity (92.3%), while ZN sensitivity and specificity were respectively 33.9% and 100%. There was good agreement between histopathology and bacterial culture, suggesting that histopathologic examination is a reliable tool for rapid diagnosis in countries where active tuberculosis eradication programs allow the prompt identification and elimination of reactor cattle.

Pathomorphology of the tuberculous granuloma

The tuberculosis (TB) granuloma is the outcome of the local interplay between the bacterium and the host cells within the site of infection. Since the first description of the “Ghon's complex” one century ago, pathologists have described the morphological parameters of these complex
structures extensively, and it is usually portrayed as a host-driven process to constrain the bacilli and prevent dissemination. More recently, however, this view point is being questioned and there is a growing appreciation of the active role played by the bacterium in this process.

The pathomorphology of the 22 granulomatous lesions was studied in HE stained paraffin embedded sections. In the present study staging of granulomas was done as per the microscopic observations of tissue sections. The stage IV granuloma was encountered with maximum frequency. Although variable number of all four stages granulomas were seen in the same lung tissue. Interestingly, both the cases of generalized tuberculosis with extra pulmonary tubercles had more numbers of microscopic features of stage III granuloma in the pulmonary tissue. Stage IV (necrotic and mineralized) granulomas were characterized by a thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contained foci of dystrophic mineralization.

Most of the workers have not described the stage of granuloma encountered in field cases of bovine tuberculosis. Palmer et al. (2007) experimentally infected cattle with M. bovis and then staged granulomas (I–IV) on the basis of cellular composition and the presence or absence of necrosis and peripheral fibrosis. Liebana et al. (2008) in field cases of bovine tuberculosis observed that stage IV granulomas, alone or in combination with other stages, constituted 63% of lesions, while 16% of lesions were stage I/II granulomas. Menin et al. (2013) have hypothesised that due to increased resistance of cattle to M. bovis infection during the natural infection, most of bovine tuberculosis lesions found in asymptomatic animals are in advanced/chronic stage of development.

The majority of pulmonary granulomas investigated in our study presented as encapsulated lesions with multiple intra granulomatous areas of caseous necrosis and the presence of dystrophic mineralization, which, can be classified as chronic bovine TB lesions (stage III/IV). Consistent with these results, previous workers have established that the process of granuloma maturation involves the migration of phagocytes and lymphocytes to the inflammation site in response to persistent mycobacterial stimuli (Cosma et
Effective anti-mycobacteria host response primarily rely on cell-mediated immune response, controlled by cytokines such as IFN-γ produced by antigen-specific T cells. Although the protective role of cell-mediated immune responses is unknown in cattle naturally infected with *M. bovis*.

The centre of the granulomas of all four stages was characterized by caseous necrosis. Caseous necrosis is related to delayed type hypersensitivity (DTH). Activated cytolytic T lymphocytes kill infected macrophages, leading to destruction of surrounding tissue. The host locally destroys its own tissue to control the uninhibited intracellular multiplication of bacilli that would otherwise be fatal. During the process, the majority of tubercle bacilli is killed, while some survive extracellularly in the solid caseous material but are unable to multiply because of anoxic conditions, reduced pH, and the presence of numerous enzymes released from the dead cells. Caseation appears to correlate with pathogen-mediated dysregulation of host lipid metabolism (Basaraba *et al.*, 2008).

Neutrophilic infiltrate was observed particularly in early stages (I and II) of granuloma and could be important for granuloma formation. Considerable controversy exists over whether neutrophils are able to kill mycobacteria and a recent review on the issue concluded that these otherwise potent anti-bacterial effector cells fail to eliminate acid fast bacilli (Korbel *et al.*, 2008). Menin *et al.* (2013) have suggested that neutrophils may play a regulatory anti-mycobacterial role.

Another characteristic feature of the tuberculous granulomas in the present study was the observation of variable number of epithelioid cells and multinucleated giant cells in accordance to the observations of earlier workers (Headley, 2002; Jeyakumar *et al.*, 2004; Kumar and Swamy, 2005; Shitaye *et al.*, 2006 and Vural and Alcigir, 2010). Typical, Langhan’s giant cells were encountered in stage III and IV granulomas. Literature cites that multinucleated giant cells (MGCs) have lost the ability to take up bacteria, because they no longer express the phagocytic receptors. However, they seem to have retained the ability to present antigens (Russell *et al.*, 2009). The loss of the phagocytosis capacity of MGCs suggests a possible role in a bacterial escape strategy driving the fusion of macrophages to form MGCs.
Menin et al. (2013) found a negative correlation between multinucleated giant cell numbers and *M. bovis* CFU counts in granulomas and suggested that activated multinucleated macrophages contribute to the control of this important bovine pathogen. Data from experimental models have demonstrated that Langhan’s type multinucleated giant cells can be found in all stages of development of lymph nodes granulomas.

