PATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES ON PNEUMONIA/ARTHRITIS OUTBREAK IN A GROUP OF FEEDLOT CALVES ASSOCIATED WITH INFECTION BY MYCOPLASMA BOVIS.

SAMEH YOUSSEF*, AND ROSER VELARDE

Dept. Of Pathobiology, Ontario Veterinary College, Ontario, Canada

*Home Address: Dept Of Pathology, Faculty Of Vet Medicine, Alexandria, Egypt

ABSTRACT *Mycoplasma bovis* (*M.bovis*) has been associated with many disease entities in cattle including pneumonia-arthritis syndrome of calves. Lately, there is an increase concern about the increasing prevalence of this pathogen in feedlot industry around the world. In this study, approximately five percent (11 calves) of 220 recently purchased feedlot calves (5-6 month of age) developed pneumonia that was associated with polyarthritis in 6 calves shortly after arrival. *M.bovis* was identified from the joint fluid swabs and/or serum of these calves using PCR and/or ELISA. A diagnosis of *M.bovis* associated pneumonia-arthritis disease had been made and the calves were treated with specific antibiotics. Calves responded poorly to treatment, six calves died and four of them sent for the post-mortem examination. The characteristic pulmonary lesions included the presence of multifocal to coalescing caseated nodules, fibrinous pleuropneumonia, degenerative and necrotizing bronchioloitis, and a cuffing pneumonia. The affected joints had lesions ranged from mild synovial and periarticular edema to severe proliferative fibrinosuppurative synovitis. *M.bovis* antigen was strongly expressed in the pulmonary caseated areas and also on the bronchial and bronchiolar epithelium. This monoclonal antibody-based immunohistochemical technique, considered efficient and an accurate diagnostic tool in detection of *M.bovis* antigen. The differences in pathologic lesions in natural and experimental *M.bovis* infection have been discussed.

INTRODUCTION

Mycoplasma bovis (M.bovis) is an important bovine pathogen and with the exception of *Mycoplasma mycoides subsp.*

Mycoides small colony biotype, the agent of contagious bovine pleuropneumonia (CBPP), M.bovis is considered the most pathogenic and invasive species of bovine mycoplasmas (**Jubb et al 1993**; Jones et al., 1997). Several bovine mycoplasmas including M.bovis are respiratory commensals and grow mostly on epithelial surfaces of the respiratory tract of clinically normal calves (Gyles and Thoen, 1993; **Jubb et al., 1993**; Jones et al., 1997). During time of stress, M.bovis can induce a variety of diseases, including pneumonia, otitis media, arthritis of calves and young cattle, mastitis and genital tract infection in adult cattle (Gouraley et al., 1989; Adegboye et al., 1996; Pfutzner and Sachse, 1996; Jones et al., 1997; **McGavin et al., 2001**). In addition, M.bovis is implicated in contributing to the bovine respiratory disease complex (BRD complex) or enzootic pneumonia in calves, the disease which often results from the interaction of viruses, mycoplasmas, bacteria, and exacerbated by environmental factors such as weather and overcrowding (**Jubb et al., 1993**; Jones et al., 1997; **McGavin et al., 2001**).

M.bovis pneumonia is usually accompanied by arthritis (pneumonia-arthritis-syndrome), however, arthritis may be the only clinical sign in some outbreaks (**Jubb et al 1993; Adegboye et al., 1996; Step and Kirkpatrick 2001**). M.bovis is refractory to antibiotic treatment (ter Laak et al., 1993; Adegboye et al., 1996; Vogel et al., 2001) and can induce pneumonia in gnotobiotic calves (Thomas et al., 1986).

