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PATHOLOGICAL AND VIROLOGICAL STUDIES ON BOVINE EPHEMERAL FEVER

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The current study submitted in four Egyptian governorates Damietta, Menofia, Fayoum and sharkia. Samples were collected in summer 2006 from dairy cattle showing signs suspected bovine ephemeral fever (BEF). Blood samples on anti-coagulant, nasal swabs and tissues (bovine liver, kidney, lymph nodes and lung) for BEFV isolation and propagation on Vero, BHK_{21} cell lines and baby mice. Identification was done by both Indirect Fluorescence Antibody Technique (IFAT) on tissue culture and inoculated mouse brain, while immunoperoxidase technique was done on bovine lung. Haematological examination revealed leucocytosis accompanied with neutrophilia (immature form) and lymphopenia. Histopathological examination clarified the most common lesion of field cases in lung in the form of pulmonary emphysema and alveolar collapse as well as interstitial pneumonia and proliferation of the cells lining the bronchioles. Severe edema and infiltration of mononuclearinflammatory cells with proliferative fibrous connective tissues were seen in the heart. Liver showed severe edema, fibrous connective tissues proliferation, various sized organized thrombus with severe infiltration by mononuclear inflammatory cells. Depletion of the white pulp of the spleen as well as edema in the lymph node. The brain of inculcated mice showed intracytoplasmic inclusion body. Electron microscopy (EM) were applied on brain of infected mice and showed intracytoplasmic small number of bullet shape particles. Virological and pathological results were discussed

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INTRODUCTION

Bovine ephemeral fever (BEF or Bovine Epizootic Fever) is a non contagious arthropod-borne disease of cattle and water buffaloes. The BEF virus is a single-stranded RNA, ether-sensitive rhabdovirus with five structural proteins. It characterized by an acute fever of short duration, with high morbidity and low mortality. Mortality varies from 1-2 percent on average. Morbidity is partly influenced by the number of susceptible cattle in the herd and partly by the intensity of the epidemic ***(St. George et al., 1993 and*** **Nandi and Negi, 1999*).***

Clinical disease has been observed only in cattle and water buffaloes, however, neutralizing antibodies to BEF virus have been found in Cape buffalo, and species of deer and antelope in Africa and deer in Australia (***Davies et al. 1975)***

Ephemeral fever was first described in South Africa in 1895, though the disease was known to have occurred previously and was referred to briefly by ***Buxton and Fraser (1977)***. The disease is now known to exist in a broad belt of tropical, subtropical, and temperate countries in Africa ***(Curasson, 1936)***, Asia ***(Burgess, 1971),*** and Australia ***(Loses, 1986)*** and to be the same disease as bovine epizootic fever of Japan ***St. George, 1994).*** It was clearly recognized in Egypt in 1895, 1924, 1991 and finally at 2002 as recorded by ***Piot (1896); Rabagliati (1924); Hassan et al.(1991)*** and ***Nawal et al.(2001 and 2002);*** respectively.

Epizootiological evidence indicates that BEF virus is spread in nature only by an insect bite. The disease will not spread from cow to cow by close contact, droplet infection, bodily excretions, or by the transfer or injection of exudates ***(Blackburn et al., 1985).***

The clinical signs are very obvious and can be quite severe . The fever of ephemeral fever is generally biphasic, sometimes triphasic, with peaks of 40-41.5° C spaced 12-18 hours apart (***Uren et al., 1992).*** Increase in respiratory rate, anorexia and some degree of lameness ***(Naqano et al., 1990)***

Sudden sharp reduction of milk production of lactating cattle were recorded ***(Farag et al., 1998; Kirkland, 2002 and Walker, 2005).*** Death can occur suddenly in the febrile or in the recovery phase. Paralysis of the limbs may persist

for days, weeks, or permanently. A temporary infertility may occur in bulls that show structural defects in spermatozoa persisting for up to 6 months, abortions do occur if the cow suffers ephemeral fever in the eighth or ninth month of pregnancy *(Bayer, 1998).*

Recovery begins 1-2 days after the overt clinical signs are first noticed and is usually complete and without sequelae in a further 1- 2 days after the overt clinical signs are first noticed. Lactating cows, bulls in good condition, and fat steers are the worst affected, and their recovery may take up to a week even without complications *(Uren et al., 1992).*

This work is amid to recorded a recent endemic infection of BEF in Egypt, investigate the most available and rapid diagnostic test for detection of BEF virus (immunofluorescence antibody technique (IFT) and immunoperoxidase test) as well as correlation with haematologiocal, histopathological and ultrastructural studies.