Together, it is clear that *M. bovis* induced granulomas in the lungs are dynamic lesions in which the cell populations change over the course of disease, stimulating a diverse milieu during infection. The physiopathology of this complex structure during natural infection of *M. bovis* merits further investigation.

Increased amount of connective tissue encapsulation could be demonstrated in the stage III and IV granulomas by special staining techniques. Cattle immune responses against *M. bovis* may be a result of several factors, such as strain resistance, infection route and encapsulation of the tuberculous lesions. Connective tissue deposition (encapsulation) is thought to limit dissemination of bacteria and play a critical role in controlling mycobacterial proliferation by entrapping bacilli inside the lesions. Liebana et al. (2008) have reported the absence of correlation between AFB numbers and stage of granuloma development and encapsulation during natural infection with *M. bovis* whereas Menin et al. (2013) found that the amount of connective tissue surrounding the granuloma (thin encapsulation - thickly fibrous encapsulation) negatively correlated with viable *M. bovis* or AFB staining, suggesting a pivotal role of granuloma encapsulation as a host response controlling mycobacterial proliferation during natural infection.

In the present study, calcium was demonstrated in the tuberculous lesions by staining the paraffin embedded sections with Von Kossa stain. There were significant differences in the range of calcium concentrations in randomly selected lesions of granuloma which reflects the heterogeneity in the amount of calcification in lesions found within and between individuals. Earlier workers have also reported calcification in tuberculous granuloma. Rhyman and Saari (1995) and Whipple et al. (1996) described that histological manifestation of typical granulomatous lesions is a
central caseation and focal calcification. Dystrophic calcification is encountered in areas of necrosis, whether they are of coagulative, caseous, or liquefactive type, and in foci of enzymatic necrosis of fat. More importantly, primary lesions characterized by calcification, are resistant to treatment with first line anti-tuberculosis drugs and may therefore contribute to the development of multi-drug resistant strains of *Mycobacterium* sp. It is concluded that the mechanisms and significance of dystrophic calcification in primary lesions of bovine tuberculosis is poorly understood and thus warrants further study through the use of appropriate animal models.

It was observed that iron accumulated within macrophages at the periphery of the primary granulomatous lesions while extra cellular ferric iron was concentrated in areas of lesion necrosis. Maximal iron accumulation was observed in stage IV granulomas. Much of the understanding of the role of iron in the growth and virulence of mycobacteria has come primarily from *in vitro* studies. Basaraba *et al.* (2008) demonstrated increased expression of host iron-binding proteins precedes iron accumulation and calcification of primary lung lesions in experimental tuberculosis in the guinea pig. The workers hypothesized that iron metabolism by macrophages is reflective of activation status and in part, determines resistance to mycobacteria infection. Since iron toxicity is detrimental to both host macrophages and the tubercle bacillus, yet each has a strict nutritional requirement for iron, it stands to reason that the successful accumulation and sequestration of iron would be an important determinant in the outcome of tuberculosis infection.

Fat was demonstrated in the tuberculous lesions in cryosections stained with Oil Red O. Maximum fat, stained, red indicating foamy macrophages was observed in stage IV granuloma followed by stage III. Russell *et al.* (2009) in their study observed that the foamy macrophage appears to be a key player in both sustaining persistent bacteria and contributing to the tissue pathology that leads to cavitation and release of infectious bacilli. Recent observations and experiments have indicated that *Mycobacteria* promotes dysregulated lipid metabolism in its host macrophages. This promotes foamy cell formation, which supports bacterial
persistence and leads, ultimately to the accumulation of caseum within the granuloma.

On the basis of above findings it can be concluded that histomorphology, offers the major advantage of producing results within two days and has a high specificity because it can characterize lesions unrelated to mycobacterial agents (e.g. parasites, neoplasia).

**Immunohistochemical demonstration of iNOS**

Immunoreactivity of inducible nitric oxide synthase (iNOS) was detected on paraffin embedded and cryo sections. Immunoreactivity for iNOS was present in all the stages of granuloma. The induction of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) have been implicated as an important microbicidal mechanism by which activated macrophages effect cytotoxicity against microbes. NO has been found to be involved in granuloma formation of both infectious and noninfectious granulomas of humans and animals. However, NO has not been implicated in all types of granulomas; for example, evidence of NO is not detectable in foreign body granulomas and nonspecific granulomatous lymphadenitis. Few studies have examined the presence of iNOS *in situ* over the course of a disease process. Fligger *et al.* (1999) determined expression of inducible nitric oxide synthase in spontaneous bovine bronchopneumonia and observed that high levels of iNOS were expressed by cells (probably leukocytes) surrounding necrotic foci. Pando *et al.* (2001) showed expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT) during the evolution of experimental pulmonary tuberculosis with high percentages of activated macrophages immunostained for iNOS during the initial phases.

