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Original Article

Pathological and clinical investigations of an outbreak of Blackleg disease due to *C. chauvoei* in cattle in Punjab, Pakistan

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Abstract

Introduction*: Clostridium chauvoei* (*C. chauvoei*) is an anaerobic, histotoxic Gram-positive, bacterium causing fatal myonecrosis in livestock with high mortalities. The disease is common in dairy animals, but little is known about the pathophysiology of the disease in exotic (non-native) animals kept under local conditions in Pakistan.

Methodology: Diagnosis of blackleg was made based on hematological and serum biochemical analysis, PCR, necropsy and histopathology.

Results: Clinically sick animals exhibited fever, lameness, subcutaneous gaseous swelling and edema particularly in hindquarter and front legs. Hematological analysis showed increases in erythrocyte sedimentation rate and reduces in number of red blood cells, packed cell volume, leukocytes and differential leukocyte count. Serum aspartate aminotransferase, lactate dehydrogenase, alkaline phosphates, alanine aminotransferase, urea, creatinine, creatine kinase, and creatinine phosphokinase were significantly ($P < 0.05$) higher in the infected animals. At necropsy, swelling areas contained straw-colored fluid with gas bubbles. The muscles were swollen, dark to black and exhibited crepitation sounds at the time of incisions with a rancid odor. Severe pulmonary edema, myocarditis along with petechial hemorrhages, as well as enlargement and congestion of liver and spleen have been observed. Microscopic examination revealed severe inflammatory reaction, edema, and disruption of the myofibrils. Examination of heart, spleen, liver, kidneys, intestine, and lungs showed congestion, severe inflammatory changes with neutrophilic infiltration and necrosis accompanied by dissociation of the normal tissue structure. PCR confirmed *C. chauvoei* in exudates and different samples of muscles.

Conclusion: The pathophysiology should be considered in diagnosis of blackleg. The disease is exist in the non-native cattle farms and biosecurity measures have to be elevated.

Key words: cattle; clostridial myositis; clinical signs; hematology; pathology.

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Introduction

Blackleg (Clostridial myositis) is an acute, rapidly progressive, non-contagious infection caused by *C. chauvoei*. The disease is commonly known as gas gangrene, Black-quarter, gangrenous myositis and/or Clostridial myositis of ruminants [1]. The causative agent, *C. chauvoei* is a Gram-positive, anaerobic and spore-forming bacterium that survives in feces, soil and surface water [2]. The pathogen enters into the host by ingestion of contaminated materials. Eventually, *C. chauvoei* enters the blood, localized and proliferate in muscles and causes myositis. Clinically, infected animals exhibit different signs of disease including fever, lameness, crepitation sounds on palpation, swelling on the neck, gaseous swelling under the skin and edematous lesions particularly in hind and front legs. Blackleg causes wide varieties of gross and histopathological changes such as fibrinous pericarditis,

myocardial myositis, and necrohemorrhagic myositis in naturally infected cattle [3]. Infection in cattle mostly appears suddenly and death occurs within 12-48 h after showing the symptoms. Molecular mechanisms of pathogenicity of *C. chauvoei* regarding the spreading of infection are largely still unknown but the toxins and neuraminidase produced by the bacteria are believed to play an important role in the mechanisms of development of the disease [4-6]. The exotoxins are capable of liquefying adjacent tissue and inhibiting local defense mechanisms. The advancing infection destroys healthy tissue in its path and spread over the course in hours. Moreover, it is reported that toxins, hyaluronidase, neuraminidase and flagella of the infectious agent are the key factors responsible for the pathogenicity of blackleg [7].

In Pakistan, cattle and buffaloes are the main dairy animals reared for milk and meat production [8,9]. These animals play a pivotal role in the livelihood of the people in terms of meat and milk production and are mainly kept under tropical and sub-tropical climatic conditions. The dairy animals in Punjab Province suffer from different viral [10], bacterial [11,12], and parasitic diseases [13] resulting in serious economic losses. Among different bacterial diseases, blackleg or myonecrosis which affects cattle, buffalo, sheep, and goats. The disease was first reported in Nigeria as a major problem for cattle and has a worldwide distribution with high mortalities and huge economic losses in livestock industry [6,14]. The prevalence of blackleg is known to be very high during years of high average annual rainfall [4,6]. Despite, blackleg is known as one of the oldest diseases of dairy animals, but rare efforts have been made to report visceral form of this disease and its blood biochemical changes in dairy animals. Therefore, this study is the first report describing the hematological changes, the clinical parameters as well as the pathological picture of the disease in naturally infected dairy cattle infected with *C. chauvoei* and reared under intensive tropical and subtropical husbandry practices in Pakistan.