MATERIALS AND METHODS

A. Materials:

1. Samples:

a. Blood samples:

Thirty-three blood samples were collected from four Egyptian governorates Damietta, Menofia, Fayoum and sharkia. Samples were collected in summer 2006 from dairy cattle showing signs suspected bovine ephemeral fever with elevated body temperature (40-41 °C). Blood samples were collected in 2 separated vials, contained anticoagulant (EDTA) used for haematological examination and virological studies.

b. Tissues samples:

Tissues samples from the heart, lungs, liver, spleen and lymph nodes were obtained and kept in neutral 10% buffered formalin for pathological studies.

tissue specimens from brain of mice at the 2nd day post inculcation was preserved in formalin with glutaraldehyde at 1:1 to be processed for electron microscopy examination.

Specimens from brain mice at the 2nd day post inoculation was immediately immersed in liquid nitrogen and stored at –

20 for IFA and indirect immunoperoxidase test.

2. Antisera:-

Reference antiserum against BEF was kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.

3. Conjugate:-

Anti bovine immunoglobulin conjugated with fluorescien Isothiocyanate stain supplied by ICN Biochemicals, Inc. Chemical Credential Costa Mesa, California, USA.

4. Tissue culture:

Vero and BHK_{21} cell line were used for virus isolation and identification.

B. Methods:

1. Blood samples preparation:

Buffy coats separated from the blood samples, which was collected on EDTA according to ***Davis and Walker, (1974)***. Smears were made from Buffy coats for antigen detection.

2. Virus Isolation and Identification:-

Baby mice inoculation:

Buffy coats diluted 1:10 in minimal essential medium "MEM" containing penicillin and Streptomycin were intracerebally I/C inoculated (50 µl) into suckling mice (1-3 days old).The mice were observed daily for any nervous manifestation or death . Impression smears were made from their brains for IFAT. The mice which had not shown nervous manifestations were killed and their brains were homogenized in 10 % W/V in MEM containing penicillin and Streptomycin for second and third I/C inoculation in baby mice.

2. Haematological studies:

The anticoagulated blood previously collected were examined for Hemoglobin (Hb) according to ***Crosby et al. (1954).*** Packed cell volume (PCV) was determined by the micro-haemtocrit method of ***Schalm (1986);*** the total erythrocytes (RBCs) and leucocytes (WBCs) were counted according to ***Thompson (1980).***

3. Histopathological studies:

Tissue specimens were collected on neutral 10% buffered formalin, were processed to obtain five micron thick paraffin sections, stained with haematoxylin and eosin according to ***Bancroft et al.***

(1996) and used for histopathological examination.

4. Ultrastructure studies:

The tissues which previously preserved in formalin with glutaraldehyde were processed according to ***Weakly (1981)*** for Electron microscopy. examination.

5. Indirect fluorescent antibody technique (IFAT):

Frozen sections from brain of mice as well as VERO cells and brain emulsion smears of inoculated mice were subjected to fluorescent antibody technique and examined with ultraviolet light according to ***Goldman (1968).***

6. Indirect immonoperoxidase test:

Frozen sections from brain of mice were subjected to indirect immunoperoxidase test was carried out according to ***Atulk (1988).***

7. Statistical analysis:

The obtained results were statistically analyzed according to ***Petrie and Watson (1999).***

RESULTS AND DISCUSSION

Bovine ephemeral fever is a viral disease of considerable importance to many countries including Egypt. This disease is mainly observed and spreading in summer from August to October with the nature is suitable to multiplication of the haematophagous insects (mosquitoes) that appear to be borne on wind allowing rapid spread of the disease. These evidence was supported by ***Yeruham (2002); Hsieh et al. (2005); Walker (2005); Merck (2006) and Yeruhm et al. (2007).***

The virus was isolated from 5 of Buffy coats when inoculated intracerebally I/C in suckling baby mice which showed nervous manifestations in the form paralysis of hind limbs, tremors, convulsions, abnormal gait and twisting, no deaths. 10 nasal swabs and 18 suspensions of different organs were inoculated on tissue culture cell lines for isolation and application of IFAT for antigen detection (Table, 1). After 3 blind passages the characteristic CPE appeared in the form of rounded cells, gaps formation with aggregation of cellular foci and sloughing of the cells sheet within 3-4 days post inoculation. The BEF virus was isolated and detected from Buffy coat samples where the virus affected the endothelium of small blood vessels

(Mackerras et al., 1940). The virus is contained in the leukocyte fraction of the blood during fever *(Theodoridis, 1969)* and more particularly in neutrophils *(Young and Spradbrow, 1985).*The results of attempts to isolate BEF virus from the Buffy coat inoculated intracerebally I/C. the suckling mice 1-3 day –old lead to obtained 3 isolates out of 34 of 8.82%. The results coincided with those with those reported by ***Inaba et al. (1968) and Doherly et al. (1969).***

