INTRODUCTION

Tuberculosis (TB) is a zoonotic most important chronic disease with high prevalence among humans, domestic and wild animals in developing countries. The diagnosis of TB in cattle can be made when typical tubercles are detected in carcasses at slaughter houses. Inspection of these organs during postmortem examination may provide a more rapid and reliable diagnosis of TB than bacteriologic culture.

Acid fastness is a physical property of certain bacteria, specifically their resistance to decolorization by acids during staining procedures. The high mycolic acid content of certain bacterial cell walls, like those of Mycobacteria, is responsible for the staining pattern of poor absorption followed by high retention. The most commonly occurring acid fast bacteria in cattle comprise members of the *Mycobacterium tuberculosis* complex (MTBC). Direct examination of acid fast bacilli (AFB) by microscopy is rapid but has a low sensitivity, requiring about $10^4$ bacteria per milliliter of tissue specimen. Furthermore it lacks specificity as it cannot discriminate different species of Mycobacteria. Culture is essential for a definite diagnosis; however, it takes weeks for identification and its sensitivity is also relatively low (Liebana *et al.*, 1996).

Acid fast bacteria can also be visualized by fluorescence microscopy using specific fluorescent dyes (auramine-rhodamine stain) which has been reported to be specific for mycolic acids. In all fluorescent methods, artifacts present a constant problem, however, fluorescent staining techniques have been improved considerably in recent years, with the virtual elimination of tissue fluorescence, reducing the problem of artifacts to a minimum. Contamination of the paraffin wax by saprophytic, fluorescent-positive bacteria has probably given rise to some false positive results in the past. Thus, many workers have recommended the use of cryostat section in fluorescent microscopy. Greenwood and Fox (1973) recommended that the fluorescent method, with its low incidence of false-negative results and its ease of performance, can be used as a screening test for bovine tuberculosis.
Bovine tuberculosis has been on the increase in developed countries and continues to occur in developing countries (Gilbert et al., 2005). Bovine tuberculosis is mainly caused by *Mycobacterium bovis* whereas *Mycobacterium tuberculosis* has been known to cause mainly human tuberculosis. The reported increase in incidence of infection by these two species which are part of the *Mycobacterium tuberculosis* complex in domesticated animals and humans has become the subject of investigation. However, there are reports that these mycobacterial pathogens are not limited to these susceptible hosts. In fact infection with *M. tuberculosis* and *M. bovis* has shown to occur across a wide spectrum of species. Besides *M. bovis*, transmission of *M. tuberculosis* from infected humans to animals and back has been reported (reverse zoonosis) (Mishra et al., 2005). Among domestic animals, infection with *M. tuberculosis* has been most frequently identified in cattle (Ocepek et al., 2005). Recent studies have reported isolation of *M. tuberculosis* in cattle with prevalence of 4.7%–30.8% in African and Asian countries (Prasad et al., 2005). The bacteriological, biochemical and genetic similarities of these two species have made it difficult to differentially identify them in clinical samples/ cultivated isolates.

Detection of mycobacterial antigens by immunohistochemistry (IHC) using polyclonal and monoclonal antibodies is an alternative to conventional acid fast staining. Immunohistochemical staining of suspect tissues using antibodies produced against *M. tuberculosis* provides a more specific means of detection than chemical staining and can discriminate tissue samples containing *M. tuberculosis* from those containing other pathogens. In addition, immunohistochemical staining of fixed tissues has been reported to be more sensitive than acid fast staining.

Thus, the study was designed with following two objectives:

1. To demonstrate the acid fast bacilli in paraffin embedded and frozen bovine tissue samples by the fluorescence microscopy technique.
2. To detect *Mycobacterium tuberculosis* in paraffin embedded and frozen bovine tissue samples by immunohistochemistry.
REVIEW OF LITERATURE

There is abundance of literature available on various diagnostic techniques for tuberculosis. The relevant literature concerned with the acid fast staining of tubercle bacilli, fluorescence microscopy technique and use of immunohistochemistry in demonstration of bacteria in tissues is being reviewed.

Acid fast staining to demonstrate mycobacteria in tissue

Milian-Suazo et al. (2000) in their study found 400 (16%) of 2,500 cattle carcasses had gross lesions typical of TB. Of the 400 infected cattle, 336 (84%) had lesions in ≥ 1 lymph nodes. Infection was confirmed in 87% of cattle with gross lesions by histological examination and demonstration of acid fast bacilli, and in 77% by bacteriologic culture at a laboratory.

HATIPOLGU et al. (2002) conducted abattoir study on genital pathology in cows. They found tuberculous endometritis. In uterine horns, corpus uteri and oviduct, yellowish grey nodules, 2 to 5 mm sized were scattered and bulged on the mucosal surface. Ziehl-Neelsen (ZN) stained sections revealed the presence of clumps of acid fast bacilli around the necrotic center, in the cytoplasm of macrophage and Langhan's type giant cells.

Headley (2002) reported tuberculosis in bovine. Small tubercles were observed within the pulmonary and hepatic parenchyma, these being more noticeable in the liver than the lung. Microscopic evaluation of all affected lymph nodes revealed the center of caseous necrosis with varying degrees of calcification and cellular infiltration. The inflammatory cells were surrounded by an extensive layer of fibrous connective tissue. Various gram-positive and acid fast positive bacteria were identified within these necrotic centers by the Gram and ZN staining techniques respectively.

Shitaye et al. (2006) determined prevalence of bovine tuberculosis by abattoir meat infection. The retrospective meat analysis showed that 699 (0.052%) of 13,36,266 cattle were positive. Firm, creamy,
whitish nodular focal lesions were predominantly (63.9%) observed in the lung cavity. 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, lymphnode (mediastinal, bronchial retropharyngeal and mesenteric), liver, spleen and intestine were observed in 97 cattle and they were positive by ZN technique. The most affected organs were lungs 48.51% and lymph nodes 25.74%.

Liebana et al. (2008) observed that among TB-confirmed cattle, 27% of reactors and 9% of in-contact animals had gross TB-like lesions in the lungs, particularly in the caudal lobes. Diagnostic sensitivity was maximized when bacteriology and histopathology were used concurrently. Caseous necrosis and calcification were common features of the granulomas encountered in natural M. bovis infections, even with pathology limited to a small number of sites. Granulomas often covered large areas of histological sections and typically contained only small numbers of acid fast bacilli.

Varello et al. (2008) evaluated the accuracy of histopathologic examination by hematoxylin and eosin and ZN staining when suspected lesions were caused by low infectious doses and detected in early stages of the disease. For this purpose, histologic methods were compared with mycobacterial culture as reference test on suspected lymph node samples from 173 cattle reacting positive in antemortem tests. Histopathology demonstrated high sensitivity (93.4%) and specificity (92.3%), while ZN sensitivity and specificity was 33.9% and 100% respectively.

Asil et al. (2012) examined 6680 bovine carcasses in abattoir, 400 (6%) had caseous lesions; 145 of them (36.3%) revealed acid fast filaments whereas 22 (5.5%) were harboring tuberculous AFB. Out of the filamentous AFB, 26 isolates were identified as M. farcinogenes, six as M. senegalense whereas the 22 non-filamentous AFB were identified as M. bovis.

Besirovic et al. (2012) euthanized 28 cows after being found positive by the comparative tuberculin skin test. 11 animals were subjected 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.

Detection of mycobacteria by fluorescence microscopy in bovine

In 1962, Wellmann and Teng studied 64 formalin-fixed tissue specimens collected from diseased lung, skin, kidney, bladder, endometrium, lymph node and adrenal gland. Three corresponding sections were cut, one for hematoxylin and eosin, ZN and auramine-rhodamine stains. Out of 64 tissue specimens, 44 were ZN negative and also fluorescent microscopy negative and remaining 20 were positive for fluorescent microscopy out of which 9 were ZN negative and 11 were ZN positive.

Silver et al. (1966) reported fluorescence microscopy, to be more accurate when applied to the detection of acid-fast bacilli, as well as easier to use than the more commonly used ZN technique. The worker stated that organisms are more readily seen and more bacilli appear to take the auramine-rhodamine stain than the carbol fuchsin stain. In material where organisms are scarce, they are more easily, as well as more frequently, found following fluorescent staining. Thus, the number of positive identifications is increased.

Smithwick and David (1971) showed that acridine orange is an effective counter stain when used with the auramine-O acid fast stain. The non acid fast material is stained orange to red and the acid fast bacilli fluorescence yellow to yellow-green. More acid fast positive smears were found by fluorescence acid fast microscopy than by the ZN method.