Methodology

Study area

Lodhran District being the part of Punjab Province and is located at longitude 71°37′54.25″E, latitude 29°32′10.83″N and altitude116.46 m. This district has three tehsils (Lodhran, Kahror Pacca and Dunyapur) and is bordered by Bahawalpur, Vehari, Multan, and Khanewal. On the northern side, this district is surrounded by River Sutlej. May to September are known as the hottest months with maximum average temperature (41 to 46 °C), minimum average temperature (23 to 32 °C) and precipitation (13-49mm). November to February are colder months with maximum average temperature (23 to 32 °C), minimum average temperature (7- 13 °C) and precipitation (1–6mm). The wheat and cotton are the main crops of this district. Moreover, this district is enriched with a huge population of livestock such as cattle and buffaloes.

Animals population

The current study was designed to investigate an outbreak of the blackleg disease in an exotic non-native dairy cattle herd kept at district Lodhran. The farm has 137 animals, and they were kept in semi-open shed. Animals were offered lush green fodder, silage mixed ration, as well as they have access to pasture. The animals were in good nutritional and management conditions. During the Month of March 2018, 15 animals having about 1-2 years of age showed different clinical ailments including lameness. These animals were treated with antipyretics, antibiotics (penicillin; 10mg/kg; colistin; 10 mg/kg BW, and gentamicin; 15mg/kg BW), anti-allergic and multivitamins. In spite of treatment therapy, all the sick animals died within12-24 hours of the appearance of clinical signs.

Hematological and serum biochemical Studies

Prior to death, blood samples were collected from all clinically morbid (n = 15) animals as well as from non-infected animals with and without an anticoagulant (EDTA; 1.2mg/mL). The control group included all the live animals those were healthy on the farm (n = 122). The samples were shifted to the laboratory of Department of Pathobiology (Pathology), University College of Veterinary and Animal Science, Islamia University of Bahawalpur, Pakistan. Blood samples with anticoagulant were used for determination of red blood cell counts, total and differential white blood cell counts, hematocrit percentage and hemoglobin concentration [15]. Serum was separated on ice from all the blood samples. Different serum biochemical parameters like total proteins, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), urea, creatinine, cardiac enzyme (CPK) and creatinine kinase (CK)were determined by kit methods as previously described [16]. The data about hematological and serum biochemical parameters obtained in this study were analyzed by ANOVA using SAS/STAT® 9.1 while the means were compared by Tukey's test on personal computer by using SAS

University Edition Software. The level $P < 0.05$ was considered as significant difference.

Necropsy and histopathological investigations

Post-mortem examination of all dead animals (n = 15) was conducted immediately after death and gross lesions have been recorded. For histopathological investigations different tissues from visceral organs such as muscles, heart, lungs, intestine, spleen, kidneys and liver were collected and fixed in 10% neutral buffered formalin solution. After few days of fixation, all the tissues were processed by using standard histopathological procedures including dehydration, infiltration, embedding, and sectioning. Histopathological sections with thickness 3-4µm were obtained from the fixed organs and then stained with Hematoxylin and Eosin (H&E). The histology slides were screened for pathological alterations, especially in the muscle, heart, liver, spleen, and kidneys. Microscopic examination was focused on signs of inflammation, necrosis and cellular alteration in tissue architecture as well as cellular infiltration.

Molecular identification of bacteria by Polymerase Chain Reaction (PCR)

For accurate diagnosis and confirmation purposes, different tissues (muscles, heart and nasal exudates) were collected in sterilized zip bags, placed in ice pack containers and shifted to laboratory for further investigations. DNA was extracted using commercially available DNA extraction kit from tissues and PCR was performed using specific primers for amplification of a 516-bp fragment of *C. chauvoei* gene according to an earlier protocol [17].