The inspection farms of Frisian dairy cattle in different governorates were exposed to hyperthermia in the first febrile phase (40-41 °C) accompanied with depression, disinclination to move and stiffness. As the disease progresses, animals became anorexia, accelerated heart and respiratory rates, ruminal stasis, serous or mucoid nasal and ocular discharges, salivation, muscle twitching or waves of shivering, a generalized stiffness or a shifting lameness. Paralysis lead to sternal recumbency (Fig. 1) for hours or days with the head turned to the flank, or in lateral recumbency with or without loss of most reflexes. Emphysematous edematous swelling was observed in submandibular, shoulder, neck and back region. These clinical signs appeared is due to the vascular inflammatory response of the viral infection *(Naqano et al., 1990; St. George, 1994 and Walker, 2005).* The early reversible lameness or paralysis is return to the event that the total serum calcium level fell from 2.55 mmol/l to 1.8 mmol/l during the febrile phases of the disease as previously recorded by ***(St. George et al., 1984 and Uren and Murphy, 1985).*** A sudden sharp drop of the milk production which may reach to 50% in some farms was also recorded which may be attributed to the subclinical mastitis developed in the febrile stage of the disease ***(Losos, 1986 ; Hassan et al., 1991 and Nandi and Negi, 1999).***

These clinical symptoms were showed among cattle without limitation to age and sex. The fattened and high producer animals showed more severe clinical manifestation and sometimes sudden death occur. Theses findings were in parallel with ***Hassan et al. (1991)*** and disagree with ***Yeruham et al. (2002)*** who reported that the animal affected by BEF were more than 3 months old age and ***Kirkland (2002)*** who

stated that BEF virus affecting mainly the mature animals..

The haematological responses that associated to BEF infection (Table 1) was an increase in the total number of leucocytic count accompanied with neutrophilia, increased number of immature neutrophils, elevation in the number of monocytes and a concurrent decline in the number of lymphocytes. There were no detectable changes in packed cell volume, red cell count and haemoglobin concentration (Table 2). Theses results coincided with those reported by ***Young and Spradbrow (1990a)*** as well as ***Hassan et al. (1991)*** who observed neutrophilia and lymphopenia in cattle infected with BEF at lower Egypt in 1991; ***Nawal et al. (2002)*** at upper Egypt in 2001 and ***Merck (2006).*** These haematological variations could be probably correlated to the viraemic stage of the disease ***(Hassan et al, 1991).***

The most conspicuous gross lesions observed were characterized by effusion of serofibrinous fluids into the pericardium, thorasic and abdominal cavities. Edematous lymph nodes and pulmonary emphysema. These lesions were also described by ***Young and Spradbrow (1990a) and Nawal et al. (2002).***

The histopathological examination of the field cases revealed severe edema dispersed the muscular bundles of the heart as well as infiltration of fibrous connective tissues which interlacing by mononuclear inflammatory cells accompanied with congeed blood vessels (Fig. 2). These results were coincided with ***Theodoridis and Coetzer (1979); Nawal et al. (2002) and Merck (2006).***

The most intensive histopathological lesions appeared in the lungs which represented by thickening in the pleural wall by serofibrinous edema as well as infiltration of mononuclear inflammatory cells (Fig. 3). Severe emphysema with areas of atlectasis (Fig. 4). Interstitial pneumonia, proliferation of the cells lining the bronchioles. Infiltration of mononuclear inflammatory cells and proliferation of fibrous connective tissues (Fig. 5). Thickening of the wall of the blood vessels and thrombus formation. Similar findings were observed by ***Young and Spradbrow (1990a); Nawal (2002) and Merck (2006).***

The microscopical examination of the liver revealed

sever formation of different sized of organized and lamelated thrombus interlacing by inflammatory cells (Fig. 6). Severe haemorrhage, edema, proliferation of fibrous connective tissue and infiltration of diffuse and focal mononuclear inflammatory cells (Fig. 7). Necrosis of some hepatocytes was observed. These results were coincided with ***St. Gerorge (2000)*** and partially agreement with ***Nawal et al. (2002)*** who didn't observed the thrombus formation in the liver.

The spleen showed depletion of the cells of the white pulp as well as swelling and proliferation of the endothelial cells lining the congested blood vessels which surrounded by proliferated fibrous connective tissues (Fig. 8). These observation was also seen by ***Burgess and Spradbrow (1977) and Nawal et al. (2002).***

The microscopical examination of the lymph node revealed edema in the capsular wall and the trapeculea which infiltrated by mononuclear inflammatory cells (Fig. 9). Similar results were recorded by ***Burgess and Spradbrow (1977) and Merck (2006).***

The histopathological lesions observed in the examined organs indicated that the BEF virus has a direct effect on the wall and the endothelial cells lining the blood vessels as well as their permeability which represented by the swelling and proliferation of the endothelial cells as well as the formation of different sized of organized thrombus. This concept was demonstrated by ***Young and Spradbrow (1990b)*** who recorded an increase in the vascular permeability in cattle infected with BEF virus and confirmed by ***St. George (2000)*** who studies the effect of ephemeral fever of cattle on the appearance and contraction of blood clots. Also the appearance of the fibrous connective tissues. Among the examined organs may be attributed to the increase of the serum fibrinogen which may exceed 3-4 times than the normal levels in cattle infected by BEF virus as previously observed by ***St. George et al. (1984); Uren et al. (1985) and Uren (1989).***

The histopathological examination of the brain of the inculcated mice showed perivascular and pericellula edema as well as neurophagia and intracytoplasmic inclusion body in the astrocyte (Fig. 10 a, b & c).

