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CLINICO-HEMATOLOGICAL, PATHO-ANATOMICAL AND MOLECULAR BASED INVESTIGATION OF BLACKLEG DISEASE IN CHOLISTANI CATTLE

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*Clostridium chauvoe*i, causing myonecrosis in livestock animals, lives in the feces, surface water and soil. The blackleg due to *C. chauvoei* is very common in dairy animals. Still, no report is available about the pathophysiology of disease in Cholistani cattle kept under tropical and desert conditions of Cholistan, Pakistan. Therefore, in this study, we report for the first time the pathophysiology of a visceral form of blackleg infection in indigenous cholistani cattle breed reared in desert conditions of Cholistan. Clinically morbid animals exhibited different signs of infection including fever, crepitation sounds, gaseous swelling and edematous lesions. Blood was collected for hematological and serum biochemical investigation. Hematological examination indicated a significant increased erythrocyte sedimentation rate, lower red blood cells, hematocrit, and total white blood cell count. Results on serum biochemistry showed significantly (p<0.02) increased creatine kinase levels, creatinine, urea, alanine aminotransferase, aspartate aminotransferase, alkaline phosphates, creatinine phosphokinase and lactate dehydrogenase in infected cattle. At necropsy level, light pink color fluid under swelling areas of skin, gas bubbles, dark to black in color of affected muscles and crepitation sounds at palpation were observed. Necropsy showed marked myocarditis, petechial hemorrhages, consolidation and severe pulmonary edema. Spleen showed petechial hemorrhages and congestion. Histological analysis of muscular tissues indicated severe inflammatory reaction comprising of cellular infiltration, marked edema, necrosis and disruption of myofibrils. PCR confirmed the presence of *C. chauvoei* in muscles, heart and exudates of the lungs. This is the first report on molecular detection of Clostridium chauvoei from a visceral form of Blackleg disease in cholistani breed of cattle naturally infected in the Cholistan desert of Punjab, Pakistan.

Keywords: Blackleg, cholistani cattle, blood, biochemistry, pathology, PCR.

INTRODUCTION

In Pakistan, dairy animals, including cattle, camels, buffaloes, sheep and goats are mainly kept for milk and meat production (Ali *et al*., 2016; Ali *et al*., 2017; Hussain *et al*., 2018; Hussain *et al*., 2020). The livestock animals in developing countries like Pakistan are routinely kept under tropical and subtropical climatic environmental conditions and suffer from various bacterial (Mahmood *et al*., 2014; Mahmood *et al*., 2017;

