Histopathological and Immunohistochemical Studies on Bovine Ephemeral Fever in Cattle

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ABSTRACT

Bovine ephemeral fever (BEF) is an arthropod-borne viral disease of cattle caused by a single stranded RNA virus that belongs to the rhabdovirus group. The outbreak was diagnosed as BEF on the bases of clinical signs and pathological lesions. In the present study, out of two hundred and fifty cows, fourteen cows are died from BEF by mortality ratio 5.6%. The diseased cows showed viraemia, inappetance, depression, salivation, lacrimation, nasal discharge, lameness, and recumbency followed by death. Formalin-fixed paraffin-embedded samples from naturally infected cows with BEFV were grossly and microscopically evaluated and tested using immunohistochemistry. The main gross findings include grayish-white streaks in the skeletal muscles, congestion and mottling of the lung surface with darkness of the lobar septa, congestion of the tracheal mucosae with the presence of mucous exudate in their lumen. The liver and kidneys were congested with edematous and enlarged lymph nodes. The microscopical examination revealed various pathological changes in different organs. Diffuse hemorrhage and pulmonary emphysema with alveolar atelectasis and catarrhal bronchiolitis were observed in the lung. The skeletal muscle showed severe hyaline degeneration and myomalacia with inter-muscular hemorrhage. Edema with marked lymphoid depletion was observed in the pre-scapular and pre-femoral lymph nodes. Necrotic changes were noticed in the renal and intestinal tissues. Diagnosis was confirmed by detecting viral antigen in the spleen, lung, muscle, kidney, heart and lymph nodes with most of the antigen appearing within macrophages and pericytes using immunohistochemistry.

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Introduction

Bovine ephemeral fever (BEF) is an infectious blood sucking arthropod born rhabdovirus disease, commonly known as three-day stiff-sickness (AL-Busaidy and Mellor, 1991). It is caused by a single stranded RNA virus that belongs to the rhabdovirus group [Bovine ephemeral fever virus (BEFV)]. BEF occurs during hot humid season, which is active period for insect vector mostly during summer and autumn (Murray, 1997). The incubation period in natural outbreaks is unknown but suspected to be as short as 36-48 hours. Meanwhile, the incubation period varies from 29 hours to 10 days and mostly averaging between 3-5 days in experimental infections (Zaghawa, 2006). However, natural infection results in sudden onset of fever with temperatures as high as 41°C, stiffness and a temporary reluctance to move, lameness, and sometimes temporary or permanent paralysis of limbs, inappetence, difficult swallowing, serous ocular and purulent nasal discharges,

salivation, followed usually by complete recovery (Uren *et al.*, 1992; Nandi and Negi, 1999). This disease is characterized by a sudden onset that can rapidly progress to severe illness with apparent recovery within a period of 3 days-which gave rise to its common name of three-day sickness (Walker *et al.*, 2005). BEF is considered as a major pathogen for cattle and water buffalo. The disease is now known to exist in a broad belt of tropical, subtropical, and temperate countries in Africa, Asia, and Australia (George, 1994). This disease appeared at East Africa in 1867, South Africa in 1908 and in Australia in 1936. BEF was clearly recognized in Egypt in 1895 and 1924, since that time the publications about the pathogenesis of BEFV in Egypt are rare. In the summer of 1991, a typical form of the disease has been recorded in different Egyptian governorates (Hassan *et al.*, 1991; Nagi *et al.*, 1992; Banoub, 1994; Hassan, 2000; Zaghawa *et al.*, 2000, Hamoda *et al.*, 2002). In summer 1991, a typical form of the disease has been recorded in different governorates in lower Egypt (Hassan *et al.*, 1991). A second outbreak of BEF occurred in summer 2000, where it included several governorates in lower and upper Egypt and characterized by 50% morbidity and 2.5% mortality, (Zaghawa *et al.*, 2000). Recently, several outbreaks of bovine ephemeral

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fever virus infections were demonstrated in seven Egyptian governorates; during summer seasons in 2006 and 2009 (Bastaweesy *et al.*, 2009).

In outbreaks of bovine ephemeral fever, the morbidity rate may be as high as 80%. Mortality is generally low and seldom exceeds 2 to 3 %. Although mortality is usually low, the disease appeared more severely in cattle that is in good condition, accordingly, high mortality rates of 30% had been reported in obese cattle (OIE, 2008).

The disease has major economic significances that attributed to mortality of stock, abortion, decreased milk production, reduced weight gain, and disruption of markets and lowered fertility of bulls and the costs of supportive care, treatment and control programs application as insect control and vaccination (Zaghawa *et al.*, 2002; Walker, 2005).

BEF diagnosis may be difficult particularly when acute and convalescent sera are not available to support recent viral exposure due to the brief viraemia (Van Der Westhuzen, 1967). Accordingly, a typical clinical history, clinical signs, post mortem and histopathological findings in association with serological and molecular identification of the viral antigen supported the diagnosis of BEF (OIE, 2008). However, there is very little information regard to the pathological changes of BEF in Egypt. Therefore, the aim of the present study was to describe the pathological lesions associated with BEF disease in different organs of naturally infected cows with BEFV in Egypt as well as detection of the distribution of the virus in different tissues using immunohistochemistry (IHC) results.