Greenwood and Fox (1973) examined 70 cases which had been diagnosed as 'tuberculosis' and compared ZN stain with three alternative methods of staining tubercle bacilli in paraffin sections: Fite's method; a modification by Armstrong and Price of Fite's method; and a fluorescent method using the auramine-phenol stain. The workers observed nine cases in which a positive fluorescent result was obtained on sections which were ZN
negative. The workers stated that fluorescent auramine phenol technique would be the optimal method for the demonstration of tubercle bacilli in histological material.

Kommareddi et al. (1984) examined biopsy specimens from 22 patients stained by the ZN or auramine-O (AO) fluorescent technique in which part of the tissue was cultured. The workers observed that the AO technique is technically simpler, allowing faster screening at lower power and showing greater sensitivity and predictive value of a negative result although less specificity than the ZN technique.

Mc CARTER and Robinson (1994) evaluated a total of 782 primary smears by the auramine-rhodamine method at both room temperature (21°C) and 37°C. Room temperature staining detected only 85.7% of the positive primary smears. Of the 30 smears positive by both methods, 13 (43.3%) had equal numbers of AFB on both smears, 13 (43.3%) had more AFB on the smear stained at 37°C, and 4 (13.3%) had greater numbers of AFB on the smear stained at room temperature (21°C). No smears were positive only when stained at room temperature. They stated that auramine-rhodamine staining at 37°C enhances the detection of AFB.

Cserni (1998) described the use of liquid organic cleaner (LOC), a liquid detergent, to replace the toxic phenol in the auramine O fluorescence method for the demonstration of acid fast bacilli. The modified method yielded similar results, but offered several advantages over the method involving phenol as lipophilic agent. Powdered auramine O dissolves better in LOC, the stock solution is utilizable for a longer time, and use of toxic phenol is avoided.

Ghenaat et al. (2007) compared the sensitivity and specificity of acid fast and auramine-rhodamine (AR) staining and Multiplex polymerase chain reaction (Multiplex PCR) for the detection of Mycobacterium tuberculosis complex and non-tuberculosis mycobacteria on formalin fixed paraffin embedded tissues (FFPE). The sensitivity of multiplex PCR was 65% and the specificity was 100%. Whereas the sensitivity of AR staining was 53% and specificity was 100%.
Annam et al. (2009) studied 102 consecutive patients with a clinical suspicion of tuberculosis presenting with lymphadenopathy. Fine needle aspirations were processed for routine cytology, the conventional ZN method, and the modified fluorescent method. Among the 102 aspirates, 44.11% (45/102) were positive for AFB on the conventional ZN method, 58.9% (60/102) were indicative of TB on cytology, while the number of positive smears increased to 81.37% (83/102) by the modified fluorescent method.

**Immunohistochemistry to identify mycobacteria in tissues**

Orrell et al. (1991) studied the tissue distribution of immunoperoxidase staining with polyclonal anti-BCG serum in lung granulomata of mice infected with *M. tuberculosis*. Mice inoculated with *M. tuberculosis*, strain H37Rv were used as a model of human tuberculosis. The microanatomical location of immunoperoxidase staining with a polyclonal anti-BCG serum was within macrophages and appeared granular rather than delineating whole bacilli.

Cancela and Marin (1993) used avidin-biotin complex peroxidase (ABC-P) method to detect *M. bovis* and the results were compared with those obtained by the ZN technique. Lesions were examined from 18 cows and 24 goats with tuberculosis. All animals showed pulmonary lesions, in which the cattle were mainly minor (i.e. primary complex) but in the goats it was 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.

Geisel et al. (1994) evaluated the specificity of immune-histological reactions of commercial polyclonal antibodies to antigens of various mycobacteria (*M. bovis, M. duvalii* and *M. paratuberculosis*)
connection with a number of pathogens and with the help of the indirect immunoperoxidase technique. Positive reactions occurred with various mycobacterial antigens and some gram-positive bacteria and fungi. These investigations indicated that when the above antibodies were used in histopathological diagnostics, false-positive results are observed.

Carabias et al. (1998) evaluated an immunohistochemical (IH) test with polyclonal antibodies raised against mycobacteria used in formalin-fixed tissue and compared it with mycobacterial specific culture. The workers used commercially available polyclonal antiserum rabbit anti-\textit{Mycobacterium bovis} to detect the presence of mycobacteria in 65 formalin-fixed, paraffin embedded tissue blocks from different organs, showing necrotizing caseous granuloma lesions on hematoxylin and eosin sections. These 65 samples were dyed using an acid fast fluorescent technique and compared using the immunohistochemical method. Both results were also compared with the mycobacterial cultures. The IH test, compared with the culture, showed sensitivity of 52%, a specificity of 76%, a positive predictive value of 61% and a negative predictive value of 69%.

Adams (2001) diagnosed \textit{M. bovis} infection by \textit{in vitro} techniques such as antibody and cell-mediated immunity-based. Following the detection of granulomatous lesions compatible with tuberculosis, the ZN acid fast stain was applied to impression smears or histosections to identify acid fast bacilli. When the bacilli were confirmed to be acid fast, then immunohistochemical staining with antibodies specific to \textit{M. tuberculosis} complex was done to specifically identify \textit{M. tuberculosis} complex organisms in tissues or paraffin embedded histological sections. Immunohistochemical staining of tissue sections had sensitivity equal to or greater than ZN staining.

Mukherjee et al. (2002) used a commercial anti-BCG antibody in their study and recorded 74% sensitivity of immunohistochemistry.

Pockevicius et al. (2003) did post mortem examination of 89 cattle, which had presumptive diagnosis of tuberculosis via delayed type hypersensitivity response to bovine tuberculin. The gross findings of tuberculosis were detected in 20 cattle (22%). After examination of tissues
with tuberculous lesions by methods of ZN, fluorescence microscopy and immunohistochemistry, it was found out, that fluorescence microscopy and immunohistochemical methods were more sensitive for *M. bovis* detection. *M. bovis* infection was further confirmed by transmission electron microscopy.

Padmavathy *et al.* (2005) evaluated mycobacterium antigen in cutaneous tuberculosis. All the tissue sections were negative for AFB, both by ZN stain and by culture. In 50 cases, immunohistochemical staining was done to locate the mycobacterial antigen in the tissues. Cells of the mononuclear phagocyte series containing the mycobacterial antigen stained brown and in some sections the brown stain could be documented within the multinucleated giant cells. Mycobacterial antigen was demonstrable in 68% of cases of cutaneous tuberculosis.

Ulrichs *et al.* (2005) used an anti-*Mycobacterium bovis* Bacillus Calmette-Guerin polyclonal antiserum (pAbBCG) to improve immune-staining, which was compared to the ZN stain in histological samples. Screening of tissue samples revealed that pAbBCG staining detects infected macrophages harbouring intracellular mycobacteria or free mycobacteria that are present at low abundance and not detected by the ZN stain. The positive pAbBCG staining results were confirmed either by PCR analysis of micro dissected stained tissue or by culture from tissue. The workers hypothesized that immunostaining approach allows precise localization of the pathogen in infected tissue.

Mustafa *et al.* (2006) investigated a novel method for diagnosis of tuberculosis that uses immunohistochemistry to detect the secreted mycobacterial antigen MPT64 on formalin-fixed tissue biopsies. With immunohistochemistry, 64% (35/55) and with PCR, 60% (33/55) of granulomatous lymphadenitis cases were positive. The observed agreement between PCR and immunohistochemistry was 87% ($\kappa=0.73$). The workers declared that immunohistochemistry with anti-MPT64 antiserum is a rapid, sensitive and specific method for establishing an etiological diagnosis of tuberculosis in histologic specimens. Immunohistochemistry has the
advantage over PCR of being robust and cheap, and it can easily be used in a routine laboratory.

Virieux et al. (2006) evaluated three additional techniques (ZN, auramine O/ rhodamine and immunostaining using polyclonal anti-*Mycobacterium bovis*) for bovine tuberculosis diagnosis on 39 samples from several slaughter houses. The workers concluded that the immunohistochemical technique is more sensitive and could detect a greater number of positive cattle.

Goel and Budhwar (2007) developed an immunocytochemical method using anti-38kDa antibody in the fine needle aspiration (FNA) of TB specimens recording a sensitivity of 96.7%.

Purohit et al. (2007) applied anti-MPT64 antibody with 80% positivity in patients with TB. The overall sensitivity, specificity, positive and negative predictive values of immunohistochemistry with anti-MPT64 was 92%, 97%, 98% and 85%, respectively while the corresponding values for anti-BCG were 88%, 85%, 92% and 78%. This study concluded that immunohistochemistry using anti-MPT64 is a simple and sensitive technique for establishing an early and specific diagnosis of *M. tuberculosis* infection.

