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Haematological and biochemical alterations in native sheep experimentally infected with bluetongue virus serotype-2

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ABSTRACT

The study was designed to determine the haematological and biochemical alterations in sero-negative native sheep following the experimental bluetongue virus serotype-2 (BTV-2) infection. The BTV infected group comprised 14 sheep inoculated with 6 ml of clarified virus containing 1×10^6/ml $TCID_{50}$ of BTV-2 by intradermal route. The uninfected control group comprised 6 animals inoculated with 6 ml of cell culture medium without virus by intradermal route. The blood and serum samples were analyzed at 0, 1, 2, 3, 7, 11, 14, 21 and 45 days post-infection (dpi). Significant changes were observed in all the haematological and biochemical parameters studied. Marked leucopenia was observed from 2 to 7 dpi in BTV infected group. Significant leucocytosis was documented during 11 to 14 dpi in infected group. Significant thrombocytopenia was observed during 2 to 14 dpi whereas significantly low packed cell volume (PCV) and haemoglobin (Hb) values were observed between 3 and 21 dpi in BTV infected group. Differential leucocyte count revealed significantly low lymphocyte percentage on day 3 and high on day 11 in infected group. The various biochemical enzymes like alanine aminotransferase (ALT) showed significantly high values during 3 to 21 dpi, aspartate aminotransferase (AST) during 3 to 21 dpi, alkaline phosphatise (ALP) during 3 to 11 dpi and creatine kinase (CK) during 7 to 14 dpi in BTV infected group. The result of our study demonstrated significantly decreased levels of total leucocyte count, total platelet count, haemoglobin and PCV values while significantly increased levels of ALT, AST, ALP and CK values in BTV infected group. On histopathological examination, spleen and lymph nodes showed depletion of lymphoid cells, liver and kidney showed degeneration, congestion and haemorrhage at many places. The BTV nucleic acid was detected from blood and tissues by RT-PCR. These findings indicated the damage to various soft tissue organs and muscles as a sequel to vascular endothelial damages caused by BTV.

Key words: BTV-2 serotype, Experimental infection, Haematology, Native sheep, Serum biochemistry

Bluetongue (BT) is an infectious, noncontagious, arthropod-borne viral disease of domestic as well as wild ruminants (Meyer *et al*. 2009). BT is multiple species disease out of 118 OIE (Office International des Epizooties) listed animal diseases (OIE 2016). It is caused by bluetongue virus (BTV) which is spread between vertebrate hosts by *Culicoides* species (Mellor 1990, Saminathan *et al.* 2016). BTV belongs to the *Orbivirus* genus in the family *Reoviridae*. Presently, at least 27 distinct BTV serotypes have been recognized worldwide which include three recently recognized novel serotypes of BTV-25 Toggenburg orbivirus in sheep from Switzerland (Hofmann *et al.* 2008), BTV-26 in goats from Kuwait (Maan *et al.* 2011) and putative BTV-27 detected in goats from Corsica, France (Jenckel *et al.* 2015) with the possible addition of two more serotypes (Maan *et al.* 2015). The first outbreak of BT was reported from India by Sapre in 1964, and then subsequently outbreaks of BT had been reported from most of the northern and southern states of India (Sapre 1964, Anjaneya *et al.* 2018). In India, 23 out of 27 BTV serotypes (except 19, 22, 25 and 26) had been reported either by virus isolation or by detection of anti-BTV antibodies (Ayanur *et al.* 2016). BTV-2 had been isolated for several years from southern states, viz. Tamil Nadu in 1982 and 2003 and Andhra Pradesh in 1993, 2007 and 2010 (Maan *et al.* 2015). BTV, similar to other viruses, causes significant alterations in haematological parameters. Usually, it leads to transient panleucopenia with maximum leucopenia preceding both the peaks of viraemia and pyrexia (Jeggo *et al.* 1986). In classical cases, it causes considerable decrease in total erythrocyte count (TEC), total leucocyte count (TLC),