Materials and methods

Samples

A total of 250 cattle of various age and sexes showing clinical signs of the disease were admitted to the clinic of the Faculty of Veterinary Medicine Benha University, some of them from the faculty farm and others from different localities in Kaluobia governorate from May to July, 2015. Out of them, fourteen cows were died, and subjected to post mortem examination in Pathology Department at Faculty of Veterinary Medicine, Benha University. Specimens were collected from skeletal muscles, lung, lymph nodes, liver, heart, spleen, kidneys and intestine and processed for histopathological and immunohistochemical studies.

Histopathological examination

Tissue specimens from skeletal muscles, lung, lymph nodes, liver, heart, spleen, kidneys and intestine were collected for histopathological examination. The tissue specimens were fixed in 10% neutral buffered formalin. The fixed tissues were rinsed in tap water, dehydrated through graded series of alcohols, cleared in xylene and embedded in paraffin wax (Bancroft *et al.*, 1996). 5 µm thick sections were cut and stained with hematoxylin and eosin (H&E) and then the tissues were examined and evaluated by light microscopy.

Immunohistochemistry

The same tissue specimens from skeletal muscles, lung, lymph nodes, liver, heart, spleen, kidneys and intestine were stained using immunohistochemistry method for definitive demonstration of BEF virus antigen. Paraffin-embedded samples were sectioned (3µm thick), sections were mounted on positively charged glass slides (Superfrost plus; Menzel-Glaser, Braunschweig, Germany), dewaxed and rehydrated. Antigen retrieval was done by heating the slides in citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was

blocked with 1.5% H_2O_2 in methanol for 30 minutes. The sections were then incubated with 1:10 dilution of a normal goat serum (Sigma, G9023) mixed with 2% bovine serum albumin for 45 minutes at room temperature in a humidified chamber, followed by an overnight incubation with the primary BEFV antibody (1:1000). To visualize the primary antibody, the sections were extensively washed in PBS and then the sections were incubated with a 1:500 dilution of biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, USA) for 30 minutes incubation at room temperature. The standard avidin-biotin-peroxidase complex (ABC) method was used according to the manufacturer's protocol (Vector Laboratories, Burlingame, USA) to demonstrate the antigen; the peroxidase substrate solution, 3,3' diaminobenzidine; (DAB) was used as the chromogen and diluted following manufacturer instructions, then the sections were counter-stained with Harris hematoxylin. The IHC results were classified as either negative or positive, and positive sections were subclassified as 1-5 cells/section (1), 1-3 foci/section (2), 4-10 foci/section (3) or more than 10 foci/section (4), as described by Meyer *et al.* (2001).

Statistical Analysis

The data was expressed as means ± standard error. Statistical analysis was performed using SPSS program for windows (Version 16) (SPSS Inc. Chicago, IL and USA).

Results

Clinical findings

High body temperature (40.5-41°C) that lasts for 2-4 days, anorexia, depression, drooping of foamy saliva, watery lacrimation, stringy and colorless nasal discharge, labored respiration, diarrhea and muscle tremors were the most prominent clinical signs, which were observed in all cases. Generally, the muscular sign such as severe stiffness in one or more limb were more evident on second day. A posture similar to parturient paresis as head turned flank was also observed. Furthermore, lameness and/or stiff gait, and complete recumbency were also seen in severely infected cases followed by death (Fig. 1).

Post mortem findings

The pre-scapular and pre-femoral lymph nodes were edematous and severely enlarged in all examined cases. Grayish-white streaks with multiple hemorrhagic areas were seen in the skeletal muscles especially in the thigh muscle. The lungs appeared congested with the presence of grayish white patches scattered on the lung surface. Kidneys, liver, spleen and heart were congested.

Histopathological findings

Severe hyalinization of the skeletal muscles that characterized by homogenous deeply eosinophilic sarcoplasm with loss of their striation with pyknosis or absence of their nuclei (Fig. 2A) was observed in six cases (43%). Interestingly, necrosis of the muscle in combination with inter-muscular hemorrhage (Fig. 2B) was seen in most cases (71%). In other cases, (57%) myomalacia was noticed in which complete destruction of muscle fiber with replacement of the muscle fiber with fibrous connective tissue in association with mononuclear leukocytic cellular infiltrations mainly lymphocytes (Fig. 2C).

The lymph nodes showed leukocytic cellular infiltrations in trabecullae (Fig. 2D), which was noticed in most examined