Sumi and Radhakrishnan (2009) evaluated the potential diagnostic application of immunohistochemistry using a panel of antibodies against mycobacterial antigens for the diagnosis of tuberculous lymphadenitis. Immunohistochemistry was performed on the formalin fixed paraffin sections of lymph node biopsies using rabbit polyclonal antibodies against four recombinant mycobacterial proteins, that is, ESAT-6, HspX, Tb8.4 and PlcA. The results of immunohistochemistry were correlated with ZN staining method. Immunohistochemistry using anti-ESAT-6 antibody was found to be highly sensitive (88.6%). Results of the study suggest that immunohistochemistry using anti-ESAT-6 antibody has potential application for the diagnosis of tuberculous lymphadenitis, in patients in whom conventional diagnostic methods did not confirm the presence of acid fast bacilli.
Vural and Alcigir (2010) examined macroscopical and microscopical lesions in 86 cattle 4-6 years old of different sex and breeds. Generally 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 (94%) comprised miliary-nodular tubercles and five (6%) were determined to be chronic organ tuberculosis. ZN stained sections revealed acid fast bacilli in lung, lymph nodes, liver and trachea (96%). 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.

Pedersen *et al.* (2011) found that the sheep antiserum, MAS-01, reacted with all 18 mycobacterial species tested, but did not react with uninfected inflammatory tissues. The antibody was more sensitive than the ZN stain for detection of tissue mycobacteria and shortened the time required to identify these infections.

Martinez *et al.* (2012) conducted a study, to develop an indirect immunoperoxidase method for bovine tuberculosis diagnosis in formalin fixed, paraffin embedded tissues and its validation compared to mycobacterial isolation and ZN staining. About 33 bovine lymph nodes with isolation of *M. bovis* and 11 negative lymph nodes from tuberculosis free ranches were used. Sections of all lymph nodes examined were stained with ZN and IHC. From the 33 positive isolation cases, all (100%) were positive by IHC. From the 11 negative cases, all were negative to mycobacterium by IHC. Regarding ZN staining of the 33 positive isolation cases, 30 (90.9%) had acid-fast bacilli and from the 11 negative isolation cases none had acid fast bacilli. Results show that IHC represents a fast, sensitive and specific diagnostic tool for bovine tuberculosis in formalin fixed, paraffin embedded tissues allowing simultaneous observation of tissue lesions and antigens.

**Presence of *Mycobacterium tuberculosis* in cattle**

Screening of available literature revealed that work done to demonstrate *M. tuberculosis* antigen in bovine tissue by IHC is negligible.
However many worker have detected the presence of *M. tuberculosis* by PCR technique in bovine tissue.

Sreedevi and Krishnappa (2003) detected *M. tuberculosis* complex organism in clinical samples of cattle by PCR and DNA probe methods. They found 60%, 15% and 40% blood, milk and nasal swabs respectively, were positive by PCR. 65%, 15% and 40% blood, milk and nasal swabs respectively, were positive by dot blot hybridization.

Mishra *et al.* (2005) examined the utility of the *hupB* gene (Rv2986c in *M. tuberculosis* or Mb3010c in *M. bovis*) to differentiate *M. tuberculosis* and *M. bovis* by a PCR-restriction fragment length polymorphism (RFLP) assay with 56 characterized bovine isolates. A nested PCR (N-PCR) assay was developed, replacing the PCR-RFLP assay for direct detection of *M. tuberculosis* and *M. bovis* in bovine samples. The N-PCR products of *M. tuberculosis* and *M. bovis* corresponded to 116 and 89 bp, respectively. The detection limit of mycobacterial DNA by N-PCR was 50 fg, equivalent to five tubercle bacilli. *M. tuberculosis* and/or *M. bovis* was detected in 55.5% (105/189) of the samples by N-PCR, compared to 9.4% (18/189) by culture. The sensitivities of N-PCR and culture were 97.3 and 29.7%, respectively, and their specificities were 22.2 and 77.7%, respectively. Mixed infection by N-PCR was detected in 22 animals, whereas by culture mixed infection was detected in one animal.

Prasad *et al.* (2005) designed a specific nested-PCR (N-PCR) assay, based on the *hupB* gene of *M. tuberculosis* (Rv2986c) and *M. bovis* (Mb3010c) as a method to differentiate these closely related species. Extra-pulmonary clinical samples obtained from cattle and humans were investigated. Pre-dominance of *M. tuberculosis* (15.7%) and *M. bovis* (26.8%) was seen in humans and cattle, respectively. However, more importantly, both mycobacterial pathogens (mixed infection) were identified in a number of samples. In humans 8.7% of the samples and 35.7% in cattle were classified as mixed infection.
MATERIALS AND METHODS

1. Place of Work

The work was conducted in the Department of Veterinary Pathology, College of Veterinary Science and A.H and the Advanced Tuberculosis Diagnostic Centre for Wild and Domestic Animals, Centre for Wildlife Forensic and Health, 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, suspected for tuberculosis after detailed post mortem examination, irrespective of age, sex or breed.

2.1 (b) Chemicals

The chemicals used in acid fast staining, fluorescence microscopy and immunohistochemistry are enlisted below:

- Ziehl-Neelsen staining kit (K005-1KT, HIMEDIA)
- Hifluo-Phenol free stain kit (K061, HIMEDIA)
- Citric acid (PCT0501-500G, HIMEDIA)
- Sodium Citrate Dihydrate (Product code: S0230, RANKEM)
- Tris buffer (Product code: T0350, RANKEM)
- Trypsin (Product code: RM612-1G, HIMEDIA)
- Calcium chloride dehydrate (Product Number: 223506, SIGMA-ALDRICH)
- *Mycobacterium tuberculosis* Polyclonal Antibody, FITC conjugate was used as a Primary antibody (Catalog Number: PA1-28997.THERMO SCIENTIFIC).
• Secondary antibody [Monoclonal Anti-Rabbit Immunoglobulins-Biotin clone RG-16, produced in mouse] (Catalog Number: SAB4200363, SIGMA-ALDRICH).

• ExtrAvidin(TM)-Alkaline Phosphatase (E2636, SIGMA-ALDRICH)

• SIGMA FAST TR/Naphthol AS-MX tablets (F4523 – 5 set)

2.1 (c) Equipments

i. For Cryosections, cryostat (LEICA, Model No.CM 1100) was used.

ii. For Fluorescent microscopy, Motic BA400-600 Upright Microscope with Epi-Fluroscence attachment EF-UPR –III was used.

3. Methods

3.1 Study period

The study was conducted for a period of eight months from August 2012 to March 2013.

3.2 Collection of samples:

The lung and lymph node samples were collected after examination of 252 bovine carcasses at slaughter house, Municipal Corporation Jabalpur, Livestock Farm, Adhartal, College of Veterinary Science and Animal Husbandry, Jabalpur and Organized Dairy Farms in and around Jabalpur. Out of these 24 cases showed nodular lesions with casseating mass suggestive of tuberculosis. Samples were taken from the lesions together with the normal adjacent tissue. Samples were also collected from 20 prescapular lymph nodes showing enlargement and casseation. Grossly healthy bovine lung tissue (n=6) was also collected and processed.

3.3 Preparation of impression smears

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 in their guidelines.
3.4 Processing of samples

3.4 (a) Fixation of tissue

The suspected tissue was cut into two parts. One part was used for cryosectioning and the remaining tissue sample was fixed in 10% formalin and processed for histopathological examination (Gridley, 1960). The tissue samples were collected and stored at -20°C for cryosectioning.

The tissue samples were also collected in buffered formalin for fluorescence microscopy and detection of *M. tuberculosis* in paraffin embedded tissue sections.

3.4 (b) Frozen sections

The collected tissue slices were kept at -20°C in deep freeze until processing. The tissue 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 at -22°C for freezing. Sections of 6-7μm thick were taken on clean glass slide. Staining was done for fluorescence microscopy and for detection of *M. tuberculosis* using immunohistochemistry.

3.4 (c) Paraffin sections

The small tissue pieces were dehydrated in three changes of acetone for 30, 30 and 45 minutes respectively. These sections were then cleared in three changes of benzene for 30, 30 and 45 minutes respectively. After that infiltration of wax was performed by keeping the tissues in four changes of hard wax in wax oven. The blocks were made after infiltration with the help of L – molds after that blocks kept in refrigerator for overnight. The 5μm thick sections were cut by rotary type microtome.