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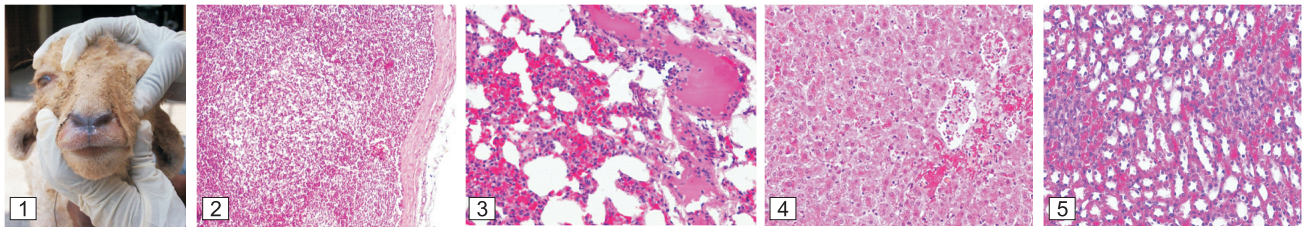

Figs 1–5. **1.** Edematously swollen face with congestion and nasal discharge in sheep. **2.** Poor population of lymphocytes in the splenic white pulp (H & E × 100). **3.** Lung section showing thickened inter-alevolar septa with congestion of capillaries and sero-sanguinous fluid in alveoli (H & E × 200). **4.** Liver section showing vascular congestion, haemorrhages and hepatocytic degeneration (H & E × 200). **5.** Kidney section showing distended and degenerated tubules with vascular congestion (H & E × 200).

packed cell volume (PCV), total platelet count (TPC) and haemoglobin (Hb) values (Singh *et al.* 2008).

Tissue injury leads to changes in various biochemistry panels (Chandra *et al*. 2002). BTV infections elicit changes in levels of several enzymes like serum glutamic oxaloacetic transaminase/aspartate amino transaminase (SGOT/AST), serum glutamic pyruvic transaminase/alanine amino transaminase (SGPT/ALT), lactic dehydrogenase (LDH) and aldolase vis-à-vis severity of viremia and with tissue or organ damage (Singh *et al*. 2008). These levels may increase and reach peak values about 8 days after the peak of pyrexia. But, significant changes are usually seen in creatinine kinase (CK) levels owing to muscular damage in later stage of infection (Sanchez-Cordon *et al.* 2013). However, the information on haematological and biochemical changes caused by bluetongue virus serotype-2 infection in sheep was not documented. Keeping the above facts in view, the present study reports the changes in haemato-biochemical values in sheep experimentally infected with BTV-2.

MATERIALS AND METHODS

Ethical approval: The study was approved by the IAEC (ICAR-IVRI) and CPCSEA (Ethical Clearance Certificate's Number 108/HRECC.FODM/VII/2017).

Bluetongue virus: Bluetongue virus serotype-2 (BTV-2) isolate was procured from Virus Repository of ICAR-Indian Veterinary Research Institute, Mukteswar Campus, Uttarkhand, India. The virus was maintained at 7th passage level in baby hamster kidney-21 (BHK-21) cells. The revived virus was clarified and titer of the stock used in the study was determined as tissue culture infective dose$_{50}$ (TCID$_{50}$) by endpoint titration assay (Reed and Muench 1938).

Screening of sheep for BTV: BTV sero-negative indigenous, non-descript sheep (20) 1 to 2-year-old, of either sex used in present experimental study were tested negative by bluetongue c-ELISA test Kit (VMRD, Pullman, Washington, USA).

Experimental animals and infection: These animals were quarantined for 1 month and then shearing, de-worming and dipping were carried out at appropriate intervals. These sheep were housed in an insect proof shed. The shed was daily washed with soda and lime with spraying insecticide once in a week. The BTV infected group comprising 14 sheep received intradermal dose of 6 ml of clarified virus containing 1×10^6/ml TCID$_{50}$ suspension and served as BTV infected group. The uninfected control group comprising 6 animals inoculated with 6 ml of cell culture medium without virus by intradermal route.

Analysis of blood parameters: For haematological parameters, 2 ml blood was collected from jugular vein by venipuncture using sodium salt of ethylene diaminetetra acetic acid (EDTA) @ 1–2 mg/ml as an anticoagulant from infected and control groups. Collections were done at 0, 1, 2, 3, 7, 11, 14, 21 and 45th day post infection (dpi). At each interval, blood samples were analyzed for TLC, PCV, Hb, TPC and differential leucocyte count (DLC) using standard manual procedures described by Benjamin (2001).