Hussain *et al*., 2017; Mahran *et al*., 2020), viral (Khan *et al*., 2018; Hussain *et al*., 2020) and parasitic infections (Mahmood *et al*., 2014b; Hussain *et al*., 2016, 2017b, 2018; Zafar *et al*., 2019; Ali *et al*., 2020; Abbas *et al*., 2020; Hamza *et al*., 2020; Salman *et al*., 2020; Zaman *et al*., 2020). Dairy animals are the main cornerstones for the livelihoods of the people of the Cholistan district. However, different infectious diseases are the key constraint in rearing dairy animals and affecting livestock productivity and food security (Zafar *et al*., 2019; Rashid *et al*., 2019; Batool *et al*., 2019; Attia *et al*., 2020; Peter, 2020; Zeedan *et al*., 2020). Among various infectious diseases, clostridial myositis/blackleg or myonecrosis is one of the fatal diseases of dairy animals (Hussain *et al*., 2019; Nasir *et al.,* 2020). The disease is characterized by an acute, rapidly progressive infection of the soft tissues commonly known as "gas gangrene (Hussain *et al*., 2019: Zaragoza *et al*., 2109) and causes huge economic losses to dairy farmers. Different synonyms of the blackleg disease known e.g.: black-quarter, gangrenous myositis of ruminants and clostridial myositis. The disease is caused by *C. chauvoe*i, an anaerobic, and spore-forming bacillus bacterium that commonly survives in surface water, soil and feces (Idrees *et al*., 2018; Abreu *et al*., 2017; Thomas *et al*., 2017). Infection of cattle by *C. chauvoei* is caused by contact of the animals to the bacterium that exists in the form of spores in the soil of "poisoned" cultures. However, *C. chauvoei* has been found in dung, which also shows a source of infection and can lead to the pollution of animals (Zaragoza *et al*., 2019). The etiological agent enters into the body of animals through ingestion of contaminated products. After exposure, the bacterium enters into the bloodstream and proliferates in muscular tissues under anaerobic conditions. The infected animals showed various clinical ailments of disease such as fever, anorexia, unilateral nose bleeding, lameness, gaseous swelling under the skin of affected areas, crepitation sounds, and edematous lesions in hind legs. Studies have determined that neuraminidase and different toxins released infectious agent play crucial to establish and progress of disease (Useh *et al*., 2003; Zaragoza *et al*., 2019). Black quarter induces different macroscopic and microscopic lesions like emphysematous skeletal muscles, fibrinous pericarditis, myocardial myositis, endocarditis, and necrohemorrhagic myositis in infected animals (Abreu *et al*., 2017; Ziech *et al*., 2018; Hussain *et al*., 2019; Nasir *et al.,* 2020). Infection in infected animals appears suddenly, and death results after showing the symptoms within 12-20 h. Susceptible cattle first ingest endospores. The endospores then cross over the gastrointestinal tract and enter the bloodstream where they are deposited in muscle tissue. They then lie dormant in the tissue until they become activated and trigger the disease in case of any damage present in the muscles. The exotoxins produced by the bacterium cause liquefaction of adjacent tissues and inhibit the defense mechanisms of infected animals. Furthermore, the toxins, DNAse, neuraminidase, hyaluronidase, CctA - a hemolysin and protective antigen and flagella of the bacterium are the major factors responsible for the rapid progress of the disease (Gupta *et al*., 2019). The black quarter was first appeared as a major disease in cattle in Nigeria and has worldwide distribution causing substantial economic loss in dairy sectors (Gizaw *et al*., 2020). Although the disease (blackleg) is known as one of the oldest infections. Still, scanty information is available in accessible literature about the pathophysiology of natural cases of blackleg infection in indigenous cholistani cattle breeds kept under desert conditions in Pakistan. Therefore, the current study describes the clinical picture, hemato-biochemical alterations, necropsy lesions, histopathology, and molecular diagnosis of the visceral form of the blackleg disease in naturally infected indigenous dairy cattle kept under desert conditions in Pakistan.

MATERIALS AND METHODS

Study area: Cholistan desert being in Southern Punjab lies between longitudes 69° 52' to 75° 24'E and latitudes 27° 42' 29°45'N and comprised of the following three districts: Bahawalnagar, Bahawalpur, and Rahim Yar Khan (Shabbir et al., 2020). It is known as a tropical desert and has approximately cattle (0.7 million), sheep (0.47 million), goats (0.38 million) and camels (0.08 million) as dairy animals. Inadequate water sources, seasonal fodder, nutrition, marketing, and animal health monitoring services are the major constraints for dairy farmers and favor different disease outbreaks in animals. The hot and humid climate favors rapid proliferation and multiplication of various infectious agents resulting in severe disease outbreaks. Prior to this study, no report could be found in the accessible published literature regarding the outbreak of blackleg infection in Cholistan.

Study Animals: The present study investigated an outbreak of blackleg in Cholistani cattle were housed under desert conditions. All the animals were kept in semi-open sheds and were offered green fodder and silage mixed ration. These animals were also offered cottonseed cake @ 0.5 kg/day/animal. During May and June 2019, young cattle calves **(**n=11) of Cholistani breeds having about 8-11 months of age exhibited different clinical signs like high fever (105°F), unilateral nose bleeding and lameness. All the morbid animals were then treated with antibiotics (Penicillin; 10 mg/kg; Amoxicillin: 10 mg/kg BW; colistin; 10 mg/kg BW, and CTC-20: 10 mg/kg BW), antipyretics, multivitamins and anti-allergic. Despite care, proper management and treatment, the sick animals did not recover and died within 12 hours after onset of clinical ailments.