3.5 Staining

3.5 (a) Hematoxylin and Eosin (H and E) staining

Routine Hematoxylin and Eosin staining was done for study of tuberculous lesions in tissue sections following the procedure of Gridley (1960).
3.5 (b) Ziehl-Neelsen (ZN) staining

ZN staining was done for demonstration of acid fast bacteria in impression smears and tissue sections as described by Chauhan (2006). This staining was performed as follows

- 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.
- Counterstained in methylene blue solution of 30 seconds.
- Dehydrated, cleared and mounted in DPX.

4. Fluorescence Microscopy (FM)

For detection of acid fast bacilli the fluorescence staining method employed was that of Kuper and May (1960) after making suitable modifications.

4.1 Samples for FM

Demonstration of acid fast bacilli by fluorescence microscopy was done in impression smears and tissue sections, both cryo sections and paraffin embedded. Impression smears and tissue sections from all the 24 cases with lesions suggestive of tuberculosis and 10 prescapular lymph nodes were observed for demonstration of acid fast bacilli by fluorescence microscopy.

4.2 Staining solutions

SO82: Auramine-rhodamine solution (Phenol free)
S099: 0.5% Acid alcohol as decolourizer
SO83: Counter stain, Potassium permanganate solution

4.2 (a) Impression smears

- One side of a clean glass slide was thoroughly flamed and allowed to cool. Impression smear was prepared on glass slides from center of the tubercle.
- Smear was prepared on the flamed side of the slide and allowed to air dry.
- Smear was fixed by gently passing the slides through flame.
- Smear flooded with auramine-rhodamine solution (phenol free) and allowed to stain for 15 minutes without drying.
- Section rinsed with distilled water for 30 seconds.
- After that the smear was flooded with decolourizer (0.5% acid alcohol) and kept for de-staining for 30 second till the smear appeared pink.
- Again smear was rinsed with distilled water for 30 seconds. After that smear was flooded with potassium permanganate solution and stained for two minutes.
- The smear was then carefully rinsed with distilled water for 30 seconds.
- The smear was allowed to air dry and observed under fluorescent microscope.

4.2 (b) Tissue sections
- The sections were cut and de-paraffinised in the usual manner. (for cryosections this is not required).
- Auramine-rhodamine solution (phenol free) was flooded on the sections and allowed to stain for 22 minutes without drying.
- Sections were rinsed with distilled water for one minute and drained.
- After that sections were flooded with decolourizer and allowed for de-staining for one minute. Section should appeared pink.
- Again sections were rinsed with distilled water for 1 minute and drained.
- After that sections were flooded with potassium permanganate solution and stained for three minutes.
- Carefully rinsed with distilled water for one minute.
- Blot dry.
- Dehydrated in absolute alcohol for 15 seconds (for cryosections step 9 and 10 were not required).
- Cleared in xylene.
- Mounted in DPX or glycerol gelatin under a coverslip.
• Observed under fluorescent microscope.

5. Immunohistochemistry to detect *M. tuberculosis*

Immunohistochemistry to detect *M. tuberculosis* was performed on tissue sections, both cryo sections and paraffin embedded from lung and prescapular lymph nodes for the localization of mycobacterial antigens by using an indirect streptavidin-biotin method as described by (Brees et al., 2000).

5.1 Preparation of solutions

1. 10 mM Sodium citrate, (Molecular weight-294.10 g/mol)
   Add 0.588 gm Sodium citrate in 200 ml distilled water.
2. 2 mM citric acid, (Molecular weight-192.12g/mol)
   For 2mM, 0.0384gm Citric acid is required in 100ml.
   Therefore for 200 ml, 0.0768 gm citric acid was added.
3. 0.05 mM Tris buffer (Molecular weight-121.14 g/mol)
   3.0285 gm Tris buffer was added in 500 ml distilled water.
4. Mycobacterium tuberculosis Polyclonal Antibody, FITC conjugate was used as a Primary antibody at 1: 100 dilution (4µl of antibody + 396µl PBS).
5. Monoclonal Anti-Rabbit Immunoglobulins-Biotin clone RG-16, produced in mouse was used as secondary antibody at 1: 20 dilution (25µl antibody + 475 µl PBS)
6. Extra Avidin(TM)-Alkaline Phosphatase as enzyme was used at 1: 20 dilution (25µl Enzyme + 475 µl PBS)
7. SIGMA FAST TR/Naphthol: In 10 ml distilled water, Tris buffer tablet was added. When it dissolved completely Sigma fast red tablet was added.
8. Mayer’s hematoxylin: five gm alum was mixed in 100 ml distilled water, to this 0.1 gm hematoxylin was added along with 0.02 gm sodium iodate and 2 ml glacial acetic acid, mixed well and filtered.

5.2 Staining protocol

• Tissue sections were mounted on glass slides deparaffinized and rehydrated in water.
• Sections were incubated in a solution of 2 mM citric acid and 10 mM sodium citrate in a microwave for four minutes.
• Sections were washed for five minutes with 0.05 M Tris buffer (pH 7.6)
• Incubated with 0.1 g of trypsin and 0.1 g of calcium chloride in 100 ml of Tris buffer for 10 minutes at 37° C.
• After incubation, the sections were washed for 5 minutes in Tris buffer. (for cryosections step 1 to step 4 were not required).
• The tissue which was prominently visible was encircled and blocked for 10 minutes with rat serum.
• The rat serum was removed from the slides, and the sections were incubated with a *M. tuberculosis* polyclonal antiserum diluted at 1:100 for two hours at 37°C.
• Following incubation, the sections were washed for five minutes in Tris buffer.
• 100μl biotinylated secondary antibody diluted at 1:20 was applied on particular tissue sections and incubated for 30 minutes at room temperature.
• After that the preparation was rinsed gently with phosphate buffer saline for five minutes.(washing)
• 100μl ExtrAvidin-Alkaline phosphatase diluted at 1:20 was applied on tissue sections and incubated for 20 minutes at room temperature.
• The slides were rinsed gently with Tris buffer for five minutes.
• Red chromogen was prepared at the time of final washing.100μl Red chromogen was applied to the slides covering the tissue sections and kept for eight minutes.
• The slides were rinsed gently with distilled water.
• Mayer’s hematoxylin was applied on tissue sections for one minute.
• Slide was washed with distilled water for 2-3 minutes.
• The slides were mounted with glycerol gelatin or DPX and observed.

**Statistical analysis**

The percent of acid fast bacilli observed in lung tissues and lymph nodes by ZN staining and fluorescent microscopy was calculated. The immunohistochemical stained slides were observed for presence of *M. tuberculosis* antigen.

**RESULTS**
The study was designed to demonstrate the acid fast bacilli in impression smears, paraffin embedded and frozen bovine tissue samples by the fluorescence microscopy technique and to detect *Mycobacterium tuberculosis* in paraffin embedded and frozen bovine tissue samples by immunohistochemistry.

**Collection of samples**

Total number of 252 necropsies were conducted. There was visit to slaughter house, Municipal Corporation, Jabalpur, Livestock Farm, Adhartal, College of Veterinary Science and Animal Husbandry, Jabalpur and Organized Dairy Farms in and around Jabalpur. Out of these 252 necropsies, 24 cases had lesions suggestive for tuberculosis i.e. caseous nodules. From these 24 cases, in 20 animals enlarged prescapular lymph nodes also showed caseous masses and samples were collected from these also.

**Post mortem examination**

The animals were subjected to detailed post mortem examination. In all 24 cases the caseous nodules were primarily observed in the lung tissues. 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.

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 only pulmonary tuberculosis was observed (Plate 1). In four cases in addition to pulmonary lesions caseous nodules were also observed in pleura, spleen, liver or peritoneum. In all the 20 cases with pulmonary tuberculosis the prescapular lymph nodes were enlarged with caseous centers (Plate 2).

**Macroscopic examination of tubercles**

The tubercles were hard, yellowish in colour. They were 1 mm to 2-3 cm in size. The lesions were classified as miliary with small sized tubercles scattered throughout the surface or chronic organ tuberculosis with
larger sized hard tubercles. Most of the tubercles were present in cranio-ventral lobe but some were also found in diaphragmatic lobe (Plate 3). They were either embedded in the organ or were hard protrusive. Tubercles showed caseous mass in the center on cutting (Plate 4). The gritty sensation was also felt. The mediastinal lymph nodes were enlarged and showed caseation and calcification. The enlarged prescapular lymph nodes also had caseous necrosis with cheesy mass oozing out.