Recently iNOS expression has been documented in granulomas from cattle naturally infected with *M. bovis* (Suarez *et al.*, 2006). Expression varied between cattle, likely due to the unknown duration of infection and stage of granuloma development.

In the present iNOS immunoreactivity was visible in the macrophages and multinucleated giant cells. iNOS was observed in the area adjacent to the caseous necrotic centre. Maximum iNOS expression was
observed in the stage I granulomas and the immunoreactivity decreased in the later stages of III and IV granulomas. iNOS was also expressed in the affected lymph nodes within the cells.

Earlier workers have observed that in other mycobacterial diseases of cattle such as paratuberculosis, iNOS expression is minimal or absent in granulomas with high numbers of acid-fast bacilli. Similar to the present study, iNOS immunoreactivity in human granulomas, caused by mycobacterial and non-mycobacterial infectious agents, is greatest in smaller granulomas and minimal in large granulomas with extensive necrosis (Facchetti et al., 1999). Interestingly, in the present study, iNOS expression was limited to cells within the granuloma and not present in surrounding tissue, suggesting that iNOS expression is dependent on conditions within the granuloma microenvironment.

In human granulomas, iNOS expression has been positively associated with the expression of Th1-type cytokines such as IFN-γ and negatively correlated with expression of Th2-type cytokines such as IL-4. Th1-type cytokines induce iNOS, which oxidizes L-arginine to form NO. In contrast, Th2-type cytokines induce an alternative pathway where L-arginine is metabolized by arginase to L-ornithine and urea, inhibiting NO production through iNOS. Thus, the increased expression of iNOS early in disease with decreased expression in advanced disease is in agreement with the generally held belief that Th1-type responses prevail early in mycobacterial infections, while Th2-type responses prevail in later disease stages.

To conclude, tuberculous granulomas in cattle represent dynamic lesions that, early in development, are characterized by iNOS-expressing macrophages and multinucleated giant cells, moderate numbers of acid-fast bacilli, minimal necrosis, mineralization, peripheral fibrosis, and heterogeneous foamy macrophages, lymphocytes, epithelioid and giant cell population. With disease progression, there is an increase in necrosis, mineralization, peripheral fibrosis with a decrease in iNOS-expressing cells. Although there is an orderly progression through lesion stages as disease advances, within a given tissue at anytime, there may be granulomas of various stages of development which likely represent lesions with differing
microenvironments. Moreover, investigations on a larger number of histologically inconclusive cases will be needed to clarify their interpretation, and to determine whether further microscopic features can be highlighted to classify them definitively as positive because they could be consistent with an initial stage of the disease, or negative because they could be consistent with other causes.

The observations presented in the study offer basic information on the host response during the natural infection with *M. bovis*, which could be utilized as a potential source for biomarkers to test novel vaccine/adjuvant molecule candidates as well as efficient diagnostic methods. In addition, our findings may be important to reveal new components to understand the pathogenesis of the bovine TB and contribute to the establishment of rational strategies for bovine TB infection surveillance and control. Furthermore, the growing concern over increasing incidence of tuberculosis/HIV/AIDS co-infection, the high incidence of extra pulmonary tuberculosis and a high risk of acquiring zoonotic tuberculosis among the majority of the population emphasizes the need for paying the necessary attention towards the control of bovine tuberculosis.
SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

Summary

Work was undertaken to study the pathomorphology of the tuberculous lesions in bovine and to demonstrate inducible nitric oxide synthase (iNOS) in tubercular granuloma immunohistochemically. For collection of tubercular lesions the post mortem examination was conducted of 252 bovine carcasses at Slaughter House, Municipal Corporation, Livestock Farm Adhartal, College of Veterinary Science and Animal Husbandry and Organized Dairy Farms in and around Jabalpur, according to the guidelines for the meat inspection. All major viscera and regional lymph nodes were examined and incised. The location and characteristic of each lesion were recorded

Out of these 24 cases (9.52%) showed nodular lesions with casseating mass suggestive of tuberculosis. Of these 24 cases, 22 were from necropsy and only two were from post-mortem examination of slaughtered animals. The mean age of the animals with tuberculous lesions was determined as five years and maximum number of cases was seen in Sahiwal breed. Eleven cases were in lactating females and in two cases the animals were pregnant. The frequency of lung involvement was in all the 24 cases (100%). The lesions were prominently observed involving all the lobes (16/24, 66.66%), caudal lobe (6/24, 25%) and caudal cranial lobes (2/24, 8.33%). In 20 cases lesions suggestive for tuberculosis were located in the lungs and regional lymph nodes. In two cases tubercles were also observed in the pleura. Only in two animals generalized lesion with involvement of liver, spleen and mesentery along with lungs and regional lymph nodes were noticed.