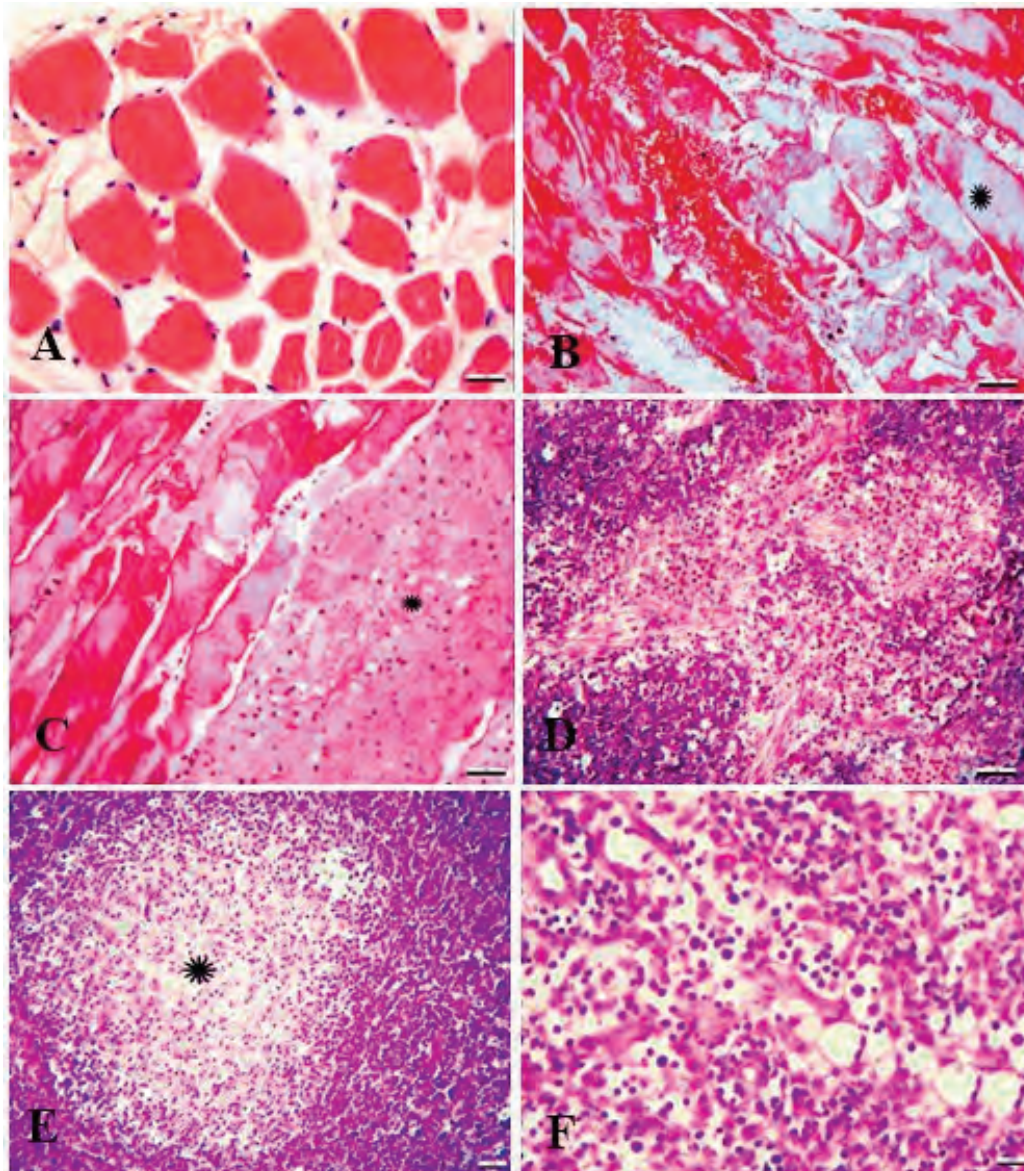Fig. 1. Cattle naturally infected with BEFV showing difficulty of standing.

Fig. 2. H&E stained sections of muscles (A-C), and lymph nodes (D-F) from naturally infected animals with TDS virus showed: A) Hyaline degeneration of muscles (Bar=100 μm). B) Inter-muscular hemorrhage with extensive necrosis of the muscle (asterisk), (Bar=100 μm). C) Extensive myomalacia of some muscle fiber (asterisk) with mononuclear leukocytic infiltrations (Bar=100 μm). D) Leukocytic infiltrations in trabeculae. (Bar=100 μm). E) Necrosis of lymphocytes of lymphoid follicles with accumulation of edematous fluid in the follicles (asterisk) (Bar=10 μm). F) Mononuclear leukocytic infiltrations mainly lymphocytes and macrophage in the medulla of lymph node (Bar=10 μm).

cases (71%). Furthermore, marked depletion of lymphocytes in lymphoid follicles with degeneration of lymphocytes in association with oedema of lymph nodes that characterized by pale eosinophilic substance in their meshes (Fig. 2E) was detected in 86% from dead cases. Moreover, hemorrhage was also seen in the cortex of lymph nodes (64%). Meanwhile, accumulation of edematous fluid in the medulla of lymph node (93%) was observed. Additionally, mononuclear leukocytic cellular infiltrations mainly lymphocytes and macrophage (Fig. 2F) was detected in the medulla of lymph node of most examined animals.

Visceral and parietal pleuritis was detected. Marked congestion of peri-bronchial and inter-alveolar capillaries with perivascular edema admixed with mononuclear leukocytic cellular infiltrations mainly lymphocytes were seen in the pulmonary tissues of most investigated cows (93%). Accidently, fibrin thrombi were demonstrated in the lumen of the congested blood vessels. Occasionally, focal areas of alveoli were consolidated with homogenous, structureless eosinophilic substances admixed with aggregates of inflammatory cells mainly macrophages and lymphocytes. Meanwhile, the inter-alveolar

septa were focally thickened by aggregates of lymphocytes. Alternative areas of emphysema and atelectasis (Fig. 3A) were noticed in 79% of studied cases. Moreover, focal areas of inter-alveolar hemorrhage were detected in 71% of cows (Fig.3B).

Additionally, bronchiolitis evidenced by distension of the bronchial lumen with desquamated epithelial cells with eosinophilic necrotic debris admixed with inflammatory cells mainly lymphocytes (Fig. 3C) were demonstrated in 93% of the dead cows. The peri-bronchiolar tissues were expanded by moderated numbers of lymphocytes and few macrophages.