**Microscopic examination**

For microscopic study from each case, impression smears and four corresponding sections were cut, one each for Hematoxylin and Eosin, Ziehl-Neelsen, and Auramine-Rhodamine stains and Immunohistochemistry. Where necessary, additional sections were prepared.

**Acid fast staining of impression smears**

Impression smears were prepared from the center of the caseating mass of lung lesions from all the suggestive 24 cases and the tuberculous lesions from ten enlarged prescapular lymph nodes and stained by the ZN method. 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 bacteria were detected in at least one section of the sample.

In impression smears from 12 lung samples (50%) acid fast bacilli in the form of clumps extra-cellularly and sometimes intra-cellularly in macrophages were observed (Plate 5). In addition, impression smears from all ten prescapular lymph nodes also showed intracellular acid fast red coloured bacilli in the macrophages and lymphocytes (Plate 6 and 7).

**Microscopic examination of tubercles**

In Hematoxylin and Eosin stained sections a classic granuloma, as a characteristic lesion of tuberculosis, composed of a central caseous necrosed area with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan’s giant cells were seen especially in
miliary TB (Plate 8 and 9). The lesions were surrounded by a rim of fibroblasts with interspersed lymphocytes. Often the center of the granuloma was necrotic and heavily calcified mostly in cases of chronic organ tuberculosis. Depending upon the microscopic features of the granuloma observed by light microscope they were placed into three sub groups (Fig. 1). Cases with minimal histological activity were characterized by little or no caseation and by distinct fibrosis (Plate 10). Marked histological activity was denoted by little or no fibrosis but by large fields of caseous necrosis (Plate 11). In tissues showing moderate histological activity, necrosis and fibrosis were intermediate in degree (Plate 12).

Minimal histological activity : Six samples
Moderate histological activity : Four samples
Maximum histological activity : Fourteen samples

Microscopic evaluation of all affected lymph nodes revealed a center of caseous necrosis 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. These inflammatory cells were surrounded by an extensive layer of fibrous connective tissue (Plate 13 and 14).

**Ziehl-Neelsen staining of tissue sections**

Ziehl-Neelsen stained sections revealed the presence of clumps of acid fast bacilli around the necrotic center, 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 ten cases which showed acid fast bacilli in direct smears also (Plate 15). In sections of lymph nodes from ten animals, also clumps of acid fast bacilli were observed (Plate 16).

**Correlation of histological stage of tubercle with demonstration of acid fast bacilli**

The correlation of tubercle stage with demonstration of acid fast bacilli is as depicted in table 1. Maximum bacilli could be observed in the tissue with marked histological lesion showing extensive areas of caseous
necrosis. Whereas in tubercles with marked fibrosis acid fast bacilli could not be demonstrated.

Table 1: Correlation of tubercle stage with demonstration of acid fast bacilli

<table>
<thead>
<tr>
<th>Number of cases (n=24)</th>
<th>Histological activity of tubercle</th>
<th>Acid fast positive</th>
<th>Acid fast negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Less than 10 bacilli identified</td>
<td>More than 10 bacilli identified or clumps of bacilli</td>
</tr>
<tr>
<td>06 (25%)</td>
<td>Minimum</td>
<td>0</td>
<td>00</td>
</tr>
<tr>
<td>04 (16.66%)</td>
<td>Moderate</td>
<td>0</td>
<td>02</td>
</tr>
<tr>
<td>14 (58.33%)</td>
<td>Maximum</td>
<td>02</td>
<td>08</td>
</tr>
</tbody>
</table>

Fluorescence microscopy for detection of acid fast (bacilli) organisms

For detection of acid fast bacilli the fluorescence staining method employed was that of Kuper and May, (1960) after making suitable modifications.

Initially, both known positive (showing acid fast bacilli in impression smears and tissue sections) and negative (healthy lung bovine tissue collected from slaughter house) were employed as positive and negative control respectively.

The impression smears and tissue sections were stained by auramine rhodamine (phenol free) solution.

The acid fast bacilli were observed in various colour depending on the type of filter used. After using both blue and green filters, we observed a better visibility of fluorescence by the blue filter. As such the blue filter was preferred.

Criteria used for differentiating the artifacts

In our study the following criteria were used for differentiating the acid fast bacilli from the artifacts
a) Typical size: Most acid fast bacilli measured 2 to 5 microns in length; in our experience, most artifacts appeared bigger.

b) Typical form: Acid fast bacilli were present as narrow rods which were sometimes curved; most artifacts were plump and more irregular.

c) Typical structure: Acid fast bacilli were either homogeneous or granular.

d) Sharp delineation was important as many artifacts had fuzzy, ill-defined borders.

e) Most acid fast bacilli showed strong and brilliant fluorescence, in contrast to many of the artifacts.

f) Atypical fluorescenting structures were not reported as positive unless they were accompanied by typical rods.

**Observations**

In impression smears, all 12 cases found positive for acid fast bacilli (Positive control) and six cases in which acid fast bacilli could not be demonstrated showed red-orange fluorescence (rods) against dark background and were now considered positive for acid fast bacilli by fluorescence microscopy (Plate 17).

Paraffin embedded tissue sections were prepared and stained by HiFluo Phenol free stain kit and observed under fluorescent microscope using blue filter. Despite repeated efforts non specific fluorescence was predominantly seen in these sections and they were not preferred for demonstration of acid fast bacilli by fluorescent technique.

In cryo sections out of the all 12 cases found positive for acid fast bacilli by ZN staining and in addition six cases in which acid fast bacilli could not be demonstrated showed red-orange fluorescence (rods) against dark background and were considered positive (Plate 18). Thus, total 18 cases (75%) revealed acid fast bacilli by FM as against only 12 cases (50%) found positive by ZN staining.

No fluorescence was observed in the impression smears and tissue sections of the healthy bovine lung (Negative control).
The non specific fluorescence was negligible in both the impression smears and cryo sections. In addition to identifying more positive cases Fluorescence microscopy also revealed higher average number of acid fast bacilli (more than 5 bacilli per field) as compared to ZN staining.

For demonstration of acid fast bacilli in the prescapular lymph nodes by fluorescence microscopy technique also the impression smears, cryosections and paraffin embedded tissue sections were prepared. In all the 10 cases fluorescence was visible in the impression smears and cryosections (Plate 19 and 20) with minimum non specific fluorescence. However non specific reactions were observed in appreciable amounts in the paraffin sections of lymph nodes also.

The comparative data for presence of acid fast bacilli by Ziehl–Neelsen (ZN) and fluorescence microscopy techniques is shown in table 2 (Fig. 2).

**Table 2: Demonstration of acid fast bacilli by ZN and fluorescence microscopy**

<table>
<thead>
<tr>
<th>Sample size</th>
<th>ZN staining</th>
<th>Fluorescence microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Lung tissue N=24</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>06</td>
</tr>
<tr>
<td>Lymph node N=10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

**Immunohistochemistry to detect *Mycobacterium tuberculosis***

Immunohistochemistry was done for the localization of antigens of *Mycobacterium tuberculosis* by using an indirect streptavidin-biotin method on 18 lung and 10 lymph nodes tissue sections which were found positive for
acid fast bacilli by ZN and FM staining (12 ZN + fluorescence positive and 6 only fluorescence positive).

Both paraffin embedded and cryo tissue sections were prepared from lungs and lymph nodes and an indirect streptavidin-biotin method was used to detect *Mycobacterium tuberculosis*. Both the techniques were found to be uniformly sensitive and with negligible non specific reactions once the technique was standardized for the laboratory. However excessive *M. tuberculosis* antigen were observed in cryosections as compared to the paraffin embedded tissues.

*M. tuberculosis* antigens were mostly seen in the macrophages of 12 samples of lung tissues (12/18., 66.66 %) (Fig. 3) mostly in the caseous necrotic debris and rarely in the Langhan’s giant cells as reddish coloured rods in the same tissues (Plates 21, 22 and 23). Whereas in only six lymph nodes the presence of *M. tuberculosis* antigens could be demonstrated in the caseous debris (Plates 24 and 25, Fig. 4).

The immunohistochemical stained slides generally showed a much clearer and more striking positivity than ZN method.