Results

Clinical observations

The sick animals were depressed, reluctant to move, lethargic, unable to feed and drink. Few animals indicated nose bleeding. The intensity of these signs was aggravated after 12 h of infection and the morbid animals showed lameness, fever (105-106° F), swollen and painful areas on the neck and shoulder muscles. The morbid animals were in arched-back position and showed congested mucous membranes. The subcutaneous muscular emphysema was observed on palpation in affected front and hindquarters and in brisket area. The intensity and frequency of different clinical ailments is presented in Table 1. In spite of antibiotic therapy and palatable diet, death occurred in all the cases (n = 15) after about 12-24 h of contracting the disease. PCR confirmed the presence of *C. chauvoei* isolates by amplification of specific gene (516bp) in a total of 09/15 (60.0%), 13/15 (86.66%) and 11/15 (73.33%) nasal exudates, muscles and heart tissues, respectively.

Hematology and serum biochemistry

The blood analysis showed significant increases in neutrophil counts and erythrocyte sedimentation rate while decreases in red blood cell counts, packed cell volume, hemoglobin concentration, monocyte and lymphocyte population in clinically infected animals. Serum analysis indicated significantly increased concentrations of cardiac enzyme (CPK), liver function tests (ALT, AST, ALP and LDH), kidneys biomarkers (urea and creatinine) and increase concentrations of creatinine kinase (Table 2).

Necropsy findings

At necropsy, bloodstained froth in the nose and copious amount of red color fluid having fibrinous material in the thoracic cavity was observed. The muscles of all infected animals appeared as dark red and wet around the ribs. The swelling areas of skin contained straw-colored serum with gas bubbles. Necropsy revealed severe hemorrhagic myositis, presence of gas bubbles in skeletal muscles and necrotizing lesions in muscles of neck, hind and front quarters.

Table 1. Intensity and frequency of clinical symptoms in cattle (n = 15) infected with *C. chauvoei* bacterium.

clinical symptoms	Intensity of ailments	Frequency	
		No.	%
Lethargic	Severe	11	73.33
Lameness	Moderate	09	60.0
Fever (105-106° F),	Severe	13	86.66
Swollen and painful areas on neck	Moderate	11	73.33
Swollen and painful areas on shoulder muscles	Moderate	13	86.66
Subcutaneous and muscular emphysema	Severe	11	73.33
Emphysema in front and hindquarters	Severe	13	86.66
Emphysema in brisket area	Moderate	07	46.66
Nose bleeding	Moderate	05	33.33

Table 2. Hematological and serum biochemical parameters (mean ± SD) of non-infected and infected cattle.

Parameters	Non-infected	Infected	P-values
Hematological biomarkers			
Red blood cell counts (10^6/µL)	5.69 ± 0.05	3.49 ± 0.11	< 0.001
Hemoglobin (g/dL)	11.28 ± 0.50	8.12 ± 0.37	< 0.01
Packed cell volume (%)	37.13 ± 1.18	28.24 ± 2.75	< 0.01
White blood cell counts (10^3/µL)	9.92 ± 0.72	7.15 ± 0.22	< 0.001
Neutrophil (%)	24.54 ± 1.69	17.32 ± 1.96	< 0.01
Monocyte (%)	4.87 ± 0.09	3.32 ±0.16	< 0.001
Lymphocyte (%)	63.71 ±3.23	45.14 ± 3.59	< 0.001
ESR (mm/24 h)	4.91 ± 0.22	6.99 ± 0.50	< 0.001
Serum biochemical parameters			
Alanine aminotransferase (unit/L)	35.08 ± 3.35	59.36 ± 3.55	< 0.001
Aspartate aminotransferase (unit/L)	117.68 ± 2.82	152.88 ± 6.44	< 0.001
Alkaline phosphatase (unit/L)	41.72 ± 3.13	68.36 ± 7.54	< 0.001
Lactate dehydrogenase (unit/L)	381.48 ± 6.58	420.98 ± 6.54	< 0.001
Creatinine kinase (unit/L)	169.48 ± 9.13	396.94 ± 23.63	< 0.001
Creatinine phosphokinase (unit/L)	92.11 ± 3.54	165.74 ± 13.61	< 0.001
Urea (mmol/L)	4.536 ± 0.39	6.826 ± 0.26	< 0.001
Creatinine (µmol/L)	86.68 ± 5.39	108.16 ± 3.37	< 0.001

Table 3. Intensity and frequency of necropsy and microscopic lesions in cattle (n=15) died of Blackleg.