The microscopical examination of the liver revealed congestion of the central, portal veins and hepatic sinusoids. Perivascular and peri-portal mononuclear leukocytic cellular infiltrations mainly lymphocytes and few macrophages were detected in 71% of the examined cases (Fig. 3D). Cholangitis that characterized by desquamation of the lining epithelium of bile duct with distension of its lumen by eosinophilic necrotic debris was observed in 64% of dead cows in association with periductal fibrous connective tissue proliferation was also demonstrated (Fig. 3E). Furthermore, marked diffuse
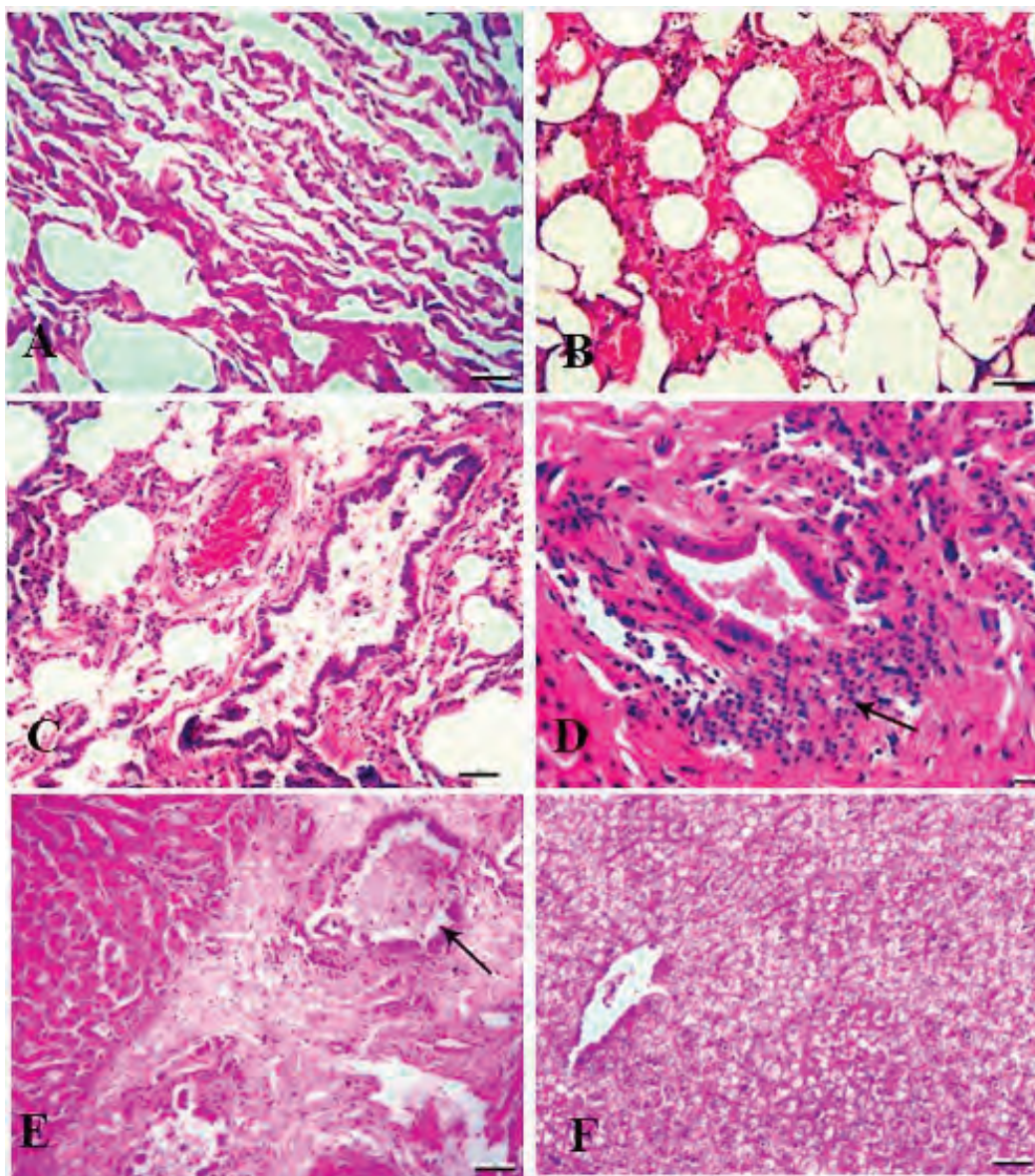

Fig. 3. H&E stained sections of lung (A-C), and liver (D-F) from naturally infected animals with TDS virus showed: A) Alternative areas of atelectasis and emphysema (Bar=100 μm). B) Diffuse inter-alveolar haemorrhage (Bar=100 μm). C) Bronchiolitis with degeneration of peribronchial blood vessels wall (Bar=100 μm). D) Mild hyperplasia of the biliary epithelium with distention of the ductal lumen with eosinophilic debris. Notice also, periductal fibrosis with mononuclear leukocytic infiltrations (arrow, Bar=10 μm). E) Cholangitis (arrow) with marked periductal fibrous connective tissue proliferation (Bar=100 μm). F) Degenerative changes of hepatocytes mainly hydropic degeneration characterized by swollen, pale, vacuolated cytoplasm with pyknosis (Bar=100 μm).

hydropic degeneration of the hepatocytes characterized by swollen, pale, vacuolated cytoplasm with pyknotic nuclei (Fig. 3F) was observed in 78% of the examined cases.