**Correlation between histological type, acid fast staining, fluorescence microscopy and *M. tuberculosis* antigen**

The correlation between histological type, acid fast staining, fluorescence microscopy and *M. tuberculosis* antigen in the affected lung and lymph node tissues is shown in table 3. *M. tuberculosis* antigen was observed only in the tubercles with maximum histological activity.
Table 3: Correlation between histological type, acid fast staining, fluorescence microscopy and *M. tuberculosis* antigen

<table>
<thead>
<tr>
<th>Histological activity</th>
<th>ZN staining</th>
<th>fluorescence microscopy</th>
<th>M. tuberculosis antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum (lung)</td>
<td>0</td>
<td>01</td>
<td>0</td>
</tr>
<tr>
<td>Moderate (lung)</td>
<td>02</td>
<td>03</td>
<td>0</td>
</tr>
<tr>
<td>Maximum (lung)</td>
<td>08</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Lymph node</td>
<td>10</td>
<td>10</td>
<td>06</td>
</tr>
</tbody>
</table>
DISCUSSION

Tuberculosis (TB) is a zoonotic most important chronic disease with high prevalence among humans, domestic and wild animals in developing countries. Mycobacterial organisms are responsible for this chronic infectious disease of man and animals. These diseases are characterized by the progressive development of tubercles in many organs in most species. Tuberculosis in humans is caused by *Mycobacterium tuberculosis* complex, an actinomycetes and characteristically acid fast. *M. bovis*, the cause of tuberculosis in cattle is also pathogenic for a number of other animal species as well as human. Diagnosis of bovine TB is usually made by the relevant clinical manifestations, supported by the characteristic histopathological features of biopsies, such as presence of granulomatous lesions (with or without cæsation). Granulomatous lesions have extensive differential diagnosis and are known to occur in granulomatous reaction in malignancies of epithelial origin, fungal infection, parasitic infection etc. At times, the histopathological features in these diseases resemble closely and can pose considerable diagnostic challenge. The present study was designed to demonstrate the acid fast bacilli in impression smears, paraffin embedded and frozen bovine tissue samples by the fluorescence microscopy technique and to detect *M. tuberculosis* in paraffin embedded and frozen bovine tissue samples by immunohistochemistry.

In the present study, total number of 252 necropsies was conducted. Out of these 252 necropsies 24 cases had lesions suggestive of tuberculosis i.e. caseous nodules in lungs. Milian-Suazo *et al.* (2000) identified tuberculosis in 16% cattle whereas Shitaye *et al.* (2006) determined prevalence of bovine tuberculosis in 0.052% cattle. Thus, the prevalence of bovine tuberculosis varies as the epidemiology in developed and developing countries differ, owing to differences in the implementation of preventive measures (WHO, 1999). Despite the ominous features of bovine tuberculosis to date there have been only projected global estimates of the disease burden. There has been no international effort to determine the actual disease burden, owing to the non availability of a reliable user-friendly technology for
early detection of *M. bovis* in clinical samples. Bovine tuberculosis has been on the increase in developed countries and continues to occur in developing countries (Gilbert *et al.*, 2005). However, there are limited reports from India (Singh and Shah, 2004) and from underdeveloped countries relating to the prevalence of bovine tuberculosis.

Neill *et al.* (1994) reported that the characteristics and lesions of tuberculosis vary according to the species of the animal affected, the species of the mycobacteria involved, the immunity of the host, the route of infection and probably certain ill defined factors. In our study all the cases showed primary lung involvement (pulmonary tuberculosis). In accordance with our findings the lung involvement was maximal in earlier studies also indicating a high incidence of pulmonary bovine tuberculosis (Cancela and Marin, 1993; Cassidy *et al.*, 1999; Pockevicius *et al.*, 2003 and Virieux *et al.*, 2006). In the present study 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%). 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. Palmer *et al.* (2007) hypothesised 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. 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.

For microscopic study from each case, impression smears and four corresponding sections were cut, one each for Hematoxylin-Eosin, Ziehl-Neelsen, Auramine-Rhodamine stains and Immunohistochemistry. Where necessary, additional sections were prepared.
In Hematoxylin and Eosin stained sections 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 were seen especially in miliary tuberculosis. Depending upon the microscopic features of the granuloma observed by light microscope they were placed into three sub groups that is minimal, moderate and maximum. Cases with minimal histological activity (6/24) were characterized by little or no caseation and by distinct fibrosis. Marked histological activity (14/24) was denoted by little or no fibrosis but by large fields of caseous necrosis. In tissues showing moderate histological activity (4/24), denoted by necrosis and fibrosis, were intermediate in degree. The classification was done as it has been stated by earlier workers and in accordance with the hypothesis that cases with marked fibrosis are histologically inactive with minimum concentration of acid fast bacilli. On the other hand a histological lesion showing increased caseation necrosis but little fibrosis is an histologically active lesion containing more number of acid fast bacilli.

Our results confirm and extend previous studies (Palmer et al., 2007) which suggest an important role for the host granulomatous responses against mycobacteria during natural infection. Tuberculous granuloma is a complex host-protective structure generated in response to persistent mycobacterial stimuli with focal accumulation of inflammatory cells, such as multinucleated giant cells and lymphocytes (Ramakrishnan, 2012). In addition, encapsulation, a process involving production of connective tissue around the granuloma, has been shown to be critical for controlling both mycobacterial growth and tissue dissemination (Gil et al., 2010).

**Demonstration of tubercle bacilli by ZN staining**

Greenwood and Fox (1973) have observed that of the various fuchsin stains used in their investigation the ZN method emerges as the best. It gives the highest yield of positive results, it is easy to interpret, and the staining technique is neither difficult nor prolonged. All fuchsin methods for the detection of tubercle bacilli suffer, however, from the drawback that sections
require prolonged and careful examination and a considerable proportion of cases are likely to be missed.

In the current study, in impression smears from 12 lung samples acid fast bacilli in the form of clumps extra-cellularly and sometimes intra-cellularly in macrophages were observed. In addition, impression smears from all ten prescapular lymph nodes also showed intracellular acid fast red coloured bacilli in the macrophages and lymphocytes. However, in tissue sections stained by ZN the presence of clumps of acid-fast bacilli around the necrotic center, in the cytoplasm of macrophage and Langhan’s type giant cells was observed in only ten sections. They were frequently observed free in the caseous debris. Cousins et al. (2004) reported marked variation in the number of acid fast bacilli or even their absence in tuberculous granuloma. 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 that the relative sensitivity and specificity of ZN staining were 33.9% and 100% respectively.

**Correlation of histological stage of tubercle with demonstration of acid fast bacilli**

In the present study maximum bacilli could be observed in the tissue with marked histological lesion showing extensive areas of caseous necrosis. Whereas, in tubercles with marked fibrosis acid fast bacilli could not be demonstrated.

Thus, our findings are in agreement with those of Palmer et al. (2007) who stated that tissue examination for acid-fast bacilli is most likely to be successful in histologically active cases, i.e. cases with little fibrosis but much caseation necrosis. 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 (Gil et al., 2010). However, Liebana et al. (2008) have reported the
absence of correlation between AFB numbers and stage of granuloma development during natural infection with *M. bovis*. Our findings are in partial agreement with those of Menin *et al.* (2013) who in their study observed that the histological analysis of granulomatous response and tissue remodeling reveals high frequency of chronic lesions in different tissues, which negatively correlated with viable mycobacterial counts. In our study more number of histologically active lesions with increased frequency of acid fast bacilli were observed whereas in lesions with minimum histological activity (marked fibrosis) the acid fast bacilli could not be demonstrated.

**Fluorescence microscopy for detection of acid fast (bacilli) organisms**

Although the diagnosis of mycobacterium induced disease is most commonly made by direct sample examination and culture, acid-fast bacilli detection has low sensitivity and specificity, and, at best, can only provide a preliminary diagnosis (Nahar *et al.*, 2011). Thus, in the present study the detection of acid fast organism was also attempted by fluorescence microscopy. Methods applying fluorochromes have been used for acid-fast staining since the introduction of an auramine O based method and an auramine-rhodamine based method by several workers (Truant *et al.*, 1962; McCarter and Robinson, 1994 and Somoskovi *et al.*, 2001).

Bachmann and Finke (1939) were the first to apply fluorescence microscopy for the detection of acid-fast bacilli in tissue sections. Gray (1953) reported the presence of up to five times as many tubercle bacilli in adjacent tissue sections stained differentially and recommended fluorescence microscopy for routine purposes. Fluorescing artifacts, while helpful in focusing, have posed a problem for some and have even been blamed as causes for false-positive interpretations. However, we agree with the observations of Greenwood and Fox (1973) that fluorescence staining techniques have been improved considerably with the virtual elimination of tissue fluorescence, reducing the problem of artifacts to a minimum. In the present study, bacterial morphology showed up clearly and only morphologically definite bacilli were counted as positive.
The basic principle of fluorescence microscopy adopted was as stated by Silver et al. (1966). A combination of auramine and rhodamine dye was used as a stain, and potassium permanganate was used as a counter stain after the material is decolorized with acid alcohol. The acid-fast organisms do not decolorize after staining. A light source producing a suitable wavelength is used to excite fluorescence of the stain retained by acid-fast organisms and this fluorescence can then be detected by use of either dark field or bright field microscopy. A filter system which absorbs background light, but transmits the light caused by fluorescence results in easily visible organisms.