Necropsy lesions	Intensity of lesions	Frequency	
		No.	%
Blood stained froth in nose	Moderate	07	46.66
Muscular edema and dark red muscles	Severe	11	73.33
Skin filled with straw-colored serum	Moderate	07	46.66
Hemorrhagic myositis	Severe	15	100.0
Crepitus sounds in infected muscles	Moderate	13	86.66
Rancid butter odor	Moderate	09	60.0
Congested and dark to black heart	Severe	13	86.66
Focal hemorrhages in heart	Moderate	13	86.66
Ventricles with marked dilation and necrosis	Severe	09	60.0
Myocarditis and endocarditis	Moderate	13	86.66
Congestion of lungs	Severe	13	86.66
Splenomegaly	Severe	13	86.66
Enteritis	Moderate	13	86.66
Microscopic lesions			
Edema and congestion in skeletal muscles	Severe	14	93.33
Myofibrillolysis and loss of cross-striations	Severe	13	86.66
Sarcoplasmic vacuolation	Moderate	11	73.33
Coagulative necrosis of myocytes	Severe	13	86.66
Neutrophilic myocarditis	Severe	14	73.33
Deposition of fibrin and hemorrhages in heart	Severe	11	73.33
Interstitial pneumonia and emphysema	Severe	14	93.33
Neutrophilic infiltration and hemorrhages in lugs	Severe	14	93.33
Hemorrhages in glomeruli	Moderate	11	73.33
Neutrophilic infiltration and hemorrhages in liver	Moderate	13	86.66
Depletion of lymphocytes and hemorrhages in spleen	Moderate	09	60.0

The affected muscles had black to dark red color along with multifocal and coalescing hemorrhagic areas (Figure 1a). Crepitus sounds, hemorrhages and edema were prominent in affected muscles. The rancid butter odor was also observed. The heart was congested, dark to black in color and had extensive necrotic areas (Figure 1b). The epicardium was extensively adhered to pleura and focal hemorrhages were observed in myocardium of infected cattle. The right and left ventricles were markedly dilated, congested and focal to multifocal pallor necrotic areas were observed. Epicarditis, myocarditis, and endocarditis were the characteristic features in all dead animals. The lungs were severely congested, red in color, edematous and were collapsed in few cases. The cut sections of lungs exhibited extravasation of large quantity of red color fluid. The spleen of infected animals was congested, dark black in color and extensively enlarged (Figure 1c). On cutting a large amount of red color fluid appeared in spleen. The mucosa of the intestine of infected animals was congested and contained red color exudates. The intensity and frequency of various necropsy lesions are indicated in Table 3.

Histological observations

Microscopic examination revealed severe inflammatory changes in skeletal muscles including edema, congestion and disruption of myofibrils. Skeletal muscles showed myofibrillolysis, loss of cross-striations, degeneration and swelling of myocytes. Extensive sarcoplasmic vacuolation, emphysematous myositis, segmental coagulative necrosis of myocytes with pyknotic nuclei were prominent features in infected cattle. Moreover, infiltrations of neutrophils along with presence of strands of fibrinous material were observed in infected muscles (Figure 2). Microscopic analysis of cardiac tissues revealed presence of degenerative changes in cardiac myocytes and neutrophilic myocarditis. Coagulative necrosis of myocardial cells along with deposition of fibrinous

Figure 1. Gross picture of organs of infected cattle with *Clostridium chauvoei*: A) black to dark red colored muscles with multifocal and coalescing hemorrhagic changes, B) congested, dark to black heart with extensive necrotic areas, C) congested, dark black and extensively enlarged spleen.