Hemato-biochemical Studies: Blood was obtained from healthy (n=11) and all morbid (n=11) animals in vacutainers with and without anticoagulant (EDTA; 1.0mg/ml). Serum was obtained from blood samples collected without

anticoagulant (Khan *et al*., 2020). Blood samples with anticoagulants were subjected to the determination of erythrocyte counts, hemoglobin, white blood cell counts and pack cell volume. Different serum biomarkers such as alanine aminotransferase, total proteins, aspartate aminotransferase, alkaline phosphatase, cardiac enzymes (CPK), urea, , creatinine kinase, creatinine and lactate dehydrogenase (LDH) were determined (Hussain *et al*., 2019).

Necropsies and histopathological investigations: Postmortem of animals (n=11) was conducted soon after death to observe different macroscopic lesions in various visceral organs. For the histopathological investigation, tissues were obtained from various visceral organs, including lungs, heart, intestines, muscles, liver, kidneys and spleen. All the collected tissues were immediately fixed in 10 % neutral buffered formaldehyde (Gherissi *et al.,* 2020). After two weeks of preservation in formaldehyde, all these tissues were processed for histopathological changes using routinely used procedures like dehydration, embedding, sectioning, mounting and staining. About 4-5µm thick sections were cut and stained with Hematoxylin and Eosin staining technique (Eyhab *et al*., 2019; Hussain *et al.,* 2020).

Polymerase chain reaction (PCR): For confirmation of bacterial agent previously specified forward and reverse primers for DNA amplification of *C. chauvoe*i such as 5′-ATCGGAAACATGAGTGCTGC-3′ and 5′-AGTCTTTATGCTTCCGCTAG-3′ were used. All the DNA amplification procedures, including initial denaturation, primers annealing, extension, and staining, were carried according to the protocols described elsewhere (Kojima *et al*., 2001).

Statistical analysis: The data on blood and serum profile were analyzed using IBM SPSS statistics software (version 20). The level $p < 0.05$ was considered as a significance level.

RESULTS

Clinical ailments: The clinically morbid animals exhibited different signs of disease, including high fever (104-105° F), anorexia and unable drink. All the morbid animals were lethargic, depressed and hesitant to move. Unilateral nose bleeding was observed in few morbid cases. The sick animals indicated facial edema, lameness, swelling, hyperemic mucous membranes, arched-back position, and painful front and hind shoulder muscles and neck areas. At the time of palpation, subcutaneous muscular emphysema was the characteristic feature in all clinical cases. The frequency and intensity of various clinical lesions are presented in Table 1. Results on polymerase chain reaction techniques confirmed the presence of *C. chauvoei* bacterium in all specimens of clinically morbid animals (Fig. 1).

Table 1. Frequency of different clinical ailments observed in cattle (n=11).

Clinical ailments	Intensity of ailments	Frequency	
		No.	%
Fever (105-106° F)	Severe	07	63.6
Unilateral nose bleeding	Moderate	04	36.6
Lameness	Moderate	07	63.6
Anorexia	Moderate	05	45.4
Emphysema in brisket area	Moderate	09	81.8
Lethargic	Severe	07	63.6
Subcutaneous emphysema	Severe	07	63.6
Hyperemic mucous membranes	Moderate	09	81.8
Swollen areas on shoulder muscles	Moderate	07	63.6
Arched-back position	Severe	03	27.2
Emphysema in front and hindquarters	Severe	05	45.4
Swollen areas on the neck	Moderate	03	27.2