In the present study the impression smears and cryosections were prepared from all twenty four lung tissues and stained by HiFluo Phenol free stain kit and observed under fluorescent microscope using blue filter under oil immersion. Earlier, Mansfield (1970) and Greenwood and Fox (1973) advocated the combination of Auramine-Phenol for fluorescent microscopy. Phenol used earlier by many workers as a lipophilic agent is also known for its toxicity. Cserni (1998) advocated other lipophilic agents like liquid organic cleaner giving even better results, to replace phenol. The HiFluo phenol free stain kit is based on the method of fluorescence microscopy devised by Truant et al. (1962) which makes inclusion of phenol in staining solution unnecessary. The sensitivity and specificity of the staining results are identical with those obtained using classical method employing phenol (McCarter and Robinson, 1994 and Somoskovi et al., 2001).

Fluorescence microscopy depends on the ability of certain dyes to radiate visible light when illuminated by light of a shorter wavelength. When auramine and rhodamine are used, ultra-violet light is not required since the excitation maxima of these dyes do not lie in the ultra-violet range. Auramine fluorescence at about 4,320 A.U. and rhodamine at about 5,560 A.U. With insertion of filters which absorb direct illuminating radiation but which transmit the differently colored light caused by fluorescence, organisms retaining the stain become visible.
In the present study, Motic BA400-600 Upright Microscope with Epi-Fluorescence attachment EF-UPR –III was used. The technique of fluorescence with epi-illuminators is based on the adaption of the vertical illuminator used in reflected light microscopy. The equipment uses externally mounted lamphouse 6volt/30watt quartz halogen Koehler illumination. With insertion of filters which absorb direct illuminating radiation but which transmit the differently colored light caused by fluorescence, organisms retaining the stain become visible. A blue light source is needed for illumination. A barrier filter, placed in the microscope ocular, filters out the blue light of the background allowing fluorescing organisms to stand out on a darker field (McCarter and Robinson, 1994).

Earlier workers have reported that focusing during FM is somewhat difficult because of the dark background although double fluorochroming and counterstaining have served to lessen this problem. With tissue sections, focusing was best accomplished at the edge of the section. It was observed that examination should not begin before the lamp performs at full capacity, which required from 20-25 minutes. A dark room was considered essential by earlier observers, but with the improved more advanced microscope dimmed light or a shield light sufficed in our study.

In the present study, auramine-rhodamine staining was done at 37°C for a period of 22 minutes. The optimum temperature and time for AO staining has remained a topic of discussion by many workers. McCarter and Robinson (1994) observed that rhodamine-auramine staining at 37°C enhances the detection of AFB compared with conventional staining at room temperature (21°C). Somoskovi et al. (2001) suggests that a longer (20 to 25 min) staining time might increase sensitivity. However, any possible influence of the staining time on the sensitivity of fluorochrome stains needs further investigation.

Somoskovi et al. (2001) observed that more concentrated acid-alcohol solution in the decolorization step, may result in over decolorization and consequently a decrease in sensitivity. However, we applied the recommended 0.5% acid-alcohol solution. Although standard
recommendations for carbol fuchsin methods state that mycobacteria cannot easily be destained, we observed that the concentration of acid alcohol with fluorochrome stains is critical. In our study more bacilli were stained by the fluorochromes than by carbol fuchsin of identical smears. Earlier workers have stated that the mycolic acid, the principal acid-fast component of tubercle bacilli, combines more easily with auramine than with carbol-fuchsin and, in the former combination, resists destaining for a much longer time. Thus, it is likely that demonstration of organisms by fluorescence microscopy includes a larger number of relatively less acid-fast bacilli.

The sensitivity of FM was much more as compared to ZN staining in our study. Positive results were found in all twelve cases found positive for acid fast bacilli and in addition six cases in which acid fast bacilli could not be demonstrated also showed red-orange fluorescence (rods) against dark background and were considered positive for acid fast bacilli by fluorescence microscopy. In addition to identifying more positive cases Fluorescence microscopy also revealed higher average number of acid fast bacilli as compared to ZN staining. Our findings are in accordance with those of earlier workers (Ghenaat et al., 2007 and Annam et al., 2009). Due to intensive phagocytic activity by macrophages in tuberculous granulomas, the morphological characteristics of AFB often get distorted. This may account for the low detectability of ZN staining (Goel and Budhwar, 2007).

Earlier, the possibility of misinterpretation and finding of false positive by inexperienced workers has been pointed out (Somoskovi et al., 2001 and Nahar et al., 2011). However, we would consider that the increased acid fast cases by FM were unlikely to be false positives and this view is reinforced by the totally negative findings in the negative control sections. It should, perhaps, be stressed that the sections in which a positive result was obtained solely with the fluorescent method showed only a very few bacilli. Moreover, the fact that none of the fluorescent negative cases were found to be positive by the acid fast methods suggests that the fluorescence microscopy was reliable technique and is unlikely to give false negative results.
Thus, we conclude that more acid fast smears would be found to be positive by the fluorochrome methods than by the ZN method.

In the present study paraffin embedded tissue sections were also prepared and stained by HiFluo Phenol free stain kit and observed under fluorescent microscope. Despite repeated efforts non specific fluorescence was predominantly seen in these sections and they were not preferred for demonstration of acid fast bacilli by fluorescence technique. It has been observed by earlier workers that contamination of the paraffin wax by saprophytic, fluorescent-positive bacteria probably gives rise to false positive results. Perhaps this could be the plausible explanation for the increased non specific fluorescence in paraffin tissue sections observed.

It has been observed by earlier workers that certain factors, such as pretreatment of specimens (e.g., centrifugation) or chemotherapy, may alter both the morphological features of the acid fast bacilli and the intensity of their fluorescence. Atypical organisms are more often seen in tissue sections than in smears. Atypical fluorescing structures should not be reported as positive unless they are accompanied by typical rods.

An extensive review of the pertinent literature and the present findings has shown many advantages of the fluorescence method in comparison to the time-honored ZN staining technique. Organisms offer much contrast, appearing as red-orange, self luminous rods against a more or less darkened background. This greatly facilitates the demonstration of single bacilli. By their dispersion halo, organisms may be detected even when slightly out of focus.

To conclude, the fluorescent auramine-rhodamine technique would therefore appear to be the optimal method for the demonstration of tubercle bacilli in histological material, its only drawback being the necessity of using a fluorescent microscope. It is further recommended that the fluorescent method, with its low incidence of false-negative results and its ease of performance, can be used as a screening test: fluorescent-negative sections can be reported as such, but positive sections can be confirmed by
staining the same sections using the ZN method and concentrating on the area of the section shown to be positive by the fluorescent method.

**Immunohistochemistry to identify mycobacteria in tissues**

In the present study immunohistochemistry was done for the localization of antigens of *Mycobacterium tuberculosis* by using an indirect streptavidin-biotin method on both paraffin embedded and cryo-tissue sections on 18 lung and 10 lymph nodes tissue sections which were found positive for acid fast bacilli by ZN and FM staining (12 ZN + fluorescent positive and 6 only fluorescent positive). In 12 samples of lung tissues presence of *M. tuberculosis* antigens was seen as extracellularly and within macrophages and multinucleated giant cells whereas in only six lymph nodes the presence of *M. tuberculosis* antigens could be demonstrated.

Many workers have evaluated the specificity of immunohistological reactions of commercial polyclonal antibodies to antigens of various mycobacteria (*M. bovis, M. duvalii* and *M. paratuberculosis*) connection with a number of pathogens and with the help of the indirect immunoperoxidase technique (Geisel et al., 1994; Carabias et al., 1998; Bonenberger et al., 2001 and Pockevicius et al., 2003).

Cancela and Marin (1993) used avidin-biotin complex peroxidase (ABC-P) method to detect *M. bovis* and reported that ABC-P technique is 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. Pockevicius et al. (2003) examined tissues with tuberculous lesions by methods of ZN, fluorescence microscopy and immunohistochemistry, and observed that fluorescence microscopy and immunohistochemical methods were more sensitive for *M. bovis* detection.