As mentioned above, M.bovis usually acts in concert with other pneumonic pathogens (e.g. Manniheima (Pasterula) haemolytica, Haemophilus somnus, Respiratory syncytial virus, and Parainfluenza III virus) to induce pneumonia. However, during the last five years there have been many indications of increase in M.bovis prevalence as the primary or even the only pathogen in some BRD outbreaks in feedlot calves (Bashiruddin et al., 2001; Step and Kirkoatrick; 2001). The pulmonary lesions in some of these outbreaks were close to CBPP (Bashiruddin et al., 2001), thus calling for a special attention to M.bovis pneumonia. In Ontario, the numbers of BRD cases and/or pneumonia-artrhritis syndrome due to M.bovis infection have been slightly increased during the years 2000 and 2001, however, there is an increase concern about the prevalence of this pathogen in Ontario beef industry (Haines et al., 2001).

Described here are the pathologic changes and immunohistochemical findings associated with an outbreak of M.bovis pneumonia/arthritis in a

farm in Ontario, Canada during the year 2001. The aims of the present study were: i) to increase the awareness of the diagnostic pathologist and practitioners about the characteristic lesions that are associated with *M.bovis* pneumonia. ii) to correlate between these lesions and the presence of *M.bovis* antigen using immunohistochemistry.

MATERIALS AND METHODS

Clinical history

Approximately five percent (11 calves) of 220 recently purchased feedlot calves (5-6 month of age) developed pneumonia that was associated with polyarthritis in 6 calves shortly after arrival. The affected calves were anorectic, had a low-grade fever and had a respiratory distress. Half of the affected animals also had diarrhea, and 6 of them had swollen joints and severe lameness due to arthritis. Joint fluid from 3 lamed calves and serum from 6 pneumonic calves were sent to "The Animal Health Laboratory", Ontario Veterinary College, University of Guelph. (AHL-OVC) for bacterial isolation and Mycoplasma species identification using PCR and/or ELISA (Ball et al., 1994; Bashiruddin et al., 2001). *M.bovis* was identified in the joint fluid of two affected calves using PCR. However, the joint fluid of three

animals had a positive titers for *M.bovis* using ELISA. The joint fluids were negative for bacterial pathogens. Also five out of six serum samples from affected calves had positive titers for *M.bovis* using ELISA. After receiving the microbiological results, a diagnosis of *M.bovis* associated pneumonia-arthritis disease had been made and the calves were treated with a combination of tilmicosin (Micotil 300-Elanco) and florfenicol (Nuflor-Schering-Plough) at doses of 10 mg/Kg BW, Subcut q72 h and 20mg/Kg Subcut q48 h . The affected animals responded poorly to treatment,

Six calves died and four of them were sent for the post-mortem examination.

Pathologic studies:

At necropsy macroscopic lesions were recorded, and three to five samples of pneumonic and normal lung tissues were placed in a neutral buffered formalin and processed by a routine paraffin embedding technique. Sections for histological examination were stained with hematoxylin and eosin. Some sections were stained with Gram stain and Ziehl-Neelsen stain for demonstration of bacterial colonies and acid-fast bacilli respectively in paraffin sections (Prophet et al., 1992). Samples

from other organs of the body were obtained when possible and processed similar to the lung tissue for the histopathological examination.

Microbiological studies:

At necropsy tissue blocks (3 X 3 cm.) from pneumonic lungs and sometimes from bronchial lymph nodes were collected in sterilized plastic bags. Swabs from infected joint were also collected. All samples were sent to the AHL-OVC diagnostic laboratories. Examination for viruses, bacteria and mycoplasmas were carried out as described elsewhere (Ball et al., 1994; Quinn et al., 2000). M.bovis isolated were identified according to the biochemical properties of cloned strains, as well as by epifluorescence and immunoperoxidase staining of colonies (Erno and Stipkovits, 1973; Bencina and Bradbury, 1992).