Analysis of biochemical parameters: For serum biochemical parameters, about 2 ml of blood was collected aseptically in vacautainer tubes kept for 10 min at room temperature and then centrifuged at 3,000 rpm for 5 min to get a clear serum. Serum samples were analyzed for enzymatic activities of ALT, AST, ALP and CK. The activity was determined spectrophotometrically using coral clinical system kits and results were expressed as international units per liter at 25°C.

Histopathology: The tissues were taken in 10% neutral buffered formalin for preparation of paraffin embedded sections of 5 µm thickness and stained with haematoxylin and eosin (H&E).

Statistical analysis: The data was analysed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA). Two-way ANOVA was conducted to examine the effect of treatment and different time on dependent variables. The value was expressed as mean±SE and significant level was kept as 0.05 (P≤0.05). Multiple comparisons were done using Sidak's post hoc tests.

RESULTS AND DISCUSSION

In present study, BT disease was produced in the infected animals with varying degrees of outcome. The infected animals exhibited rise in body temperature (103 to 104.5°F) on second day of BTV infection and slowly regained normal temperature on 7th day onwards. The swelling of nose, lips, face, congestion of mucous membranes, cyanosis and coronary band congestion with associated lameness in few animals were seen on third day onwards (Fig. 1). Grossly, spleen and lymph node were congested and edematous, and

Table 1. Different haematological values at various time intervals (Mean±SE)

Group	Day 0	Day 1	Day 2	Day 3	Day 7	Day 11	Day 14	Day 21	Day 45
Total leucocyte count (×10^3)									
BTV infected	9.09±0.41	11.18±1.11	9.32±0.61	6.91±0.21*	10.55±0.53	13.23±0.25 ^{a}	11.32±0.83	10.16±0.54	10.6±0.44
Control	9±0.53	9.78±0.95	10.3±0.55	10.12±0.36	10.3±0.47	9.87±0.34	10.07±0.52	10.2±0.55	10.1±0.15
Total platelet count (×10^5)									
BTV infected	3.29±0.18	2.98±0.2	2.44±0.13^{a}	2.23±0.12^{*a}	1.78±0.07^{*a}	1.8±0.09^{*a}	2.68±0.17^{a}	3.16±0.19	3.6±0.36
Control	2.79±0.21	2.87±0.12	2.66±0.16	3±0.1	2.85±0.09	3.29±0.19	3.17±0.16	3.13±0.25	3.14±0.18
Haemoglobin (g %)									
BTV infected	12.1±0.5	11.1±0.6	10.5±0.7	9.8±0.7^{*a}	9.4±0.5^{*a}	8.8±0.5^{*a}	8.4±0.4^{*a}	9.8±0.5*	11.2±1.2
Control	13±0.2	12.6±0.7	12.4±1	12.6±0.6	12.8±0.5	12.8±0.3	13.1±0.6	12.4±0.4	12.2±0.6
Packed cell volume (%)									
BTV infected	34.29±1.37	34.07±1.89	32.92±1.12	32.92±1.31	28.09±0.88^{a}	27.22±1.18^{a}	27±1.17^{a}	31.43±1.82	35±0.58
Control	34.17±2.3	37±2.7	37.2±2.87	34.2±2.42	35.25±2.56	34.33±2.91	32±3.51	32.33±3.93	34.67±1.33
Lymphocytes (%)									
BTV infected	56.57±2.8	55.71±4.54	46.31±3.02	46.67±2.58*	51.27±2.24	67.22±2.27	60.11±5.99	59.57±1.56	61.33±1.76
Control	55.5±4.57	56.33±7.82	59.8±3.68	63.8±3.02	58.25±2.29	61.67±1.45	58±2.31	60.33±1.86	58.33±4.84
Neutrophils (%)									
BTV infected	40.5±2.72	40.79±4.65	51.69±3.28	49.33±2.82*	46.64±2.36	30.67±2.19	36.22±6.11	37.86±1.86	36±1.53
Control	38.67±4.7	38.67±7.89	36±3.78	30.8±3.01	36.5±2.63	33±1.73	37±2.52	35±2.08	36.67±5.46
Monocytes (%)									
BTV infected	2.36±0.23	3.07±0.47	1.92±0.47^{a}	3.92±0.4	1.91±0.41	2.11±0.26	3.11±0.51	2.57±0.37	2.67±0.33
Control	4±0.52	3.17±0.48	2.4±0.81	4±0.71	3.5±0.5	4±0	3.67±0.88	3.67±0.33	4±0.58