These result was in agreement with ***Holmes and Doherty (1970).***

The ultrastructural examination of the brain of the inculcated mice revealed that the virus particles are bullet- shaped, 70 by 145 nm and slightly tapered toward the round end. The outer envelope is closely apposed to an electron dense shell about 12nm thick but no other internal structure is visible (Fig. 11). These structure as previously described by ***Holmes and Doherty (1970) and Naqano et al. (1990).***

The BEF virus was also detected in VERO cells and brain emulsion smears of inoculated mice as well as other organs such as lungs, spleen and lymph nodes by using indirect immunoflurscence antibody technique (IFA) which showed intracytoplasmic fluorescent granules in the infected cells with variant intensity (Fig. 12). Similar results were observed by ***Nawal et al. (2002).*** This positive results indicate that the viral growth takes place mainly in reticuloendothelial cells of the lung, spleen and lymph nodes as previously recorded by ***Burgess and Spradbrow (1977).***

The immunoperoxidase techniques was applied on different organs, the positive results revealed brown granules in the infected tissues with different intensity (Fig. 13), as previously discovered by ***Nawal et al. (2002).***

Although BEF isn't a contagious disease and cause low mortality rates, it cause high morbidity rates may reach to 85% of animals of one herd and lead to enormous economic losses such as sudden reduction in the milk production, disruption of national and international trade and finally a variety of complication resulting from possible secondary infection.

So, it must be, firstly, irradiate the insect which is the most responsible for disease transmission and spreading, housed the diseased animals in an insect-proof area. The treatment of diseased animals is depend on a long acting anti-inflammatory drugs, redehydration with isotonic fluid, intravenous administration of calcium must be considered with a broad spectrum antibiotic to avoid any secondary infection.

Finally, the advice concerning farmers that they must vaccinated their cattle annually to prevent BEF outbreak in the future but should be used only in the endemic area.

Table (1) Virus isolation on VERO & BHK_{21} cell lines and inoculation in baby mice and isolates identification by IFAT.

Governo-rates	Number of samples	Type of samples	Isolation		IFAT	
					Cell culture	Baby mice
			Cell culture	Baby mice	+Ve	+Ve
Damietta	5	Buffy coat	-	2	-	2
Menofia	4	* Pooled organs	3	-	3	-
Fayoum	8	Liver, lung	2	-	2	-
	10	Nasal swabs	-	-	-	-
Sharkia	6	Lymph node	3	-	3	-
Total	33	-	8	2	8	2

* pooled organs (liver, spleen and lung).

Table (2): Leucogram of cattle infected with BEF in different governorates (Means ±S.E.).

Governorate	Group	WBCs (x $10^3/\mu$)	Neutro. (x $10^3/\mu$)	Staph (x $10^3/\mu$)	Eosino. (x $10^3/\mu$)	Lympho. (x $10^3/\mu$)	Mono. (x $10^3/\mu$)
Damietta	Infected	8.36 ±0.22*	3.97 ±0.06*	0.14 ±0.006	0.17 ±0.003	3.73 ±0.06*	0.33 0.008*
	Apparent healthy	7.04 ±0.10	2.31 ±0.04	0.13 ±0.002	0.18 ±0.001	4.16 ±0.04	0.23 ±0.004
Menofia	Infected	8.09 ±0.22*	3.98 ±0.05*	0.26 ±0.005	0.18 ±0.004	3.53 ±0.06*	0.32 ±0.006*
	Apparent healthy	7.02 ±0.11	2.31 ±0.03	0.14 ±0.002	0.18 ±0.003	4.13 ±0.04	0.23 ±0.002
Fayoum	Infected	8.55 ±0.23*	3.97 ±0.04*	0.16 ±0.006	0.18 ±0.004	3.89 ±0.07*	0.33 ±0.005*
	Apparent healthy	7.04 ±0.10	2.30 ±0.02	0.14 ±0.002	0.18 ±0.003	4.23 ±0.04	023 ±0.001
Sharkia	Infected	8.25 ±0.21	4.02 ±0.14*	0.16 ±0.006	0.19 ±0.004	3.54 ±0.06*	0.31 ±0.007*
	Apparent healthy	7.12 ±0.12	2.35 ±0.04	0.14 ±0.002	0.18 ±0.003	4.19 ±0.04	0.23 ±0.003

* Significant at $P < 0.001$.