Immunohistochemistry:

Paraffin blocks from lungs that showed lesions were selected for immunohistochemistry. A previously described technique for immunostaining of M.bovis, was used but after several modifications (Adegboye et al.,1995). Tissue sections were mounted on Probe-On glass slides (Sigma Diagnostics, St.Louis, MO) and coated with poly-L-lysine and deparaffinized using Hemo-De (Fisher Scientific, NJ). Endogenous peroxidases were inactivated with H_2O_2. Tissues were incubated in Protienase K (DAKO Corp, Carpinteria, CA) for 3 min at room temperature. After washing with distilled water, sections were treated with 10% normal bovine serum to block non specific-protein binding sites. The primary antibody was a monoclonal antibodies (mouse) to M.bovis (QED Bioscience Inc. San Diego, CA), the antibody was used in dilution of 1:3000 and incubated for 45 min at room temperature. The secondary antibody was a universal goat anti-mouse immunoglobulin conjugated to a peroxidase-labeled polymer (En Vision $+^{TM}$, Dako). The chromagen 3,3-diaminobenzidine-4HCL, was incubated for 3 minutes at room temperature. Sections then counterstained with Mayer's hematoxylin , dehydrated, and covered. Pneumonic lungs known to be infected with M.bovis and normal lungs were also included in each assay as positive control sections.

RESULTS

Microbiological findings:

M.bovis was isolated and identified from the submitted lung tissues of all calves and from the synovial swabs of Calves 2, 3 and 4. No other bacterial or viral pathogens

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have been identified either from the lung or synovial swabs.

Pathological results:

The pulmonary lesions were generally similar in all calves, but differed in severity and distribution. However, the gross findings of each calf were as follows:

Calf I:-

The right parietal pleura was diffusely granular as a result of fibrin deposition. On the right thoracic wall there was an area 4 x 5 cm of fibrinous adherence between the parietal and visceral pleura. Approximately the entire surface of the right lung and 50 % of the left lung were severely consolidated and failed to collapse. The entire right lung, had an alternation of congested dark red areas and multifocal to coalescing grey-white elevated nodules. These nodules were variable in size (0.5 to 4 cm in diameter) and on cut sections, they contained grayish white caseous materials and were sharply demarcated from the adjacent lung tissue by a thin (0.1 cm) fibrous capsule. On the left lung, particularly on the cranial lobe, there were few nodules similar to those present in the right lung; but the dark red congested areas predominated.

The caudal left lobe had numerous emphysematous bullae of various sizes. The thoracic segment of trachea and the tracheobronchial airways were filled with coagulated blood. The submucosa of the trachea had extensive hemorrhages. The nasal mucosa was congested with small hemorrhagic areas. The respiratory lymph nodes were edematous and enlarged 3 to 4 times their normal size. No significant lesions were present in other organs including body joints.

Calf 2:

There were few fibrinous adhesions of the cranial lung lobes to the parietal pleura. The caudo-ventral part of the lung was severely consolidated and had multifocal to coalescing randomly distributed grayish-white nodules (Fig. 1a). The affected part occupied approximately 80% of the right lung surface and 60% of the left lung. On cut sections, these nodules contained grayish-yellow pasty to caseous material. Two large (4 X 6 cm. in diameter) sequestra were present in the cranio-ventral part of the right cranial lobe (Fig. 1b). On cut section these sequestra contained a very dry grayish-white necrotic tissue and were lined by a thick (1 cm) connective tissue capsule. The caudal parts of both lungs were mildly

emphysematous. Submandibular and tracheo-bronchial lymph nodes were moderately congested and markedly enlarged. Both carpal and left stifle joints were swollen and contained turbid yellowish synovia with no fibrin. The periarticular soft tissue and synovial membranes of these joints were markedly edematous. The liver was diffusely pale tan. There were no other internal lesions.

Calves 3 and 4:

The gross lesions of these two calves were almost identical and will be described together.

The cranio-ventral parts (approximately 40% and 50% of lung surface in calves 3 and 4 respectively) of the right and left lung lobes was mildly congested , failed to collapse and were very firm in texture, On cut section, enormous amount of small (0.2- 1 cm) pale tan friable caseated nodules were present. The caudo-dorsal portion of the lung was pale, failed to collapse, and was meaty on palpation, but no nodules were present. Both stifle, hock and knee joints were greatly swollen with marked edema and congestion of the periarticular tissues. The synovial fluid of these Joints was cloudy and had numerous fibrin tags. The articular surfaces of these joints had no degenerative or ulcerative

lesions, but were only covered with fibrin tags.