Meanwhile, marked congestion of the renal blood vessels and inter-tubular capillaries was seen in most cases (85.7%). Additionally, vascular changes in the renal blood vessels including endothelial hyperplasia with perivascular leukocytic cellular infiltrations mainly lymphocytes and macrophage (Fig. 4A) was observed in some examined cows (50%). Multifocally, marked vacuolar and hydropic degeneration of the lining epithelium of renal convoluted tubules was demonstrated in 36% of examined cases. Necrosis of the tubular epithelium was characterized by more eosinophilic cytoplasm with shrunken, pyknotic nuclei and loss of cellular details was demonstrated in 64% of examined cows (Fig. 4B). Cellular and hyalinized eosinophilic casts in the lumen of most renal tubules was seen. Moreover, the renal tubules in both cortex and medulla of most cases exhibited cystic dilatation and lined by attenuated epithelium (Fig. 4C). Multifocally, peri-glomerular cellular aggregates mainly lymphocytes, macrophages and few neutrophils. Moreover, necrosis of endothelial cells of glomerular tuft was observed with shrinkage of glomerular tufts and widening of the Bowman's space with thickening of the Bowman's capsule (Fig. 4D) were noticed in 86% of the examined cows. Accidently, hypersegmentation of the tuft was also detected in other cases (50%).

The examined intestine showed coagulative necrosis of the superficial layer of the intestinal villi was seen in 64% of the investigated cows with distension of the intestinal lumen with desquamated enterocytes, necrosed eosinophilic debris and inflammatory cells mainly neutrophils, lymphocytes and macrophages (Fig. 4E). Rarely, necrosis of the intestinal glands with by inflammatory cells infiltration was seen in few cases. Moderate numbers of inflammatory cells mainly lymphocytes and few macrophages was observed in the lamina propria with hyalinization of submucosal muscles (Fig. 4F).

The histopathological examination of the heart revealed severe hemorrhage in between the cardiac muscles with moderate leukocytic celluar infiltrations mainly lymphocytes, macrophages and few neutrophils was noticed in some cases (50%). Furthermore, hyaline degeneration of the cardiac muscles were also detected in some cases (50%).
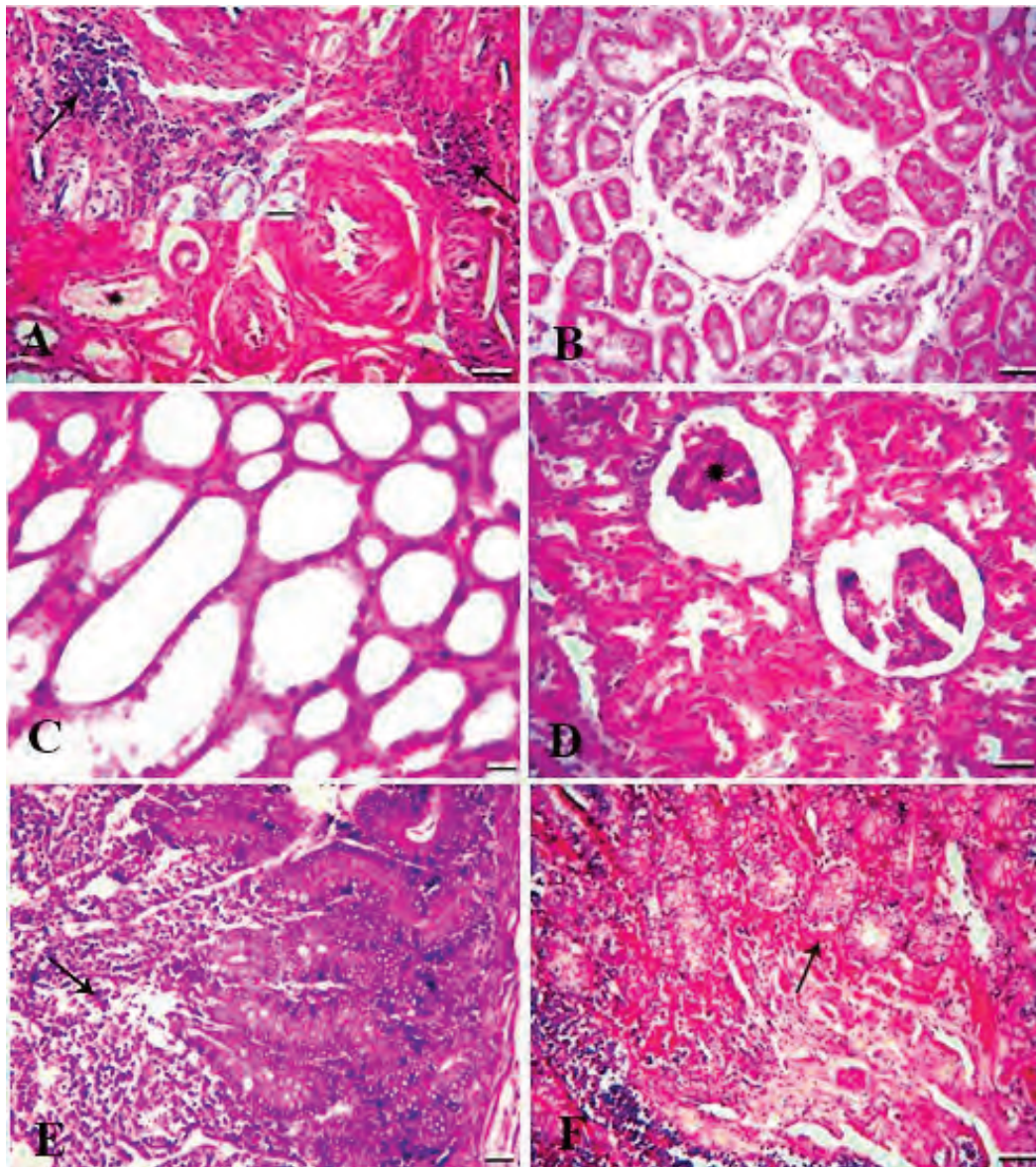