Pathomorphologically, 20 cases (83.33%) comprised military nodular tubercles and only four cases (16.67%) were determined to be chronic organ tuberculosis. Gross lesions of 18 cases were classified as miliary–nodular and in maximum cases involved all the lobes of lungs with several small sized nodules of 2-6 mm in diameter. Conglomerate tubercles
located particularly in the cranioventral lobes and lymph nodes were observed in four cases of miliary nodular tuberculosis. The upper surface of the tubercles was formed due to mergence of small nodules with irregular boundaries.

Gross lesions in four cases were classified as chronic organ tuberculosis. The lungs revealed nodules which were fibrotic, hard, yellowish in colour. They were 2 mm to 2-3 cm in size and were embedded in the organ. Nodules showed caseous mass in the centre on cutting. The gritty sensation was also felt. In addition to this tubercles of varying sizes localized in a narrow area in the form of a chain were observed scattered in the mucosa of the trachea in only one case of chronic tuberculosis. Tubercles were observed in the bronchial and mediastinal lymph nodes in all cases. The cut section of the enlarged lymph nodes had caseous calcified areas particularly in the cortex.

Only two cases of generalized tuberculosis with tubercles on lungs, pleura, spleen and peritoneum were recorded. These nodules were hard, yellow in colour and embedded in organs. They were of same size, about 1-2 mm in diameter. Nodules showed caseous mass in the centre on cutting. Little gritty sensation was felt. The regional lymph nodes were enlarged and showed caseation and calcification. The peritoneum was studded with these small sized nodules. Spleen had nodules of 2 cm in size while liver had small sized numerous nodules.

Impression smear was prepared from the lesions of 24 lung tissues suspected of tuberculosis and stained by Ziehl-Neelsen. From the 24 cases examined only 12 cases showed acid fast bacilli in the impression smear of lung tissue whereas in remaining 12 cases acid fast bacilli were not observed. Impression smears were also prepared from the mediastinal lymph nodes of all these 24 cases and from enlarged prescapular lymph nodes observed only in twenty animals. Microscopic sections of 5-6 µm thickness were prepared from different areas of the affected lung and lymph node of all the 24 cases and stained with haematoxylin and eosin.

The histopathologic findings were evaluated microscopically and classified as positive, inconclusive or negative. The positive cases comprised
a classic granuloma as a characteristic lesion of tuberculosis composed of a central caseous necrosis with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan’s giant cells and were observed in 18 cases. Inconclusive lesions characterized by irregular unencapsulated clusters of epithelioid macrophages but not Langhan’s type multinucleated giant cells and necrosis, consistent with an initial stage granuloma was observed in four cases. Negative features not consistent with tubercular granuloma including significant eosinophilic infiltrates and lymphoid hyperplasia were seen in two cases.

The eighteen positive cases of the pulmonary granulomas were further staged from I to IV. The division of granulomas was based on accumulation of epithelioid macrophages, lymphocytes, and presence of multinucleated giant cells, connective tissue rim, central necrosis and mineralization.

For better understanding of the pathomorphology of tubercular lesions the sections prepared from different parts of the granuloma were also stained with special stains for demonstration of acid fast bacilli, connective tissue, calcium, fat and iron. Ziehl-Neelsen stained sections revealed the presence of clumps of acid-fast bacilli around the necrotic centre, in the cytoplasm of macrophage and Langhan’s type giant cells in only 10 cases. The stage III and IV granulomas exhibited extensive fibrosis as evident from thick bands of red collagen around the granulomatous regions whereas in stage I and II granulomas the fibrosis was mild and a thin band of collagen fibres was visible. Maximum calcification, iron and fat indicative of foamy macrophages was observed in the stage IV granulomas followed by stage III.