Histopathologic Results:

The histologic lesions of lungs from all studied animal were almost similar; but with a variable degree of severity. The most characteristic lesion was the presence of variably-sized multifocal to coalescing caseated areas (Fig.2). Each caseated area consisted centrally of a structureless eosinophilic caseated material (contained nuclear and cellular ghosts) that was surrounded in order by an inflammatory zone, occasionally a remnant of bronchiolar epithelium and a thin (100-200 microns) layer of fibrovascular membrane (Fig.3). The inflammatory zone was made primarily from lymphocytes, plasma cells and few macrophages (Fig.4). Neutrophils were present sporadically. The caseated areas appeared to be developed or started in the bronchioles or bronchi, as the smallest caseated areas were present in the bronchiolar lumens and also some of the larger ones were lined by a bronchiectatic bronchiolar or bronchial epithelium. In general, there were a dramatic inflammatory and degenerative bronchiolar changes. Most of the bronchioles were dilated, had denuded epithelium and were packed with caseated materials (Figs 5 and 6). Peribronchiolar aggregation of

mononuclear round cells (cuffing pneumonia) was a frequent observation. In contrast to the severe bronchiolar lesions, the alveolar lesions were generally mild and characterized by proliferation of pulmonary alveolar macrophages and intraluminal infiltration with few fibrin and neutrophils. However, in calf 2, few sections showed moderate to severe alveolar lesions. The alveoli in these sections were packed with fibrin and high numbers of oat-shaped cells (swirling neutrophils) (Fig.7). Few Gram-negative bacterial colonies were. Ziehl-Neelsen stain was negative and no acid-fast bacilli were present in the sections examined from all calves.

Articular Lesions ranged from mild synovial and periarticular edema to severe proliferative fibrinosuppurative synovitis. In calves 3 and 4, where the joints gross lesions were more severe, synovial membranes were hyperplastic (villous hyperplasia)and edematous (Fig.8). Perivascular lymphoplasmacytic infiltrates, focal loss of epithelium, and exudation of fibrin and neutrophils into the joint space was also marked.

No significant lesions were present in liver, kidneys, spleen and heart of the all examined calves.

Immunohistochemistry:

M.bovis antigen was strongly expressed at the periphery of the necrotic caseated areas (Fig.9). Also, M.bovis antigen was strongly expressed in bronchial and bronchiolar epithelium especially those that had the cuffing lesion (Figs 10 and 11). Occasionally M.bovis antigen was present in macrophages. Rarely, M.bovis antigen was present in the alveolar epithelium.

DISCUSSION

The present study describes an outbreak of pneumonia/arthritis syndrome in a group of feedlot calves on a farm in Ontario, Canada with the isolation of M.bovis from joints and/or the lung of these animals. M.bovis was first isolated in USA in 1962 and then in the Middle East in 1964 (ter Laak et al., 1992). M.bovis is now believed to be prevalent worldwide and may also have a high undetected incidence (ter Laak et al., 1992; Bashiruddin et al., 2001; Step and Kirkpatrick, 2001). Mycoplasma diseases generally including M.bovis pneumonia/arthritis are typical examples of multifactorial diseases, where factors such as intercurrent infection and stress from transportation and handling are important determinants of the

final outcome of natural infection *(Gyles and Thoen 1993; Jones et al., 1997; McGavin et al., 2001).* In the present study, the calves were recently castrated and transported shortly before developing of the clinical signs. During time of stress *M.bovis* can breach the pulmonary innate resistance and invades the lower respiratory system causing a disease *(Jones et al., 1997; McGavin et al., 2001).* It is not clear how the stress makes lung more susceptible to infection with commensal pathogens *(McGavin et al., 2001).* once succeed to infect few animals in the herd, coughing and sneezing conceivably promote areosolization of respiratory secretions and thereby promote the spread of *M.bovis* infection *(Gyles and Thoen 1993).* Systemic infection with joints localization and the development of arthritis may follow mastitis in adult cow and pneumonia in calves *(Gyles and Thoen 1993; Pfutzner and Sachse; 1996).*

During the last two years, the prevalence of *M.bovis* had increased in many parts of the world (Stipkovits et al., 2000; Haines et al., 2001; Bashiruddin et al ., 2001). We believe this increase is misleading and indicates a previous under diagnosis rather than increase in the pathogenicity of *M.bovis*. The fact that, testing for the presence of Mycoplasma is not included in the routine bacteriological

examination of clinical samples, indicate that certainly not all cases caused by *M.bovis* are actually recognized as such. In addition, the recent advance in diagnostic facilities by introduction of PCR, and immunohistochemistry, facilitates the identification of *M.bovis*.