Table (3): Erythrogram of cattle infected with BEF in different governorates (Means ±S.E.).

Governo -rate	Group	RBCs (x 10^3/µl)	Hb (gm/dl)	PCV (%)	MCV (Fl)	MCH (Pg)	MCHC (%)
Damietta	Infected	6.41 ±0.36	10.4 ±0.09	38.0 ±0.30	59.54 ±2.27	16.17 ±0.55	27.23 ±0.71
	Apparen t healthy	6.25 ±0.31	10.2 ±0.06	38.1 ±0.21	60.96 ±0.35	16.32 ±0.30	26.76 ±0.43
Menofia	Infected	6.22 ±0.24	10.3 ±0.36	38.3 ±1.17	62.01 ±2.49	16.80 ±0.58	27.13 ±0.61
	Apparen t healthy	6.26 ±0.21	10.3 ±0.05	38.5 ±0.31	61.50 ±1.36	16.16 ±0.31	27.2 ±0.45
Fayoum	Infected	6.41 ±0.32	10.31 ±0.07	38.21 ±0.11	59.54 ±2.38	16.24 ±0.54	27.25 ±0.81
	Apparen t healthy	6.455 ±0.23	10.15 ±0.09	38.42 ±0.21	59.61 ±1.32	15.73 ±0.19	26.38 ±0.41
Sharkia	Infected	6.15 ±0.37	10.92 ±0.010	39.2 ±0.31	63.12 ±2.51	17.25 ±0.50	27.81 ±0.44
	Apparen t healthy	6.19 ±0.31	11.08 ±0.09	39.2 ±0.22	63.15 ±1.41	17.88 ±0.21	28.33 ±0.48

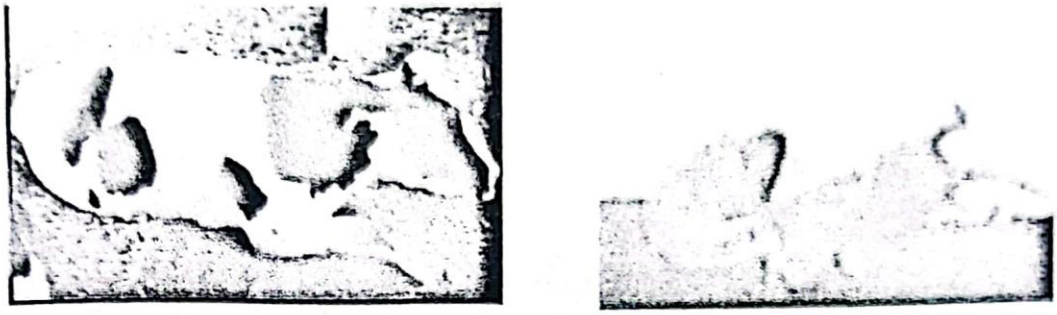


Fig. (1): Cow showing paralysis lead to sternal recumbency and nervous manifestation on baby mice (tremor, convulsion and paralysis).

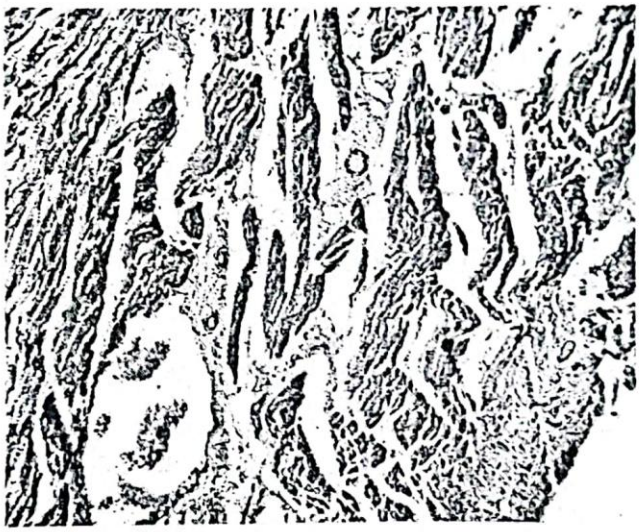


Fig. (2): Heart of cow infected by BEF showing severe edema dispersed the muscular bundles as well as infiltration of fibrous connective tissues which interlacing by mononuclear inflammatory cells accompanied with congeed blood vessels. (H & E, x 200).