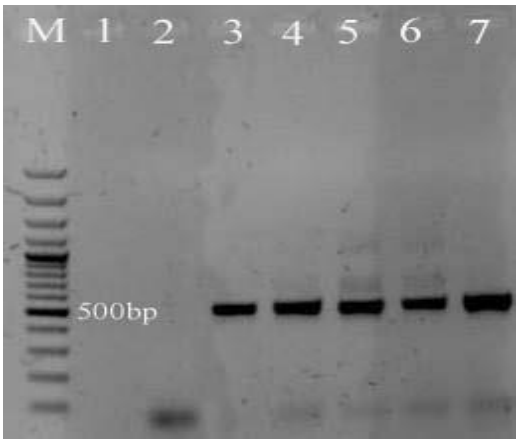

Figure 1. Photograph showing PCR-based confirmation of *Clostridium chauvoei*. M is molecular marker (100bp), Lanes 1-2 negative samples and lanes 3-7 are positive samples (515 bp) product size.

Hematological and serum biochemical parameters: The results on hematological and serum biochemical levels are presented in Table 2. Results revealed significantly lower values of erythrocytes, leukocytes, monocytes, lymphocytes, hematocrit %, neutrophil population, hemoglobin quantity in morbid animals compared to healthy animals. The significantly higher values of different serum biochemical parameters such as glucose, aspartate aminotransferase, alkaline phosphates, alanine aminotransferase, lactate dehydrogenase, creatine kinase, creatinine phosphokinase, creatinine, urea and cardiac biomarkers such as triglycerides, CPK and CK-Mb were observed when compared to non-infected animals.

Table 2. Blood and serum biochemistry analyses of healthy and infected cattle.

Parameters	Healthy (n=11)	Infected (n=11)	P-Values
Hematological biomarkers			
Leukocyte (10^3/µl)	8.75±1.33	5.32±1.11	<0.01
Erythrocytes (10^6/µl)	5.13±1.05	3.22±0.02	<0.01
Monocyte (%)	6.11±0.03	3.31±0.22	<0.01
Lymphocyte (%)	57.10±2.22	39.10±4.10	<0.01
Neutrophil (%)	21.30±3.30	14.30±2.10	<0.01
Packed cell volume (%)	35.70±2.80	24.30±3.20	<0.01
Hemoglobin (g/dl)	12.30±0.20	7.80±0.40	<0.01
ESR (mm/24 h)	5.11±0.31	7.13±0.21	<0.01
Serum biochemical parameters			
Creatinine kinase (unit/L)	143.30±5.30	313.30±15.5	<0.01
Creatinine (µmol/L)	73.40±3.90	99.30±5.30	<0.01
Urea (mmol/L)	5.22±1.11	7.90±0.90	<0.01
Creatinine phosphokinase (unit/L)	77.20±4.40	117.90±9.30	<0.01
Alkaline phosphatase (unit/L)	46.30±4.20	73.10±3.30	<0.01
Alanine aminotransferase (unit/L)	33.20±5.20	53.50±4.30	<0.01
Lactate dehydrogenase (unit/L)	349.70±10.7	403.30±7.30	<0.01
Aspartate aminotransferase (unit/L)	107.30±3.30	137.70±5.50	<0.01

***Gross and histopathological lesions*:** Necropsy examination indicated hyperemic trachea, presences of blood mixed exudates in the trachea and nasal cavity. The classical muscular lesions, including myositis of sublingual muscles and muscles of the heart, neck, hindquarters and forequarters, were observed in all cases. The pectoral and pelvic girdles muscles were involved and appeared dark black to red and contained gas bubbles. The straw-colored fluid was observed beneath swelling areas of skin. Edema, congestion, crepitus sounds, necrotizing lesions and hemorrhagic myositis were the main postmortem lesions observed in the muscles of infected animals (Fig. 2).