The characteristic pulmonary pathologic lesions of the present studies included the presence of multifocal to coalescing caseated nodules, fibrinous pleuropneumonia, degenerative and necrotizing bronchioloitis, and cuffing pneumonia. These lesions are very close to those described previously (Adegboye et al.,1995; Adegboye et al., 1996; Rodriguez et al., 1996; Stipkovits et al., 2000; Bashiruddin et al., 2001). The multifocal necrotic areas which are associated with natural *M.bovis* infection either in the present study or in previous studies were described previously as abscesses, abscesses contain caseated materials, coagualtive necrosis or caseated nodules (Adegboye et al., 1995; Rodriguez et al., 1996; Step and Kirkpatrich, 2001; Bashiruddin et al., 2001). Given the histological characteristic of these necrotic areas (absence of both cellular and tissue details besides the lacking of neutrophils), we preferred to describe these necrotic areas as caseated nodules . Although very unlikely

in this age and in this part of the world, tuberculosis (TB), should be considered in the differential diagnosis of any caseated lung lesions. In this study, acid-fast stain for TB bacilli was negative and the histologic appearance of the current pulmonary caseated areas were not typical of TB granuloma.

On the other hand, the gross and microscopic lesions of experimentally induced *M.bovis* are quite different than those occurred in natural cases (Rodriguez et al., 1996; Step and Kirkpatrich, 2001). The multifocal pulmonary necrotic (caseated) areas were not recorded in the experimentally induced *M.bovis* pneumonia, instead, the pulmonary lesions in experimentally infected calves included suppurative bronchioloitis and varying degrees of peribronchial mononuclear cell cuffing (cuffing pneumonia) (Rodriguez et al., 1996; Step and Kirkpatrich, 2000). This difference in lesions between the natural and experimental *M.bovis* infection may be due to the fact that the naturally occurring disease is a complex phenomena and mostly there is interaction of other organisms (e.g. *Manniheima haemolytica* or *H.somnus)* in the early stage of the natural disease. No bacteria were isolated in the current study, and only few gram-negative bacterial colonies were present in few lung sections in calf 2. The excessive treatment of the calves in this study with antibiotics might interfered with the bacterial isolation.

M.bovis pneumonia in calves is usually associated with polyarthritis, and in a very rare occasion arthritis can be the salient clinical feature of *M.bovis* infection (Adegboye et al., 1996; Stokka et al., 2001; Step and Kirkpatrick 2001). In the current study three out of four submitted calves had moderate to severe arthritis. The gross and histologic lesions recorded in these joints, are almost identical to those described before (Adegboye et al., 1996; Pfutzner and Sache, 1994; Henderson and Ball, 1999; Stokka et al., 2001) .

We have used a monoclonal antibody for *M.bovis* to localize the *M.bovis* antigen. *M.bovis* antigen was strongly expressed in bronchial and bronchiolar surface epithelium, caseated necrotic areas and associated macrophages. Similar results were reported previously (Adegboye et al.,1995; Rodriguez et al., 1996; Haines et al., 2001). The immunohistochemical stain was positive and strong in all examined cases with efficient

level of accuracy. This result suggests that using monoclonal immunohistochemical staining to M.bovis is a fast, efficient and a promising diagnostic method.