Fig. 4. H&E stained sections of kidney (A-D), and intestine (E-F) from naturally infected animals with TDS virus showed: A) Perivascular mononuclear leukocytic infiltrations (arrow) with eosinophilic hyaline casts in the lumen of some renal tubules (asterisk), (Bar=100 μm, inset Bar =10 μm). B) Coagulative necrosis of the epithelial lining of renal tubules with pyknotic nucleus (Bar=100 μm). C) Cystic dilatation of renal tubules (Bar=10 μm). D) Coagulative necrosis of the endothelial lining of glomerular tuft (asterisk) with widening of Bowman's space (Bar=100 μm). E) Desquamation of the lining epithelium of intestinal villi with distension of the lumen with desquamated enterocytes admixed with mononuclear leukocytic infiltrations (arrow, Bar=10 μm). F) Hyalinization of the muscular layer in submucosa (arrow, Bar=100 μm).

Necrosis of the pancreatic acini and pancreatic islets (Fig. 5A) was seen in the pancreas of some examined cows (71%). Additionally, hyalinization of pancreatic islets (Fig. 5B) was demonstrated in some samples (50%). Leukocytic cellular infiltrations mainly lymphocytes and neutrophils was detected in the pancreatic duct of some cows (43%).

The spleen showed subcapsular hemorrhage with congestion of splenic blood vessels. Marked lymphoid depletion of the whit pulp and periarteriolar sheeths that characterized by low cellular density of the small lymphoid cells population (Fig. 5C) was observed in most cases (86%). The red pulp showed marked congestion of sinusoids with multifocal areas of hemorrhage as well as mononuclear leukocytic cellular infiltrations. Moreover, perivascular hemorrhage in the splenic trabecullae (Fig. 5D) was noticed in 50% of the examined samples.

Immunohistochemistry findings

Positive immunohistochemical staining signals of BEFV was demonstrated in different organs (Fig. 6). Post using of polyclonal antibody, the viral antigen was noticed in high percentage of lymph nodes as positive reaction was detected in 98.8% of the examined lymph nodes. Moreover, positive signals were observed in spleen (85.7%), pulmonary blood vessels, alveolar wall, bronchial lumen as well as in interstitial pulmonary tissues (83.3%). Additionally, viral antigen was also seen inside the tubular epithelium, glomerular tuft and in the interstitial renal tissues (83.3%), in muscle fiber (64.3%), cardiac muscles (35.7%) and in pancreas and liver (21.4%). On the other side, intestine did not show any positive staining in all examined samples. Generally, quantitative analysis of BEFV antigen positive immunolabelling was summarized in Table (1).

Table 1. Quantitative analysis of BEFV antigen positive immunolabelling

Organ	Score of BEFV antigen positive IHC reaction
lymph nodes	3.08±65
spleen	2.58±45
lung	2.3±63
kidney	2.1±25
heart	1.4±42
skeletal muscle	1.44±35
pancreas	1
liver	1.2±25
intestine	0

Data were expressed as mean±SE

Discussion

Bovine Ephemeral fever (BEF) is an inflammatory disease affecting mainly cattle and buffaloes. The maximum prevalence of BEF occurs during hot humid season, which is active period for insect vector that resulting in high rates of virus transmission. This disease has major economic effect through marked reduction in milk production in dairy herds, infertility, loss of condition in beef cattle, disruption of markets and costs of control programs application as insect control and vaccination. These economic losses could be attributed to the wider distribution of the disease than that recorded due to the relatively mild symptoms and good recovery rate in many cases. Additionally, BEF for the same reasons may also be misdiagnosed or the actual diagnosis may not be confirmed by practitioners. Besides that, point, persistence of the BEF virus beyond the acute phase of the disease and the potential viral replication sites in peripheral tissues have not been established. Therefore, the current study was conducted to investigate the pathological alterations associated with this disease
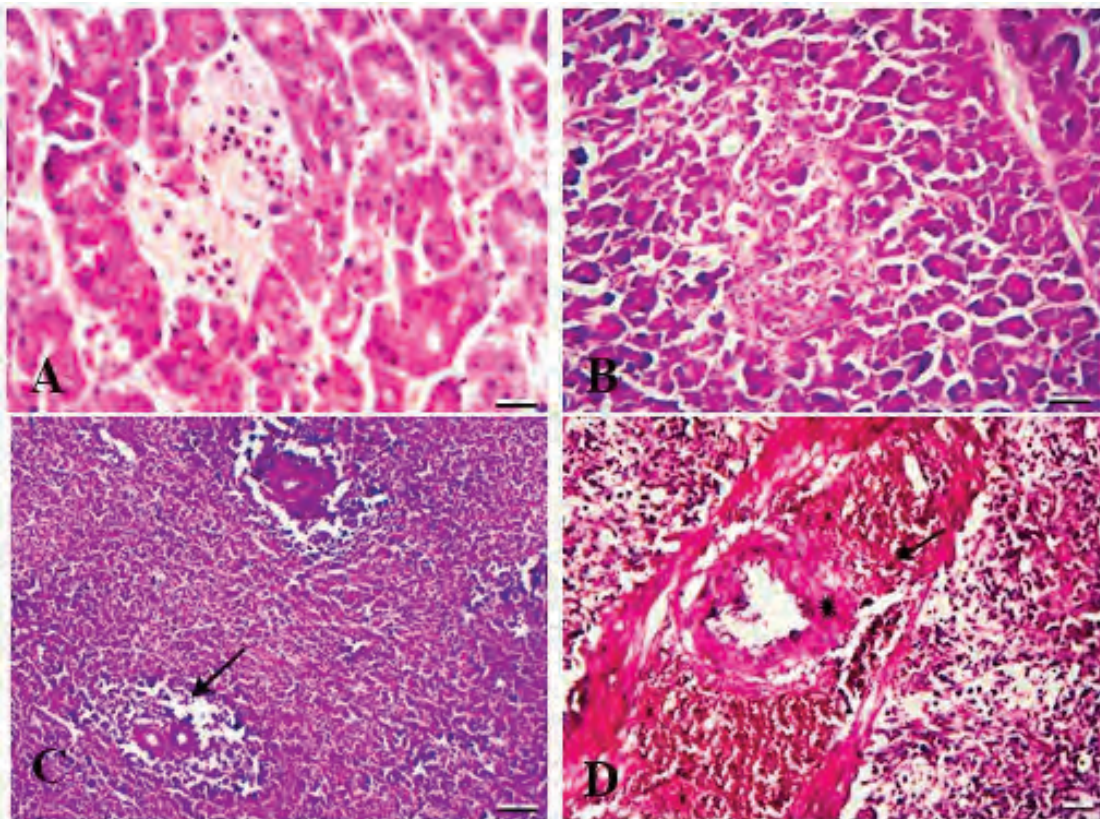