*****Values bearing asterisk differs significantly (P≤0.05) between groups within same day. **[a]**Values bearing latter differ significantly (P≤0.05) from 0 day within group.

microscopically showed depletion of lymphoid cells (Fig. 2). On histopathological examination, lungs showed thickening of interalveolar septa, presence of serosanguinous fluid in alveolar lumen, congestion and haemorrhage were found at many places (Fig. 3). In liver, congestion and diffuse degeneration of hepatocytes were seen (Fig. 4). Kidney showed distended and degenerated tubules with vascular congestion (Fig. 5). In present study, the disease was confirmed by detection of BTV nucleic acid in blood and tissues of lungs, pulmonary artery, spleen, lymph node and skin by RT-PCR using group specific primers of BTV.

Blood parameters: Infected group showed gradual decrease of total leukocyte count (Table 1) from 2 to 7 dpi, reaching significantly lower values on 3 dpi (P≤0.05). Thereafter, counts significantly increased on 11 and 14 dpi with respect to pre-inoculation values. Subsequently on 21 and 45 dpi TLC values were normal. In present study, TLC values significantly decreased in BTV infected group at the height of temperature on day 3 followed by increased trend in the values on regaining the normal body temperature. The high body temperature was recorded in infected group from 2 to 4 dpi which indicated the development of viraemia and resultant leucopenia. These findings were similar to those described by Luedke *et al.* (1964), who reported leucopenia in sheep during day 3 to 5 post-infection, whereas some researchers found that leucopenia was observed around 5 to 7 dpi (Jeggo *et al.* 1986, Ellis *et al.* 1990, McColl and Gould 1994), which were a little late response as compared to present study. The cause of the leucopenia during early period of BT infection was probably due to the replication of BTV in leucocytes or in stem cells of haemopoietic system (Singh *et al*. 2008).

TPC values revealed decreasing trend in infected groups. TPC values differs significantly (P≤0.05) in BTV infected group between 2 to 14 dpi compared to pre-inoculation values as well as control group. From 3 to 11 dpi, the values were significantly low (P≤0.05) in BTV infected group with respect to uninfected control group. However, the values returned to normal level on day 14th onwards. Similar observation was also made by Luedke *et al.* (1964), who reported marked thrombocytopenia in the naive sheep on days 8 and 11 after inoculation. BTV-induce suppression of bone marrow has not been established however, because virus has direct association with circulating platelets, it was postulated that both virus and platelets may originate from the same site that is megakaryocytes (Ellis *et al.* 1990). In addition, McColl and Gould (1994) supported the association between platelets and BTV by PCR. BTV replication in the blood platelets during viraemic stage might also responsible for thrombocytopenia (Gibbs and Greiner 1988, McColl and Gould 1994). Virus-mediated endothelial injury leading to consumptive coagulopathy (disseminated intravascular coagulation) can also contribute to the thrombocytopenia (MacLachlan 2004). Haemorrhages and congestion observed in the lungs and kidney of all the infected animals also supported the thrombocytopenia. A number of *in vitro* (DeMaula *et al.* 2002) and *in vivo* studies (Sanchez-cordon *et al*. 2013) suggest that BTV-induced vasoactive cytokines may contribute to endothelial cell dysfunction and increased vascular permeability during BT. Further research should be carried out in order to clarify the influence of these

chemical mediators on platelet function and determine the role of thrombocytopenia in the appearance of vascular lesions in BT. Further BTV replicate in the blood platelets and leucocytes during viraemic stage, which is responsible for leucopenia and thrombocytopenia (McColl and Gould 1994, Gibbs and Greiner 1988).

Haemoglobin values in BTV infected animals revealed decreasing trend between 3 to 21 dpi and significantly low (P≤0.05) values were noticed on 3, 7, 11 and 14 dpi as compared to pre inoculation values within group. PCV values also showed similar decreasing pattern from 7 to 14 dpi as compared to 0 dpi in BTV infected group. Values were significantly low (P≤0.05) on 7, 11, and 14 dpi in BTV infected group. However, the PCV values of BTV infected group were not significant from uninfected control group but it was decreasing in trend over time interval in BTV infected group with respect to uninfected group. The experimentally infected group registered significantly low haemoglobin and PCV values from 3 to 14 dpi and then regained normal levels by 21 dpi. The low values for these parameters were as per the expectation as BTV virus remained attached to RBC where it might have caused destruction of these cells. Similarly, Luedke *et al.* (1964) reported haemolytic anaemia based on packed cell volume, haemoglobin and icterus index.