The current histological and imnmunohistochemical results indicate that M.bovis colonize and target bronchial and bronchiolar epithelium and starts its characteristic lesions from there. Similar result was reported previously (Adegboye et al.,1995; Rodriguez et al., 1996; Haines et al., 2001). It is not known yet why M.bovis target bronchiolar not alveolar epithelium, however, M.bovis might have the same pathogenesis of Mycoplasma hyopneumoniae, which colonize the bronchiolar epithelium also in swine. Mycoplasma hyopneumoniae adhere to the cilia of the bronchi by a unique adhesive protein and produces ciliostasis, which facilitates its bronchial colonization (McGavin et al., 2001).

In conclusion, (i) the gross and histologic lesions associated with M.bovis pneumonia in calves is the presence of multifocal pulmonary caseation with degenerative and necrotizing bronchiolitis, (ii)this lesions are characteristic; but not pathognomonic. Confirmation of diagnosis depends on the microbiological results and/or immunohistochemistry using monoclonal antibodies.

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Description of Figures

Fig.1a: Lung, Calf 1 : The Lung failed to collapse and is covered with a fibrinous exudate (arrow) besides the presence of numerous multifocal grayish white caseated nodules (arrow heads).

Fig.1b: Cross section of the same lung in Fig.1a: Multifocal to coalescing grayish-white areas of Caseation (arrow heads) and a sequestrum formation (arrow).

Fig.2: Lung, Calf 1: Multifocal caseated areas (A) that are surrounded by an inflammatory zone (arrow). Note that the adjacent alveoli (B) are almost normal and devoid of inflammatory cells. H&E (X=60).

Fig.3: Same lung section of Fig.2: Caseated area consists of a caseated center (A) and a peripheral inflammatory zone (arrow). Alveoli are devoid of inflammatory cells (arrow) H&E (X=160)

Fig.4:Lung, Calf 3 : Area of caseation (A) consists of a caseated center and lined by nuclear debris (arrow heads), and inflammatory zone made from mononuclear inflammatory cells (B). H&E (X=400)

Fig.5:Lung, Calf 3: Denuded necrotized bronchiolar epithelium (arrow) and a peribronchiolar Fibrosis. H&E (X=250).

Fig.6:Lung, Calf 4: Bronchiectatic dilated bronchial epithelium (arrows), plugged with caseated contents. inset: Bronchiole (arrow) completely occluded with caseated material, besides peribronchial fibrosis and cuffing. H&E (X=160).

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Fig.7: Lung, Calf 2: Alveoli (arrow) filled with fibrin and oat cells. H&E (X=250).

Fig.8: Joint capsule, Calf 4: A villar-like hyperplastic synovial membrane with mononuclear cells infiltration. H&E (X=250).