Fig. 5. H&E stained sections of pancreas (A-B), and spleen (C-D) from naturally infected animals with TDS virus showed: A) Coagulative necrosis of pancreatic islets in association with necrosis of pancreatic acini (Bar=100 μm). B) Hyalinization of pancreatic islets (Bar=100 μm). C) Lymphoid depletion of lymphocytes of lymphoid follicles characterized by reduced cellular density of mature small lymphocyte (arrow) (Bar=100 μm). D) Perivascular haemorrhage in splenic trabeculae (arrow) with hyalinization of the blood vessels wall (asterisk) (Bar=10 μm).
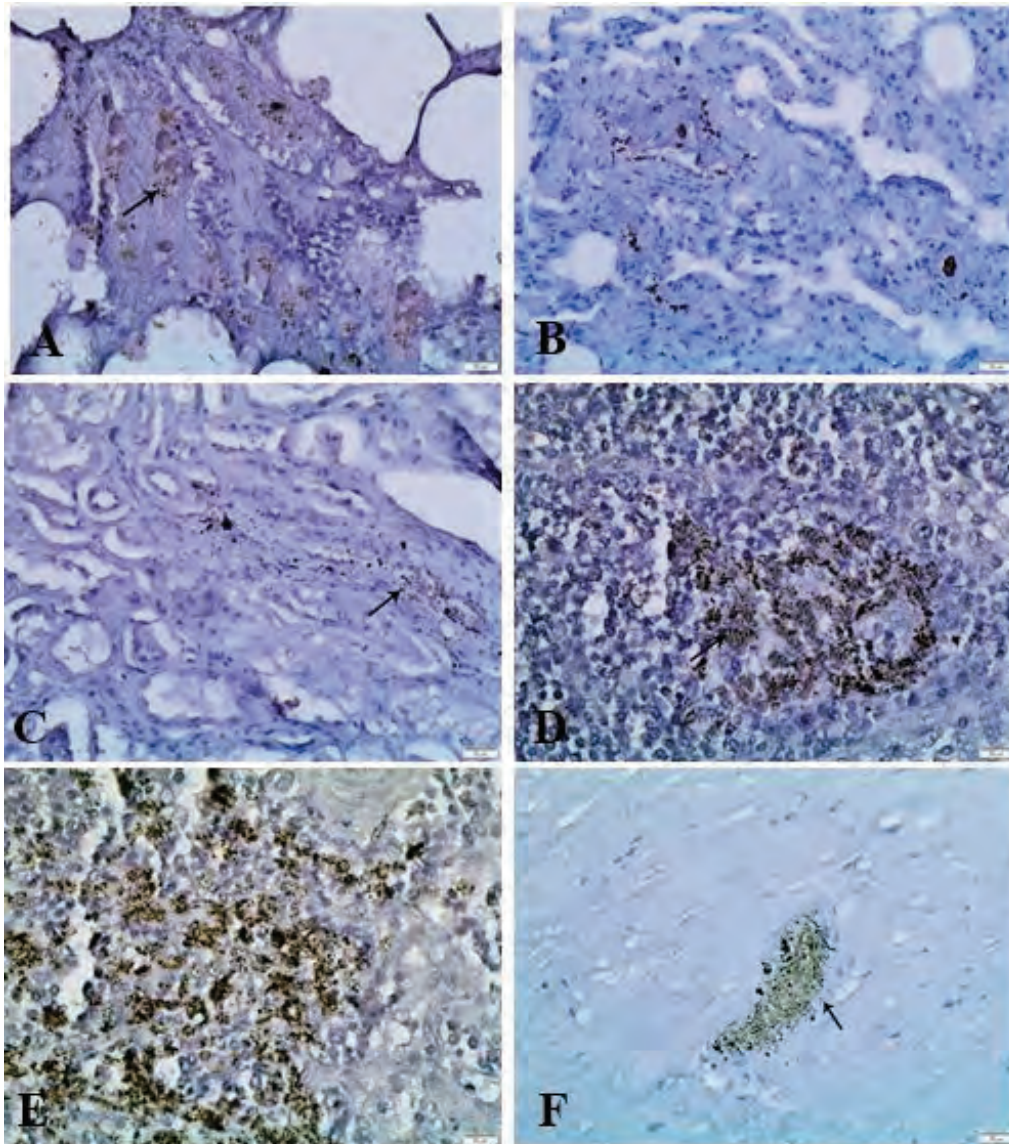
Fig. 6. Positive immunoperoxidase reaction of BEFV antigen in (A) bronchioles (arrow) and pulmonary blood vessels (asterisk). (B) interstitial tissues of lung (C) The lining epithelial cells of renal tubules and in interstitial renal tissue. (D) The lymphoid follicles of lymph node. (E) spleen, (F) heart (arrow) (Bar=20 µm).