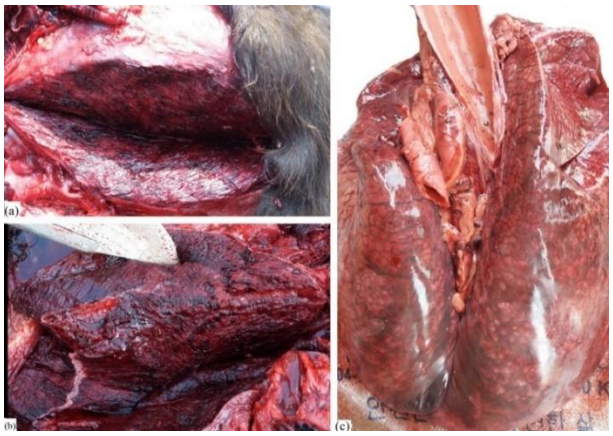

Figure 2. Photograph of muscles of Cholistani cattle (a-b) showing severe congestions, edema and necrosis of *Clostridium chauvoei* and lungs (c) showing congestion, frothy exudate, edema and bilateral enlargement.

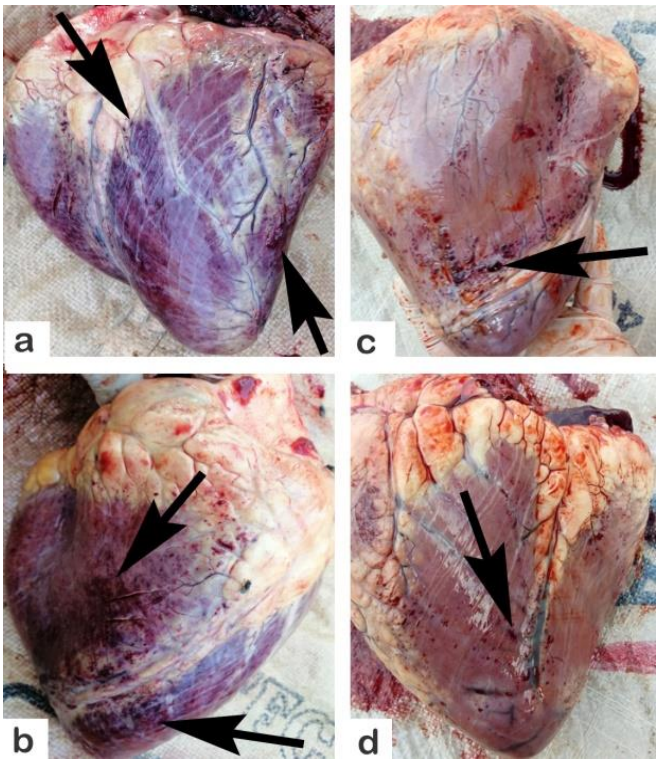

Figure 3. Photograph of the heart of Cholistani cattle (a-d) died of *Clostridium chauvoei* showing severe congestion, myocarditis and petechial hemorrhages (arrows).

In few animals, the lesions of skeletal muscles of the abdominal wall, lumbar area, brisket, thoracic wall and

diaphragm were observed. Necropsy analysis showed edema, congestion, emphysema and red hepatization of the lungs (Fig. 2). The heart of infected animals was congested, marked by dilatation of ventricles, necrotic areas, myocarditis, epicarditis and endocarditis. The heart exhibited congestion, hemorrhagic and extensive necrotic areas (Fig. 3).

The liver and kidneys were swollen, dark to black and severely congested. The spleen was severely increased in size, congested and had dark black color in infected animals. The intestine contained mucoid exudates and was severely congested and hemorrhagic.

At histological levels, different sections of skeletal muscles showed severe changes, including the presence of fibrinous strands, hemorrhages, absence of cross-striations, edema, cytoplasmic vacuolation, disruption of the myofibrils, myofibrillolysis, emphysema, leukocytic infiltrations, sarcoplasmic vacuolation and necrosis of myocytes (Fig. 4). Microscopic sections of the liver showed leukocytic infiltrations, sinusoidal spaces and hemorrhages. Histological sections of kidneys showed hemorrhages, degeneration of renal tubules, necrosis of renal tubules, increased urinary spaces and necrosis of tubular epithelial cells (Fig. 5).