Figs.9-11: Monoclonal antibody-based immunoperoxidase staining of lung from calves 1, 3 and 4 respectively. DAB chromagen with hematoxylin counter stain. 9. M.bovis antigen is strongly expressed at caseated area (A) especially on its periphery (arrow) (X=160). 10 and 11. M. bovis antigen is strongly expressed (arrows) in two degenerated bronchioles (arrow heads). X=160.
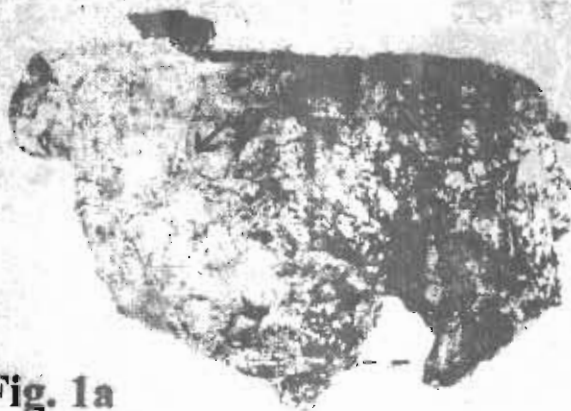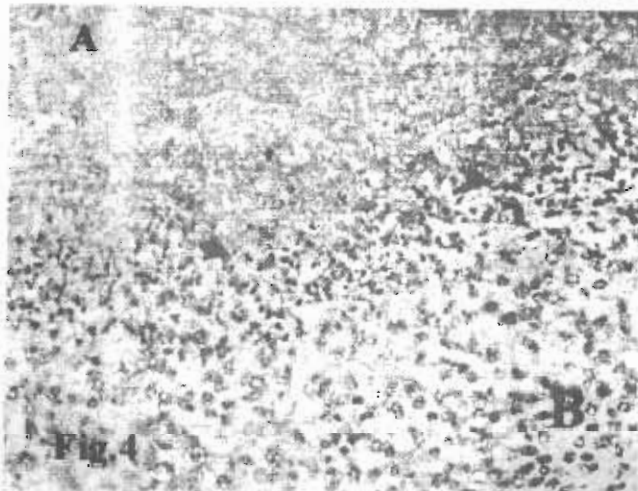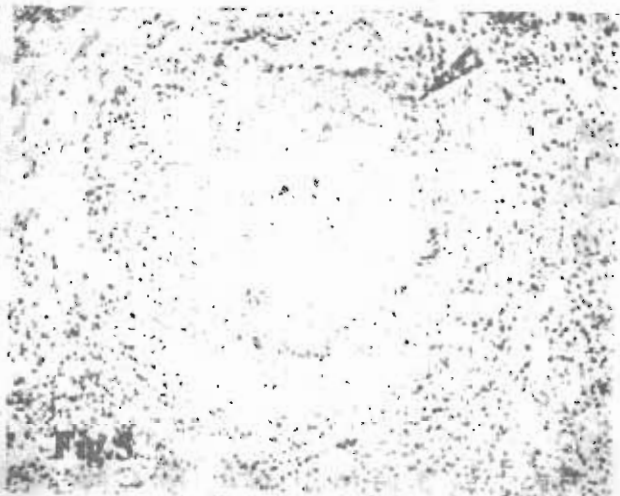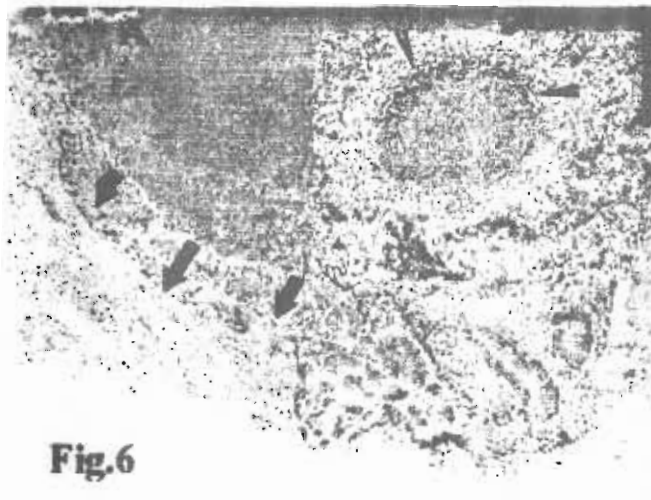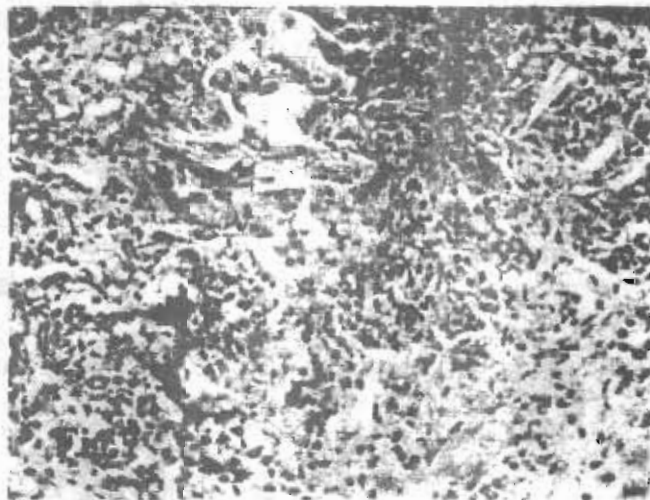

Fig. 1a

Fig. 1b

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Fig.8

Fig.9

A

Fig.10

Fig.11