in various internal organs with detection of virus antigen using IHC to facilitate the control programme of BEF disease.

In the present work, the mortality was higher (5.6%) than that was reported previously in several governorates in lower and upper Egypt that characterized by 2.5% mortality (Zaghawa *et al*., 2000). Although the mortality is usually low, cattle in good condition are affected more severely; mortality rates as high as 30% have been reported in obese cattle (OIE, 2008). Generally, the outbreak was diagnosed as BEF on the bases of clinical signs, pathological lesions, virus isolation (OIE, 2008).

In the current work, variable clinical signs and pathological changes were demonstrated in different investigated organs obtained from dead animals. Generally, the clinical signs and the pathological alterations associated with BEF could be related to massive interferon production that resulting in cellular damage as confirmed by the obtained results. Accordingly, the hosts response to the infection are considered as a reflection of the effects of the virus replication on the host tissues (Burgess and Spradbrow, 1977). The clinical characteristics of the disease are the expression of mediators of inflammation common to various numbers of acute febrile disease with a secondary hypocalcaemia (Georg, 1994).

However, in the present study, the main clinical signs observed in the diseased animals are labored respiration, diarrhea and muscular stiffness that was matched with Nandi and Negi (1999). It was suggested that, the muscle tremors and lameness associated with bovine ephemeral fever are related to the hypocalcaemia resulting from suppression of parathyroid hormone, which seems to be mediated by respiratory alkalosis caused by the disease (Uren and Murphy, 1985). Furthermore, hypocalcaemia may be contributed to anorexia, decreased ruminal and intestinal motility and recumbency. Moreover, extensive necrosis and myomalacia of skeletal muscles that was observed in the examined animals in the present study could also clarify the abnormal gait and recumbency of the infected animals with BEFV. However, the post-mortem examination is important to rule out other acute febrile diseases that often occur under the same conditions as ephemeral fever, such as tick fever.

Meanwhile, the labored respiration could be attributed to pulmonary edema as proved by the microscopical examination of pulmonary tissues collected from dead animals included in the present work. Furthermore, alternative areas of emphysema and atelectasis was also detected in the pulmonary tissues in association with bronchiolitis. However, bronchiolitis that characterized by occlusion of bronchioles by inflammatory exudate as well as necrosis of the bronchiolar walls causing rupture of bronchioles and alveoli, giving rise to interstitial and subcutaneous emphysema. Partial blockage of

the air passages by exudate, as well as necrosis of the bron-chiolar walls causing rupture of bronchioles and alveoli are all proposed mechanisms resulting in emphysema. Air may then disperse into the connective tissue septa and lymphatics of the lungs extending subpleurally to reach the mediastinum, spreading through the thoracic inlet to the subcutaneous tis-sue (Theodoridis and Coetzer, 1979, Young and Spradbrow, 1985). Moreover, the detected pleuritis and discharges from nasal and ocular mucosal surfaces may be related to the ability of the virus to target the endothelium of blood vessels result-ing in effusion of fibrin-rich fluid into pleural cavity, and dis-charges from nasal and ocular mucosal surfaces (Uren and Morphy, 1985).

The site of initial replication of BEFV following infection is largely unknown, but, as the virus has been observed at high titres circulating in the blood approximately one day prior to the onset of fever and associated neutrophilia, it seems that the virus is specifically present in the neutrophils (George, 1993). In the present work, the obtained results of IHC re-vealed detection of the viral antigen in the interstitial tissue, endothelial cells and alveolar macrophages of the lungs as well as in the reticulo-endothelial cells of the spleen and lymph nodes. It was detected also in other organs such as kid-ney, muscle, pancreas and heart. Interestingly, viral antigen was also observed in the lumen of blood vessels, which could clarify the systemic spreading of the virus in different organs as revealed by IHC positive reaction. These findings could help in understanding the complex pathogenesis of the virus. These findings are matched with the previous research that revealed BEF viral antigen could be detected at the time of peak clinical response in individual cells in the lungs, spleen and lymph nodes as well as neutrophils using specific fluorescence (Burgess and Spradbrow, 1977).

Conclusion

It could be concluded that, this work can provide a good knowledge about the pathogenesis of the disease that can lead to better management and proper treatment.

Acknowledgment

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