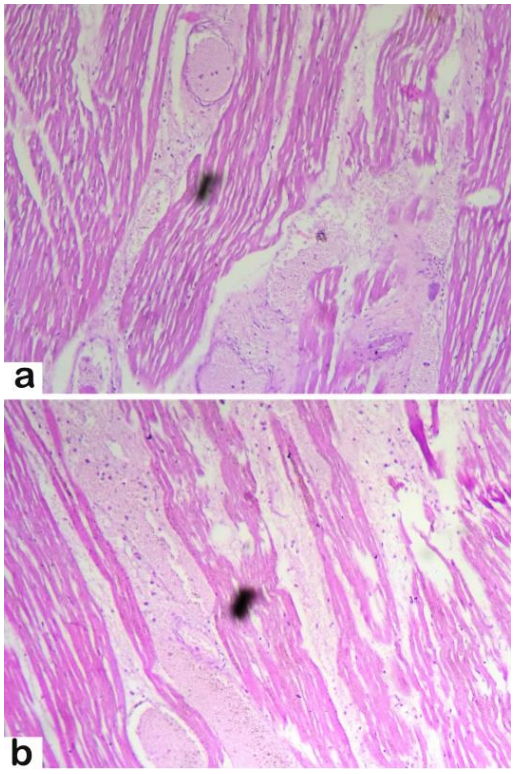

Figure 4. Photomicrograph of skeletal muscles (a & b) of cattle died of *Clostridium chauvoei* exhibiting loss of cross-striations, severe infiltration cells, edema, necrosis of myofibrils, emphysema and accumulation of fibrinous material. H&E Stain, 200X.

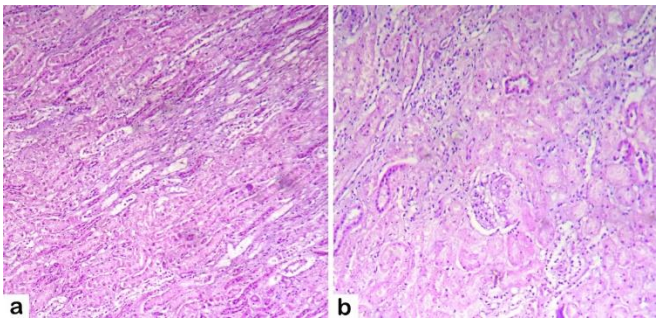

Figure 5. Photomicrograph of kidneys of cholistani cattle (a & b) died of *Clostridium chauvoei* exhibiting severe degeneration of renal tubules, necrosis of tubular epithelium, cellular infiltrations and necrosis of tubular cells. H&E Stain, 200X.

Hemorrhages, interstitial pneumonia, edema, leukocytic infiltrations and emphysema were the main lesions in lung tissues of infected animals (Fig. 6). Congestion, hemorrhages and leukocytic infiltrations were observed in the spleen and intestinal sections of infected animals. Severe inflammatory changes in different sections of hearts include neutrophilic myocarditis, leukocytic infiltrations, necrosis of myocytes, coagulative necrosis of myocytes, and hemorrhages were observed (Fig. 7).