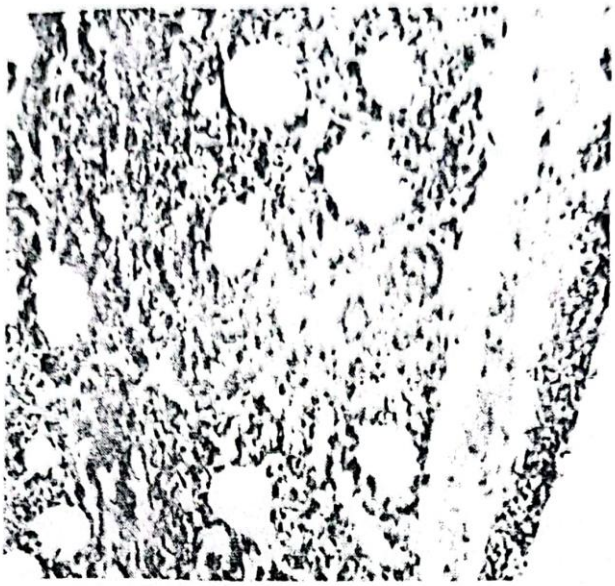


Fig. (3): Lung of cow infected by BEF showing thickening in the pleural wall by serofibrinous edema as well as infiltration of mononuclear inflammatory cells. (H & E, x 200).

Fig. (4): Lung of cow infected by BEF showing Severe emphysema with areas of atlectasis . (H & E, x 100).

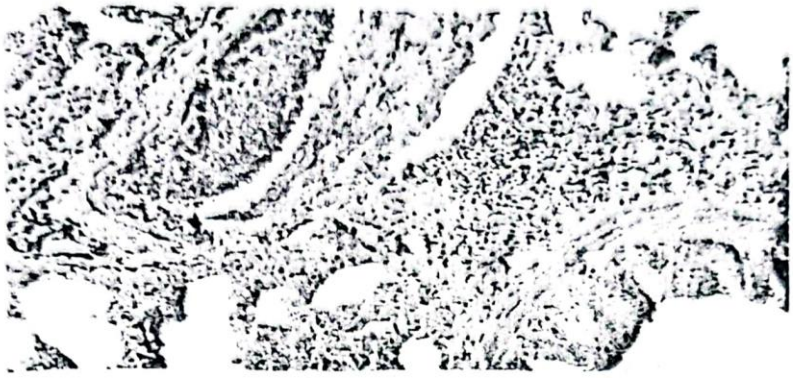


Fig. (5): Lung of cow infected by BEF showing Interstitial pneumonia, proliferation of the cells lining the bronchioles. Infiltration of mononuclear inflammatory cells and proliferation of fibrous connective tissues. (H & E, x 200).

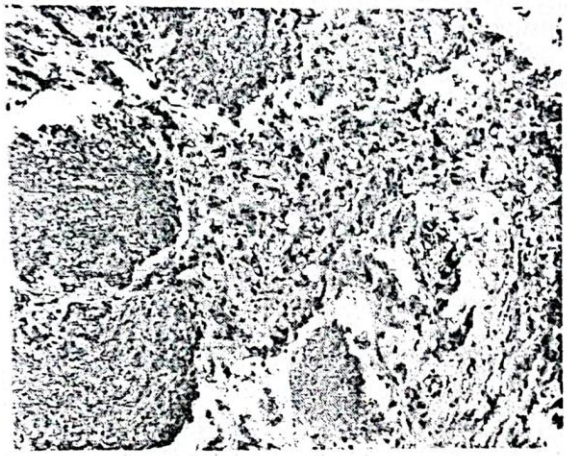


Fig. (6): Liver of cow infected by BEF showing sever formation of different sized of organized and lamelated thrombus interlacing by inflammatory cells. (H & E, x 200).

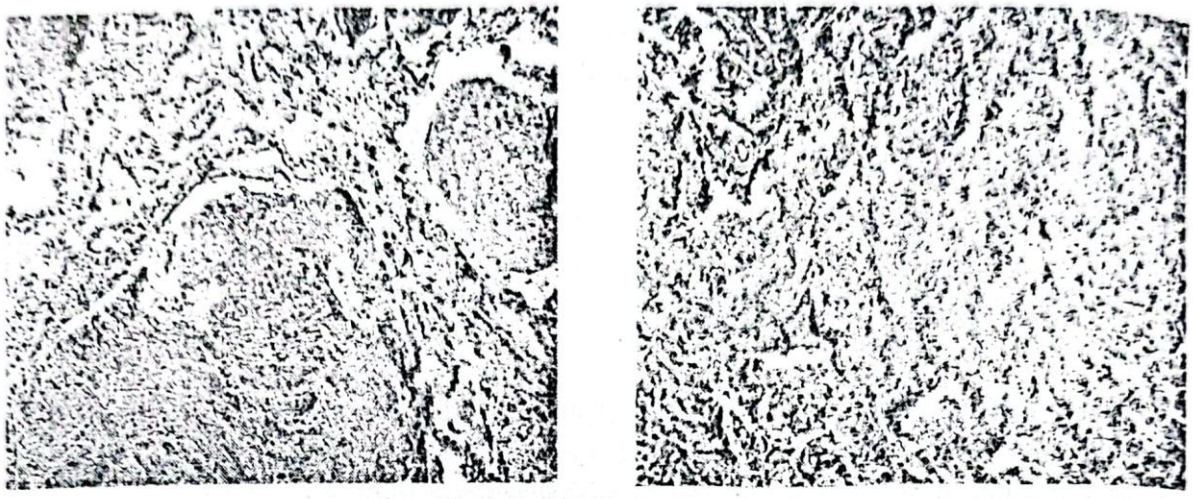


Fig. (7): Liver of cow infected by BEF showing Severe haemorrhage, edema, proliferation of fibrous connective tissue and infiltration of diffuse and focal mononuclear inflammatory cells (H & E, x 100).