Immunoreactivity of inducible nitric oxide synthase (iNOS) was detected on paraffin embedded and cryo sections. The cryosections were found better for demonstration of iNOS with less number of non specific reactions. Maximum iNOS expression was observed in the stage I granulomas and the immunoreactivity decreased in the later stages of III and IV granulomas. iNOS was also expressed in the affected lymph nodes within the cells.
CONCLUSIONS

1. Prevalence of bovine tuberculosis was found to be 9.52% with 100% lung involvement.

2. Pathomorphological study of the tuberculous lesions in bovine revealed that the different stages of the granuloma are found in naturally occurring cases with a predominance of stage IV granuloma.

3. ZN staining in tissues has low sensitivity and lesions determined to be tuberculous upon examination of HE stained section with no acid fast bacilli can be regarded as suggestive tuberculosis.

4. Maximum calcification, iron and fat indicative of foamy macrophages were observed in the stage IV granulomas followed by stage III.

5. Maximum iNOS expression occurs in the stage I granulomas and the immunoreactivity decreased in the later stages of III and IV granulomas.
Suggestions for further work

1. Work can be undertaken to develop histochemical techniques to differentiate an active tubercular lesion from the latent infection.

2. Evaluation of host response during the natural infection with *M. bovis*, at molecular or ultrastructural level, can be studied, which could be utilized as a potential source for biomarkers to test novel vaccine/adjuvant molecule candidates as well as efficient diagnostic method.
REFERENCES


APPENDIX I

Staining Solutions

i. Kinyoun’s Carbol Fuchsin solution:

Basic Fuchsin 4gm.
Phenol 8gm.
Ethanol 20ml.
Heat to dissolve and add distilled water to make volume 100 ml.

ii. Acid Alcohol:

Concentrated, hydrochloric acid 3 ml.
70% Ethanol 97ml.

iii. Methylene Blue Stain:

Methylene Blue 0.3gm.
95% Ethanol 30ml.
Add distilled water to make volume 100 ml.

iv. Weigert’ Iron Hematoxylin:

Solution A
Hematoxylin 1.0 gm.
Absolute alcohol 100.0 ml.
Solution B
29% ferric chloride 4.0 ml.
Distilled water 95.0 ml.
Hydrochloric acid, concentrated 1.0 ml.
Working solutions
Equal parts of solution A and solution B were mixed.

v. Van Gieson’s Solution:

Acid fuchsin, 1% aqueous solution 2.5 ml.
Pircric acid, saturated aqueous solution 97.5 ml.

vi. Bouin’s solution:

Pircric acid, saturated aqueous solution 5.0 ml.
Formaldehyde, 37 - 40% 25 ml.
Glacial acetic acid 5.0 ml.
vii. Biebrich Scarlet- Acid Fuchsin Solution:

Biebrich scarlet, aqueous 1% 90ml.
Acid fuchsin, aqueous 1% 10ml.
Glacial acetic acid 1 ml.

viii. Phosphomolybdic-Phosphotungstic Acid Solution:

Phosphomolybdic acid 5.0 gm.
Phosphotungstic acid 5.0 gm.
Distilled water 200ml.

ix. Aniline Blue Solution:

Aniline blue 2.5 gm.
Acetic acid 2.0 ml.
Distilled water 100ml.

x. 1% Acetic Water:

Glacial acetic acid 1.0 ml.
Distilled water 100 ml.

xi. Mallory Heidenhain stain:

Orange G 1gm.
Aniline Blue 2gm.
Acid Fuchsin 1gm.

The above ingredients were mixed and dissolved in distilled water. Volume made 100ml. Keep the stain for months prior to use.

xii. 5% Silver Nitrate Solution:

Silver Nitrate 5gm.
Distilled Water 100ml.

xiii. 5% Sodium Thiosulphate:

Sodium Thiosulphate 5gm.
Distilled Water 100ml.
xiv. Nuclear Fast Red:

    Dissolve 0.1 gm. of nuclear fast red in 100 ml., aluminum sulfate with aid of heat, cool, filter and add grain of thymol as a preservative.

xv. 5% Hydrochloric Acid Solution:

    Hydrochloric Acid, concentrated 5ml.
    Distilled Water 95ml.

xvi. 5% Potassium Ferrocyanide Solution:

    Potassium Ferrocyanide 5gm.
    Distilled Water 95ml.

xvii. Oil red O in propylene glycol:

    Oli red O stain 3.5 gm.
    Propylene glycol 100.0 ml.

    Add a small amount of propylene glycol to oil red O and mix well. Crush larger pieces. Gradually add the reminder of propylene glycol stirring periodically. Heat gently until the solution reaches 95 °C. Do not allow to go over 100 °C. Stir while heating. Filter through coarse filter paper while still warm.

xviii. Propylene Glycol, 85%

    Propylene glycol 65.0 ml.
    Water 15.0 ml.