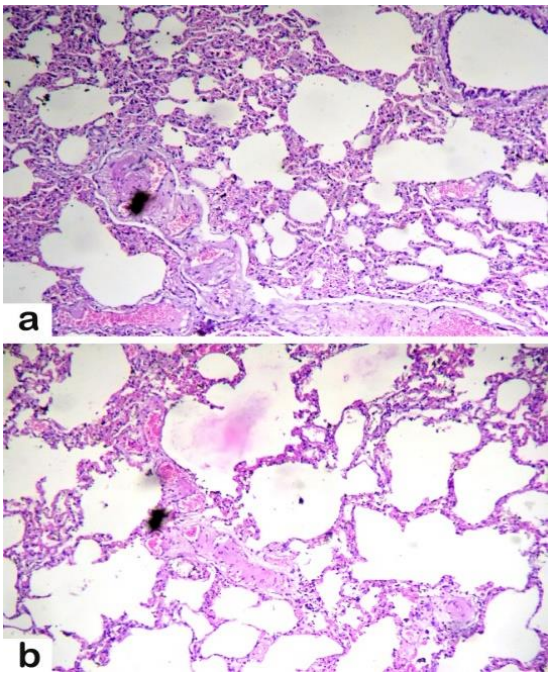

Figure 6. Photomicrograph of lungs (a & b) of cattle who died of *Clostridium chauvoei* exhibiting severe congestion, interstitial pneumonia, cellular infiltrations, fibrinous exudate, edema and atelectasis. H&E Stain, 200X

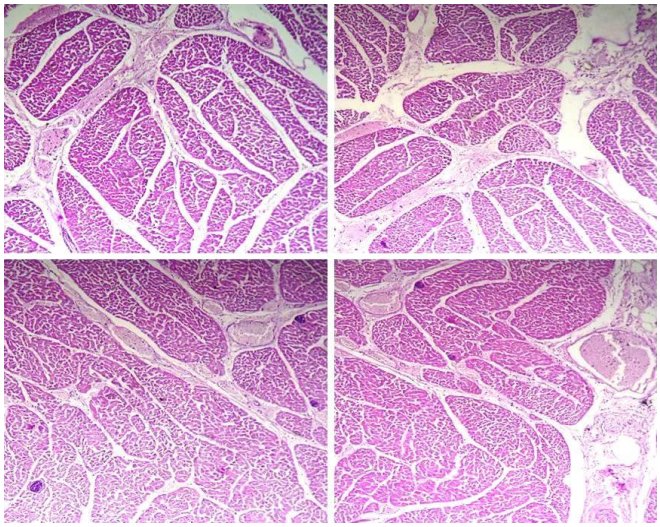

Figure 7. Photomicrograph of cardiac muscles of cholistani cattle died of *Clostridium chauvoei* exhibiting neutrophilic infiltrations, inflammatory exudate, edema, severe fibrinous carditis and necrosis. H&E Stain, 200X

DISCUSSION

The underestimated blackleg disease is a substantial economic limiting factor in commercial dairy animals and neglected control measures and prevention. Therefore, the understanding of the pathogenesis of blackleg infection (classical skeletal muscle pathology) in dairy animals is of vital importance for its control strategies and enhances the farmers' economy (Abreu *et al*., 2017; Pires *et al*., 2017; Idrees *et al*., 2018; Nasir *et al.*, 2020). Previously in published literature, no information could be found about the nose bleeding in infected animals. However, different clinical ailments such as swelling and crepitus of affected muscles, anorexia, fever, lameness, myonecrosis and death (Useh, 2003), mucous membranes, lameness, recumbency, muscle emphysema in hindquarters (Abreu *et al*., 2017) in naturally and experimentally infected cattle have been reported earlier (Uzal *et al*., 2003; Snider *et al*., 2011). The superficial muscles in all infected cases were congested, swollen, and crepitus sounds were evident, as reported earlier (Wickramasinghe *et al*., 2014). Moreover, gangrenous and dry skin over the center of the swollen area has also been observed in infected animals (Wickramasinghe *et al*., 2104).