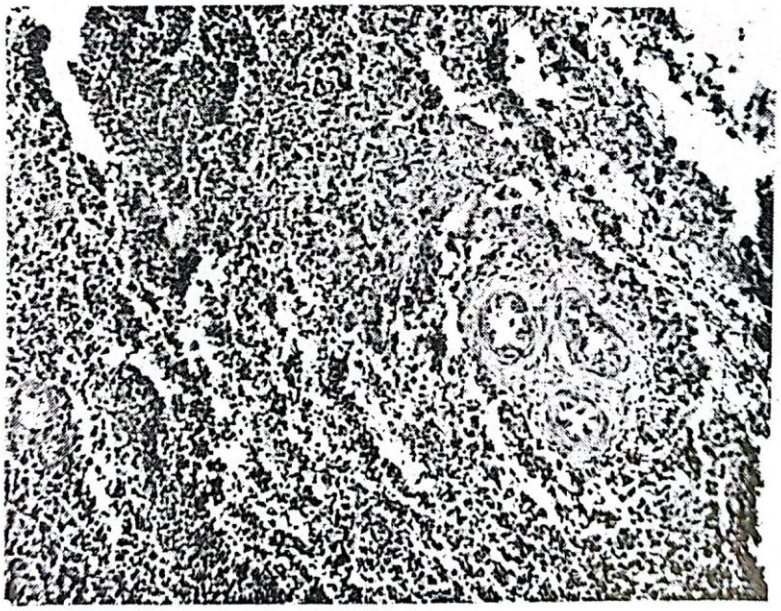


Fig. (8): Spleen of cow infected by BEF showing depletion of the cells of the white pulp as well as swelling and proliferation of the endothelial cells lining the congested blood vessels which surrounded by proliferated fibrous connective tissues. (H & E, x 200).

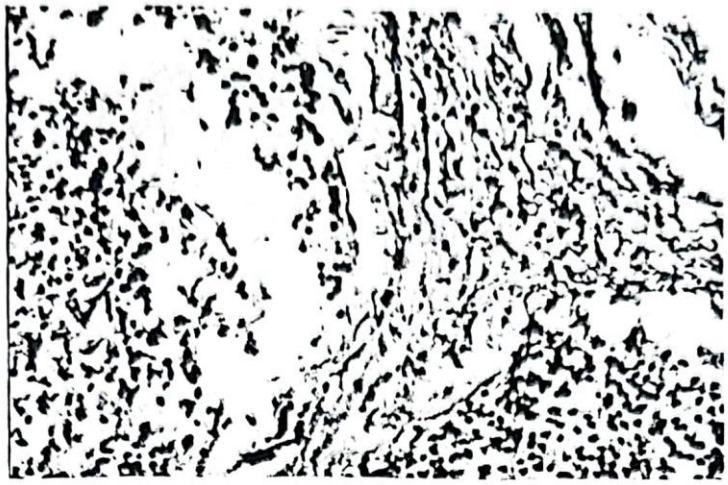
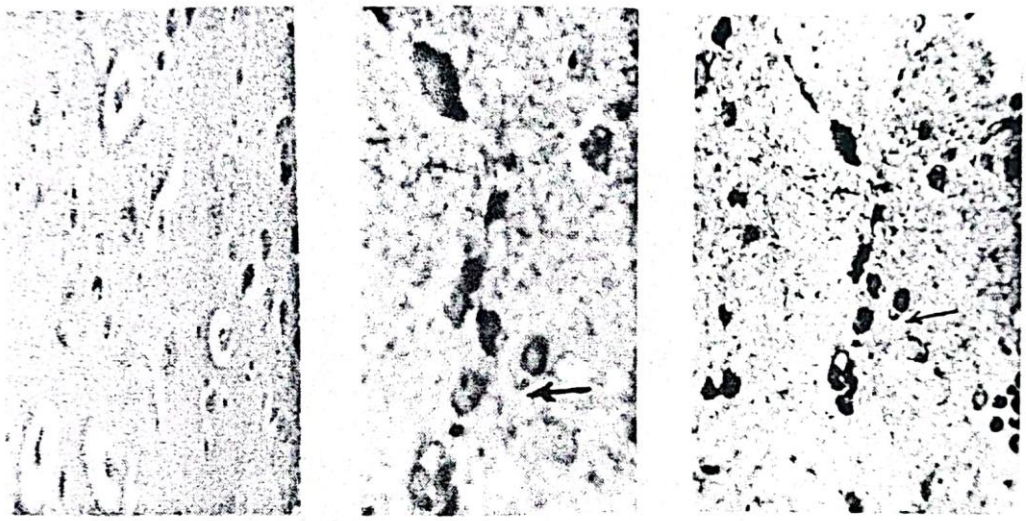


Fig. (9): Lymph node of cow infected by BEF showing edema in the capsular wall and the trapeculea which infiltrated by mononuclear inflammatory cells. (H & E, x 400).