On DLC, lymphocytes count showed decreasing trend over time interval in the BTV infected group on 2 dpi (46.31%) and 3 dpi (46.67%), and it was significantly low (P≤0.05) at 3 dpi as compared to uninfected control group. Then, lymphocytes counts recovered from 7 dpi onwards following a similar progress to that shown by leukocytes. Subsequently, marked lymphocytosis was observed on day 11 dpi. Whereas, neutrophil count in infected group increased on 2 and 3 dpi and significantly increased (P≤0.05) on day 3 as compared to uninfected control group. Subsequently, neutrophil count decreased and marked neutropenia was observed on 11 dpi in BTV infected group. Overall, in BTV infected group, there was lymphocytosis and count varied from 46 to 67% with the corresponding decrease in neutrophil count (30–49%). Monocyte count showed a decreasing trend over time in BTV infected group on 2 and 7 dpi as compared to control group. However, total eosinophil and basophil counts remained unchanged in infected sheep throughout experiment. Following infection with BTV serotype 2, transient leukopenia during initial stage of the disease (between 2 to 7 dpi) was observed which was associated with an intense lymphopenia in BTV infected group during the same period of time. Transient leukopenia and lymphopenia had been reported by few authors in sheep during infections with several BTV serotypes (MacLachlan 2004, Singh *et al*. 2008). The present study constitutes the first detailed characterization of haematological changes in sheep infected with BTV-2. However, the mechanisms involved in lymphopenia have not been understood yet. One of the mechanisms proposed for BTV-induced immuno-suppression and lymphoid depletion includes lymphocyte apoptosis (Owens *et al.* 2004)

Serum biochemistry: AST, ALT, ALP, and CK (Table 2) showed increasing trend in the infected group over a period of time. The amount of any single enzyme in serum indirectly reflects its concentration in the cell, extent of cell injury, normal cell death, and its degradation in plasma (Kaneko *et al.* 1997). The higher concentrations of the serum enzymes noticed in present study are not unusual, similar higher activity of some enzymes like lactate dehydrogenase, aldolase, CK and AST in BT infected sheep were observed by Jeggo *et al.* (1986). Similar activity of some enzymes, viz. ALT, AST, ALP, CK in experimentally BT infected sheep was recorded by Singh *et al*. (2008).

The AST values showed increasing trend over time from 3 to 21 dpi and significant increase (P≤0.05) was noticed at 11 dpi (118.67 IU/l) and 14 dpi (109.78 IU/l) in BTV infected group as compared to uninfected control group. The values were significantly high (P≤0.05) on 3 to 21 dpi and varied between 92.67 to 118.67 IU/l as compared to day 0 of BTV infected group. AST is known to present in various tissues like liver, heart, skeletal muscle, kidneys, brain, pancreas, lungs and in white and red blood cells. The presence of AST is a sensitive marker of soft tissue damage

Table 2. Different serum values at various time intervals (Mean±SE)

Group	Day 0	Day 1	Day 2	Day 3	Day 7	Day 11	Day 14	Day 21	Day 45
Aspartate amino transferase (AST) (IU/l)									
BTV infected	81.5±1.8	80.93±1.42	83.69±5.31	92.67±1.43[a]	105.45±2.73[a]	118.67±3.31[*a]	109.78±2.97[*a]	94.71±5.1[a]	77±1.53
Control	85±2.02	82.67±1.38	84±2.51	87.2±2.4	89.75±6.5	83.33±5.78	88±3.79	87.67±4.37	82.67±5.24
Alanine amino transferase (ALT) (IU/l)									
BTV infected	24.36±0.98	25.57±1.03	25.85±0.95	32.75±1.35[a]	46.45±1.65[*a]	58.78±3.21[*a]	58.89±2.94[*a]	51.71±2.46[*a]	29.67±1.67
Control	22.17±1.25	23±2.27	21.4±0.98	25±2.35	24±1.78	22.67±2.33	23.67±4.26	24.33±3.93	29.67±0.67
Alkaline phospatase (ALP) (IU/l)									
BTV infected	155.93±2.35	156.29±3.05	169.85±3.73	184.17±3.04[*a]	202.91±4.17[*a]	201.89±2.54[*a]	175.56±5.21[a]	153.29±8.73	146.67±9.7
Control	158.67±8.47	155±9.19	161±9.07	155.8±7.08	158.75±8.53	166.67±5.36	176.33±4.26	168.33±6.49	154.33±2.73
Creatine kinase (CK) (U/l)									
BTV infected	107.43±2.19	107.14±2.91	104.31±1.78	110.83±2.01	181.55±7.65[*a]	186.44±7.72[*a]	187±5.35[a]	109.29±4.31	96.67±6.69
Control	113.83±3.67	114±2.14	117.6±6.27	111.8±4.47	116.5±7.96	122±6.08	118±9.64	108±2.31	112.67±3.18