At necropsy level, crepitus sounds, hemorrhagic myositis, hemorrhages, gas bubbles, edema, multifocal and coalescing necrotic areas along with necrotizing lesions in muscles were the prominent lesions in infected animals. Besides, the muscles around the ribs appeared dark red and wet. Similarly, neutrophil necrotizing myositis (Wickramasinghe *et al*., 2014; Casagrande *et al*., 2015), visceral myonecrosis (Casagrande *et al*., 2015; Assis *et al*., 2010), subcutaneous edema, crepitus sounds on palpation and emphysematous, and necrohemorrhagic lesions in skeletal and gastrocnemius muscles have been reported (Abreu *et al*., 2017; Pires, *et al*., 2107). Different cardiac lesions observed in this study have also been reported in the visceral form of the blackleg disease in cattle, buffaloes and cattle calves (Abreu *et al*., 2017; Uzal *et al*., 2003; Snider *et al*., 2011; Casagrande *et al*., 2015; Assis *et al*., 2010). In contrast to cardiac lesions observed in the present study, diffuse necro-hemorrhagic myocarditis has been reported (Uzal *et al*., 2003). The cardiac lesions in infected animals might be associated with germination and release of toxins by *C. chauvoei* in the myocardium (Uzal *et al*., 2003). In the present study, grossly spleen lesions could be due to the release of toxins by *C. chauvoei.* Previously, inaccessible published literature, no report is available about lesions induced by *C. chauvoei* in the spleen. The severe lesion in the spleen might be due to hematogenous infection by *C. chauvoei*. Grossly, the lung lesions observed in our study have also been reported (Casagrande *et al*., 2015). These lesions might be due to severe toxemia and histo-toxicity of toxins released by *C. chauvoei*. Previously severe acute necrotizing enteritis has been reported. In contrast to the findings of this study, lesions in sublingual muscles, diaphragm, and fibrinous pleuritis due to blackleg have been reported (Casagrande *et al*., 2015; Assis *et al*., 2010).

At the histopathological level, various lesions in skeletal muscles of cholistani cattle could be due to cytopathogenicity of thermostable proteins and toxins of *C. chauvoei*. Similar microscopic lesions, including emphysematous skeletal myositis and necrohemorrhagic, have been reported (Abreu *et al*., 2017; Casagrande *et al*., 2015; Ziech *et al*., 2018). The microscopic tissue changes in skeletal muscles might be due to the action of toxins of *C. chauvoei* resulting in the formation of pores in plasma membranes of myocytes. Histologically, different lesion in the heart of different animals like myocardial necrosis, diffuse neutrophils infiltration, coagulative necrosis, diffuse fibrin deposition and mesothelial proliferation large aggregates of fibrin deposition has also been reported in the visceral form of myonecrosis (Abreu *et al*., 2017, Snider *et al*., 2011; Casagrande *et al*., 2015; Pires *et al*., 2017). The microscopic changes in liver and kidneys in the present study have not been reported previously due to *C. chauvoei*. At Histopathological levels, acute neutrophilic infiltration, interstitial pneumonia, edema, emphysema and hemorrhages have also been observed in lung tissues (Abreu *et al*., 2017; Snider *et al*., 2011). Histologically, intestinal lesions observed in current research have not been reported due to *C. chauvoei* in cholistani cattle. On histopathological evaluation, spleen tissues of various cattle showed different changes which previously have not been reported. In the present study, the histopathological lesions in different visceral organs might be due to the rapid spread of toxins of *C. chauvoei*, resulting in the damage of

intracellular matrix leading to tissue damage (Useh *et al*., 2006; Frey *et al*., 2015).

In the current study; significantly decreased hematological parameters might be due to various toxins released by the bacterial agent in tissues. The significantly increased values of erythrocyte sedimentation rate can be linked with leakage of fluid from injured tissues in body cavities. The lower hematological values in infected animals could also be due to the release of different toxins such as neuraminidase by the bacterium (Hussain *et al*., 2019; Chilek *et al*., 2019]. Similarly, in previously published literature, lower blood values have also been reported due to natural cases of blackleg infection in dairy cattle (Useh *et al*., 2006). The significantly higher serum biochemical parameters include alanine aminotransferase, creatine kinase, lactate dehydrogenase, aspartate aminotransferase, alkaline phosphates, creatinine phosphokinase, urea and creatinine have also been reported (Hussain *et al*., 2019; Radostits *et al*., 2000). The increased serum biochemical parameters can be related to liver, kidneys, cardiac and skeletal muscle damage.

***Conclusion*:** From the results of current study, it can be suggested that blackleg disease is existing in Cholistani cattle and causes multiple organ dysfunctions. Therefore, continuous monitoring and investigation is needed to develop reliable treatment protocol to control the disease.

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