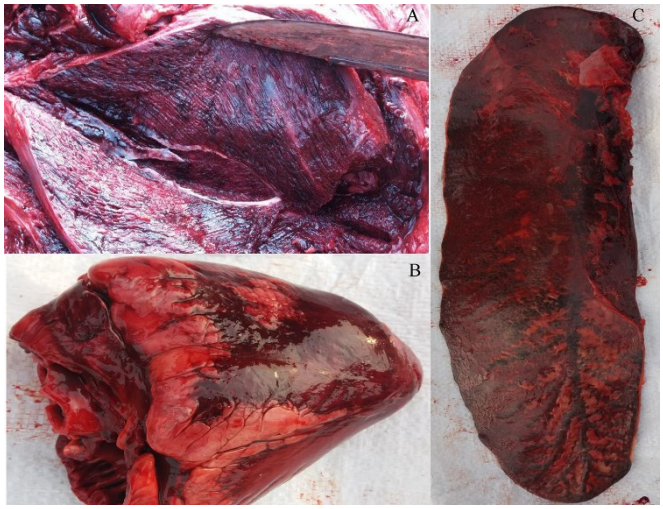


Figure 2. Microscopic sections of skeletal muscles of cattle infected with *Clostridium chauvoei* showing extensive deposition of fibrinous material (asterisk), disruption of myofibrils (arrows), loss of cross-striations, emphysema (arrow heads) along with severe inflammatory reaction, edema, necrosis and infiltration of neutrophils.

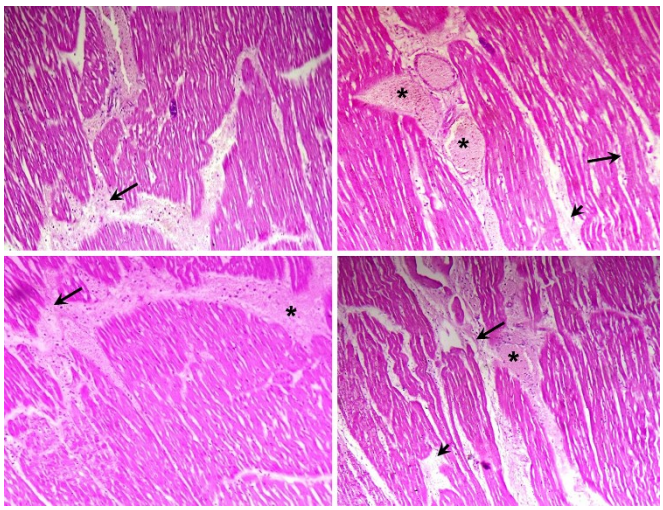
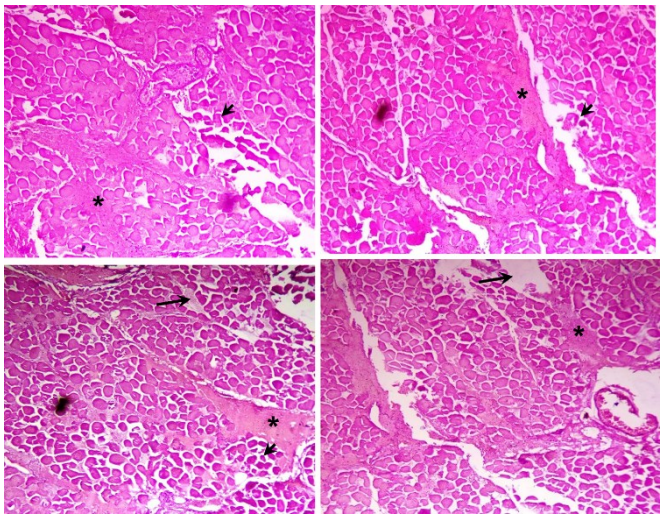


Figure 3. Microscopic sections of cardiac muscles of cattle infected with *Clostridium chauvoei* showing fibrinous carditis (asterisk), edema (arrow), disruption of cardiac muscles (arrowheads), coagulative necrosis and severe inflammatory reaction comprising of neutrophilic infiltrations.



materials and hemorrhages was observed (Figure 3). Lungs of infected animals showed severe inflammatory changes including acute neutrophilic infiltration, interstitial pneumonia, edema, emphysema and hemorrhages (Figure 4). Kidneys showed congestion and hemorrhages in glomeruli, severe necrosis and marked degenerative changes in the tubular epithelium (Figure 5). Congestion, increased sinusoidal spaces, neutrophilic infiltration and hemorrhages were observed in liver tissues. Spleen showed marked depletion of lymphocytes, dilation of sinusoidal space and hemorrhages (Figure 6). Frequency and intensity of various microscopic lesions in different tissues of infected cattle is presented in Table 3.