*****Values bearing asterisk differs significantly (P≤0.05) between groups within same day. **[a]**Values bearing latter differ significantly (P≤0.05) from 0 day within group.

although it is not an organ-specific enzyme (Boyd 1983). In present study, the high levels of AST in BTV infected group indicated the damage to soft tissues like lymphoid organs (spleen and lymphnodes), kidney, liver, lungs and striated muscles. These findings were in agreement with the results of earlier workers (Jeggo *et al.* 1986, Singh *et al*. 2008) and further supported by gross and histopathological findings in present study. Grossly, spleen and lymph node were enlarged and microscopically showed depletion of lymphocytes followed by hyperplasia of germinal follicles (Fig. 2).

The ALT values were significantly high (P≤0.05) in BTV infected group from 7 to 21 dpi which varied between 46.45 to 51.71 IU/l and peak values noticed on 11 and 14 dpi as compared to uninfected control group. The ALT values were significantly high (P≤0.05) on 3 to 21 dpi and varied between 32.75 to 58.89 IU/l as compared to day 0 of BTV infected group. ALT and AST are two of the most reliable markers of hepato-cellular injury or necrosis. However, higher levels of AST and ALT were also documented to be associated with muscle damage (Valentine *et al*. 1990). In present study, high levels of ALT observed in the BTV infected group are indicative of hepatic injury. Of the two, ALT is thought to be more specific for hepatic injury in primates, dogs and cats, whereas it is of little diagnostic importance in ruminants (Boyd 1983). In present study, the high levels of ALT and AST indicated hepato-cellular and muscle injury which was proved on microscopic findings of liver, trapezius muscles and tongue musculature.

ALP values also showed increasing trend from day 3 to 14 and then returned to the normal levels in BTV infected group. But, a highly significant increase (P≤0.05) was recorded in BTV infected group on day 3 (184.17 IU/l), 7 (202.91IU/l) and 11 (201.89 IU/l) as compared to uninfected control group. Though ALP is known to be present in all tissues, the greater activity of this enzyme is seen in kidney, liver and intestinal tissues. Singh *et al*. (2008) found lesions in kidney and liver, which might have been contributed to the high level of this enzyme in the serum. During present study, values of ALP were significantly higher in BTV infected group than uninfected control group, indicating damage of kidney and liver tissues.

CK values also revealed an increasing trend in the infected group from day 7 to 14. The values were significantly high (P≤0.05) in BTV infected group on days 7 (181.55 IU/l) and 11 (186.44 IU/l). CK is reported to be present in many cell types but its highest activity seen in skeletal muscles. Skeletal muscle degeneration and necrosis are related to increased activity of CK in BTV infection (Jeggo *et al*. 1986, Singh *et al*. 2008). In our study, the muscle changes like hyaline degeneration and necrosis with infiltration of mononuclear cells were seen and these were possibly responsible for significantly high level of CK in the BT infected animals. The muscle damage was further supported by increased level of AST. Flanagan *et al*. (2008) also reported significant changes in plasma enzyme concentrations of AST and CK in the infected sheep. The higher concentrations of these serum enzymes are not unusual and were in close agreement with the findings of Jeggo *et al.* (1986). The increased CK, AST levels were apparently related with muscle damage and the increased ALT values appeared to be due to hepatic damage.

In conclusion, changes in haematological parameters, mainly TLC, PCV, Hb, TPC and differential leucocyte count under the study related to BTV induced vascular endothelial damage resulted in changes to capillary permeability and fragility with lymphoid depletion in spleen and lymph nodes. Significant alterations in biochemical parameters, namely ALT, AST, ALP and CK were observed in the experimentally infected sheep indicative of cellular and tissue injury in liver, skeletal and cardiac muscles damage caused by BTV infection.

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