(a) (b) (c)

Fig. (10): Brain of the inculcated mice by BEF virus showing perivascular and pericellula edema as well as neurophagia and intracytoplasmic inclusion body in the astrocyte (H & E, (a) x 400, (b) x 400 & (c) 650).

Fig. (11): Ulterastructure of brain of inculcated mice showing the virus particles in a bullet- shaped appearance. (x 30 000).

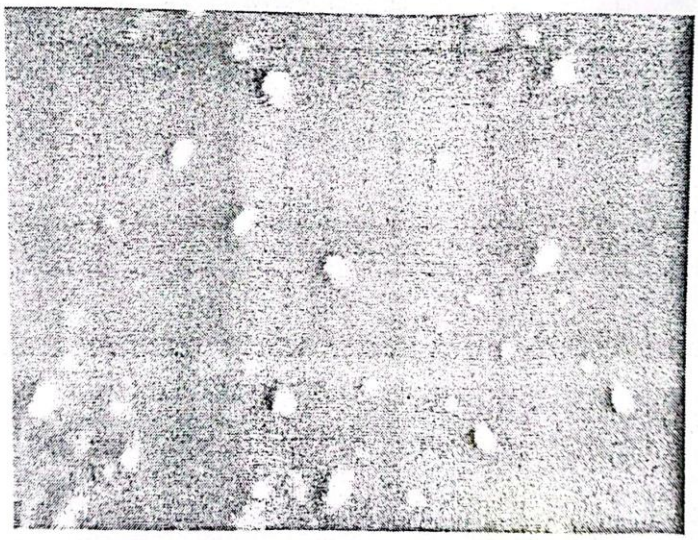


Fig. (12): Infected cells showing intracytoplasmic fluorescent granules in the infected cells with variant intensity.

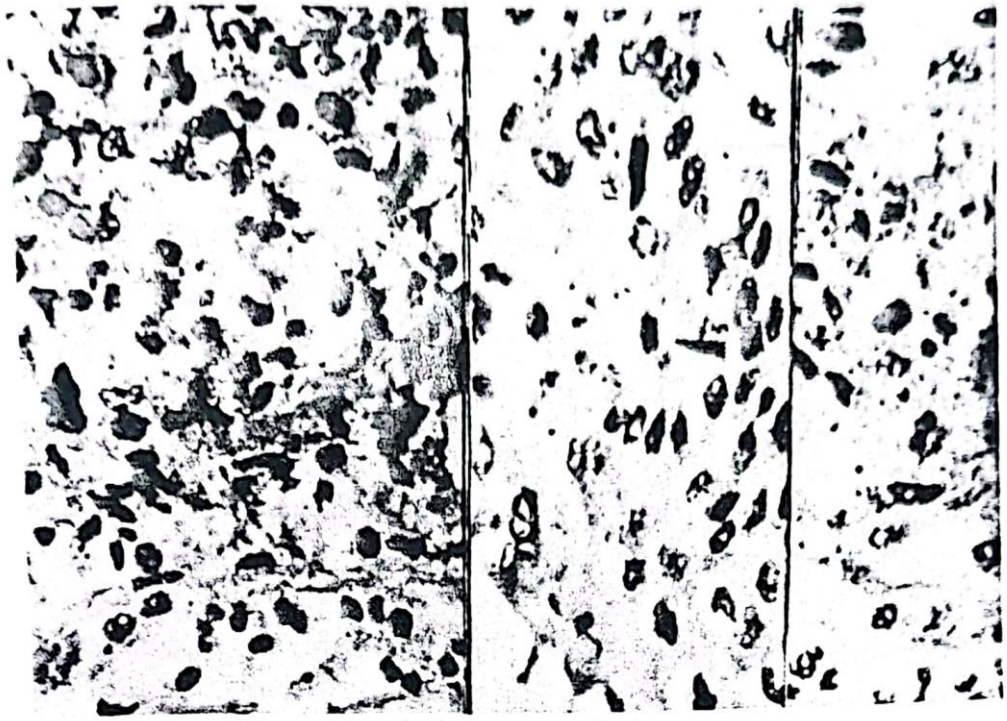


Fig. (13): Brown granules in the infected tissues with different intensity with IPS technique (x 400).

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