Discussion

Blackleg is an infectious, non-contagious bacterial disease caused by *C. chauvoei.* Animals get an infection by ingestion of bacterial spores during grazing. The bacterial spores are able to penetrate the intestine and then disseminate via the bloodstream to the skeletal muscle and induce damages. The spores germinate, multiply and produce the toxin that results in necrosis and hemorrhage. The blackleg disease is causing huge economic losses and is a limiting factor in commercial dairy animals. Previous studies on blackleg infection usually revolved around molecular identification [18,19], and classical skeletal muscle pathology [20]. For diagnosis of blackleg infection, clinical ailments and necropsy lesions may be sufficient, however, histopathological and blood biochemical investigations in this study are useful for the understanding of pathogenesis, therapeutic procedures and control strategies of visceral blackleg in dairy animals. In addition to classical clinical signs of black disease, a few of the sick animals showed nose bleeding which is unusual clinical signs in naturally or experimentally infected cattle. At necropsy, the pathognomic lesions of black disease were prominent and all dead animals showed hemorrhagic myositis, crepitus sounds, rancid odor and necrotizing lesions in muscles of the neck, hind and front quarters [21,22]. Severe cardiac lesions have been observed; mainly

Figure 4. Microscopic sections of lungs of cattle infected with *Clostridium chauvoei* showing fibrinous materials (arrows), edema (asterisk), extensive inflammatory reaction (arrowheads), interstitial pneumonia, bronchopneumonia, atelectasis, and emphysema.

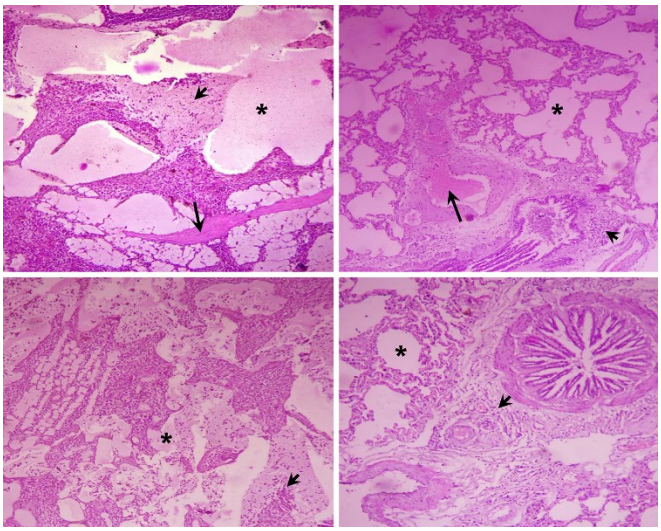


Figure 5. Microscopic sections of kidneys of cattle infected with *Clostridium chauvoei* showing severe necrosis and degeneration of renal tubules and glomeruli (arrows), sloughing of tubular epithelium and severe inflammatory response.