Orrell et al. (1991) stated that immunohistochemical techniques should provide an alternative method of estimating the extent of bacillary load; this approach may also provide evidence of mycobacterial infection from residual antigen deposits in the tissue when whole bacilli have been successfully cleared. Vural and Alcigir (2010) also observed that 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. Similarly Martinez et al. (2012) stated that IHC represents a fast, sensitive and specific diagnostic tool for bovine tuberculosis in formalin fixed, paraffin embedded tissues allowing simultaneous observation of tissue lesions and antigens.

Thus, our findings are in accordance with those of earlier workers as the mycobacteria antigens could be easily visualized in both the paraffin embedded and cryosections in 12/18 lung tissues.

*M. tuberculosis* and *M. bovis* infect animals and humans. Their epidemiologies in developed and developing countries differ, owing to differences in the implementation of preventive measures (WHO, 1999). Identification and differentiation of these closely related mycobacterial species would help to determine the source, reservoirs of infection, and disease burden due to diverse mycobacterial pathogens. The identification of the closely related members of the *Mycobacterium tuberculosis* complex (MTC) has remained a challenging task in diagnostic laboratories (Mishra et al., 2005). MTC includes a variety of closely related mycobacteria, namely, *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. africanum* and *M. microti*. A panel of classical tests based on microbiological features such as growth rate and phenotypic and biochemical characteristics has conventionally been utilized to distinguish members of MTC (Niemann et al., 2000). However, these tests are slow, cumbersome, unreliable, and time-consuming.

In our study the observation of *M. tuberculosis* antigen otherwise considered a human strain in the bovine pulmonary tissues clearly indicates the increased probability of human sources as the cause of bovine pulmonary infection. In accordance with our findings, Sreedevi and Krishnappa (2003); Mishra et al. (2005) and Prasad et al. (2005) also detected and identified of *M. tuberculosis* in bovine samples by a novel nested PCR assay.

It is well known that animals including wild ones, do suffer from *M. tuberculosis* infection (De Lisle et al., 2002) we endorse the observations of earlier workers that to establish the human sources as the cause of animal
infection, *M. tuberculosis* isolates from the farm workers should be typed by molecular methods. The pattern of the animal isolates then must be matched with human isolates to establish transmission from humans to animals. Until this is established, it remains a hypothesis only that animals are infected by *M. tuberculosis* from human sources.

The probability of mixed infection of both *M. tuberculosis* and *M. bovis* in bovine pulmonary tuberculosis cannot be ruled out. Earlier, Mishra *et al.* (2005) and Prasad *et al.* (2005) using PCR identified mixed infection in a number of cases of both human as well as bovine tuberculosis. Interestingly, in the present study, *M. tuberculosis* antigen could be demonstrated in six prescapular lymph nodes from same animals. This raises the probability of mixed infection with more dissemination of *M. bovis* to regional lymph nodes. However, a larger sample size is definitely require to validate such hypothesis.

Thus, the present findings clearly record the presence of *M. tuberculosis*, usually known to cause human infections, in bovine tuberculous granuloma. However, the probability and incidence of mixed infection needs to be established.
SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

Summary

Tuberculosis (TB) is a zoonotic most important chronic disease with high prevalence among humans, domestic and wild animals in developing countries. At times, the histopathological features of granulomatous diseases resemble closely TB and can pose considerable diagnostic challenge. The present study was designed to demonstrate the acid fast bacilli in paraffin embedded and frozen bovine tissue samples by the fluorescence microscopy technique and to detect *M. tuberculosis* antigen in paraffin embedded and frozen bovine tissue samples by immunohistochemistry.

Total 252 necropsies were conducted. Out of these 252 necropsies 24 had lesions suggestive of tuberculosis i.e. caseous nodules in lungs. From these 24 cases in 20 animals enlarged prescapular lymph nodes also showed caseous masses. The tubercles were hard, yellowish in colour. They were 1 mm to 2-3 cm in size. The lesions were classified as miliary with small sized tubercles scattered throughout the surface or chronic organ tuberculosis with larger sized hard tubercles. Most of the tubercles were present in cranioventral lobe but also found in diaphragmatic lobe. For microscopic study from each case, impression smears were prepared and four corresponding tissue sections were cut, one each for hematoxylin-eosin, Ziehl-Neelsen, auramine-rhodamine stains and immunohistochemistry. Where necessary, additional sections were prepared.

Impression smears were prepared from the centre of the casseating mass of lung lesions from all the suggestive 24 cases and the tuberculous lesions from 10 enlarged prescapular lymph nodes and stained by the ZN method. They were considered positive when one or more acid fast bacteria were detected in at least one section of the sample. In impression smears from 12 lung samples acid fast bacilli in the form of clumps extra-cellularly and sometimes intra-cellularly in macrophages were observed. In addition all 10 prescapular lymph nodes also showed intracellular acid fast red
colioured bacilli in the macrophages and lymphocytes. In H and E stained sections a classic granuloma, as a characteristic lesion of tuberculosis, composed of a central caseous necrosed area with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan's giant cells were seen especially in miliary TB. Depending upon the microscopic features of the granuloma observed by light microscope they were placed into three histological sub groups that is minimal, moderate and maximum. Cases with minimal histological activity (6/24) were characterized by little or no caseation and by distinct fibrosis. Marked histological activity (14/24) was denoted by little or no fibrosis but by large fields of caseous necrosis. In tissues showing moderate histological activity (4/24), necrosis and fibrosis were intermediate in degree. On ZN stained tissues the acid fast bacilli stained bright red were observed in lungs for ten cases which showed acid fast bacilli in direct smears also. In sections of lymph nodes from ten animals, also clumps of acid fast bacilli were observed.

For detection of acid fast bacilli the fluorescence staining method was standardized in the laboratory. Impression smears were prepared from all 24 lung tissues and stained by HiFluo Phenol free stain kit and observed under fluorescent microscope using blue filter under oil immersion. Out of these all the twelve cases which were found positive on ZN staining and six new cases in which acid fast bacilli could not be demonstrated showed red-orange fluorescence (rods) against dark background and were considered positive for tuberculosis by fluorescent microscopy. Similarly, in cryosections out of these all twelve cases found positive for acid fast bacilli and in addition six cases in which acid fast bacilli could not be demonstrated showed red-orange fluorescence (rods) against dark background and were considered positive.

Fluorescence microscopy was also done on the impression smears and the tissue sections of 10 prescapular lymph nodes and in all of these red-orange fluorescence (rods) against dark background were observed and they were considered positive for acid fast bacilli by fluorescence microscopy.
Paraffin embedded tissue sections were prepared and stained by HiFluo Phenol free stain kit and observed under fluorescent microscope using blue filter. Despite repeated efforts non specific fluorescence was predominantly seen in these sections and they were not preferred for demonstration of acid fast bacilli by fluorescent technique.

Immunohistochemistry was done for the localization of antigens of *M. tuberculosis* by using an indirect streptavidin-biotin method on 18 lung and 10 lymph nodes tissue sections which were found positive for acid fast bacilli by ZN and FM staining (12 ZN + fluorescent positive and 6 only fluorescent positive). In 12 samples of lung tissues presence of red stained *M. tuberculosis* antigens were seen extra-cellularly and within macrophages and multinucleated giant cells whereas in only six lymph nodes the presence of *M. tuberculosis* antigens could be demonstrated.

Correlation between histological type, ZN staining and fluorescence microscopy revealed that increased number of tubercle bacilli are observed in tubercles with maximum histological activity. Fluorescent microscopy was more sensitive in detecting the acid fast bacilli then ZN staining. *M. tuberculosis* antigen in maximum cases (12/18) indicates more chances of animal being infected by the human species of Mycobacteria.
Conclusions

The following conclusions can be drawn from the present study:

- The fluorescent method, with its high sensitivity, low incidence of false-negative results and its ease of performance, can be used as a screening test wherever the facilities of microscope exist.

- It is further concluded that the fluorescent method can be used as a screening test for diagnosing bovine tuberculosis. Fluorescent-negative sections can be reported as such, but positive sections can be confirmed by staining the same sections using the ZN method and concentrating on the area of the section shown to be positive by the fluorescent method.

- For demonstration of acid fast bacilli by FM the impression smears or cryosections should be preferably used.

- There is a high incidence of pulmonary tuberculosis in cattle caused by *M. tuberculosis* or a mixed mycobacteria infection.
Suggestions for further work

1. Work can be undertaken to evaluate the potential for using a fluorescence polarization assay for the detection of antibodies to Mycobacterium in cattle sera.

2. Identification and differentiation of closely related mycobacterial species in bovine pulmonary tuberculous lesions would help to determine the source, reservoirs of infection, and disease burden due to diverse mycobacterial pathogens.
REFERENCES