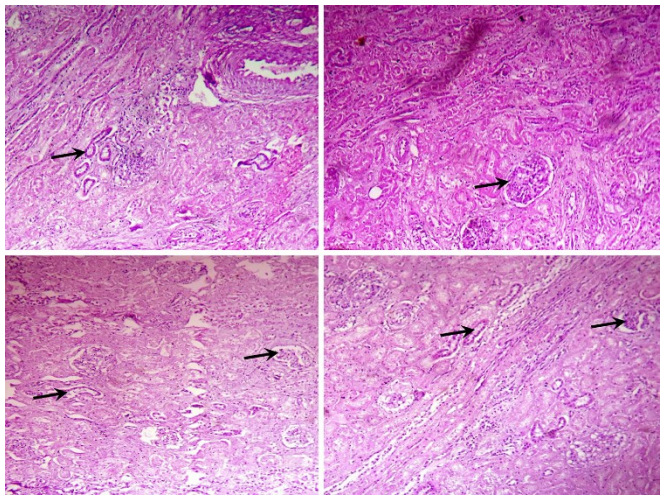
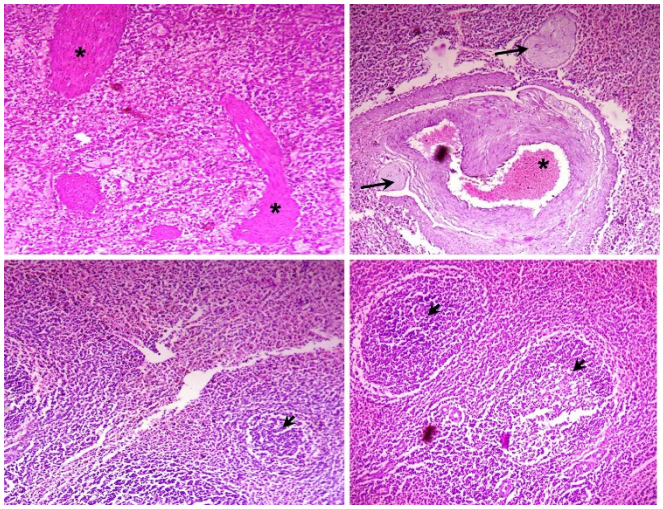


Figure 6. Microscopic sections of the spleen of cattle infected with *Clostridium chauvoei* showing severe fibrinous deposition (asterisk), edema (arrows), necrotic changes and depletion of lymphoid cells along with inflammation (arrowheads).



diffuse necrohemorrhagic myocarditis associated with extensive enlargement of the spleen. The severity of lesions might be due to release of toxins by *C. chauvoei* in the myocardium [23]. Presence of fibrinous material in thoracic cavity with reddish exudates in the intestine were clear and might be due to serious toxemia and histotoxicity produced by toxins released by *C. chauvoei*. Different toxins released by *C. chauvoei* are responsible for nuclear fragmentation and degradation of myocytes leading to myonecrosis [4]. Histopathological examination revealed the presence of degenerative changes and sloughing of intestinal villi accompanied by necrosis of enterocytes and congestion, which are unusual histopathological changes in natural infection of *C. chauvoei* in cattle. Presence of classical but sever lesions in the current study in different visceral organs might be due to the rapid spread of toxins of *C. chauvoei* which leads to damage of intracellular matrix [24]. In addition to the presence of a clear clinical and pathological picture of the blackleg disease, PCR used to confirm the presence of *C. chauvoei* in different clinical samples including tissues and exudates [17].

In the present study, significantly lower values of different hematological parameters such as erythrocytes counts, pack cell volume, the total leukocytes count (leucopenia) and differential leukocytes count (neutropenia, monocytopenia, and lymphopenia) could be due to actions of different toxins. The significantly ($P < 0.05$) higher values of erythrocyte sedimentation rate could be due to leakage of fluid from damaged tissue in body cavities. Moreover, these changes can also be attributed to the increased release of neuraminidase by *C. chauvoei*. Moreover, lower hematological parameters could be attributed to cytotoxic activity and hemolytic genes of *C. chauvoei* [14]. Similar hematological results have also been reported in experimentally induced blackleg infection in cattle [5,6]. The significantly ($P < 0.05$) increased values of serum parameters including creatine kinase, aspartate aminotransferase, lactate dehydrogenase, alanine aminotransferase, alkaline phosphates, urea, creatinine, and creatinine phosphokinase are in agreement with previous reports [25]. The higher values of serum biochemical changes in this study are suggestive of damage to skeletal and cardiac muscles and increased ALT and AST could be due to liver damage.

Conclusion

It can be concluded that the blackleg disease not only induces serious ailments in skeletal muscles but also involves different other vital tissues of the infected animals. Therefore, the investigation of the pathophysiology status of infected animals gave a complete picture of pathogenesis of visceral form of blackleg disease in cattle. The appearance of an outbreak in Pakistan is alarming and biosecurity measure has to be elevated. Application of suitable vaccine and preventive measure early in the outbreak is obvious.

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