Evaluation of oxidative stress via total antioxidant status, sialic acid, malondialdehyde and RT-PCR findings in sheep affected with bluetongue

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ABSTRACT

Introduction: Bluetongue (BT) is a non-contagious infectious disease of ruminants. The disease agent bluetongue virus (BTV) is classified in the *Reoviridae* family *Orbivirus*.

Aims and objectives: The aim of this study was to determine serum malondialdehyde (MDA), total antioxidative stres (TAS), total sialic acid (TSA), ceruloplasmin, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), cholesterol, creatinine, albumin, and total protein levels in sheep with and without bluetongue (BT).

Materials and Methods: The study included 13 Sakiz crossbreed sheep, aged 1–4 years and usually in the last stage of pregnancy, as the BT group and a control group consisting of 10 healthy sheep. All sheep were clinically examined before collecting blood samples. Serum ALT, AST, cholesterol, triglyceride, albumin, GGT, total protein, creatinine and TAS levels were measured using commercially available kits as per manufacturer's recommendations using a Biochemistry Auto Analyzer (Sinnowa D280, China). Serum lipid peroxidation was estimated through a previously described method in which MDA reacts with thiobarbituric acid (TBA) to form a coloured complex at a maximum absorbance of 535 nm. The TSA value was measured at 549 nm using the method described by Warren (1959): sialic acid was oxidised to formyl-pyruvic acid, which reacts with TBA to form a pink product. The ceruloplasmin concentration was measured according to Sunderman and Nomoto (1970): ceruloplasmin and p-phenylenediamine formed a coloured oxidation product that was proportional to the concentration of serum ceruloplasmin. Real time RT-PCR and conventional RT-PCR were performed as described by Shaw and others (2007).

Results: Biochemistry analysis of serum showed that in the BT group, TSA, MDA, triglyceride and ALT and AST were higher and that ceruloplasmin and TAS were lower than in the control group. Serum albumin, cholesterol, creatinine, total protein and GGT did not differ significantly between the two groups.

Conclusions: Serum triglyceride, ceruloplasmin, TSA, MDA and TAS concentrations may prove beneficial to the diagnosis, prognosis and biochemical analysis of BT.

INTRODUCTION

Bluetongue (BT) is a non-contagious infectious disease of ruminants and camelids (Elbers and others 2008). The disease agent bluetongue virus (BTV) is classified in the *Reoviridae* family *Orbivirus* and has 26 serotypes (Maan and others 2011). BTV is transmitted by *Culicoides* biting midges (Elbers and others 2008). BTV is widely distributed in the tropics and subtropics due to the existence of *Culicoides* species (Afshar 1994). Although BTV infection can be endemic, epidemic or non-existent at lower latitudes and altitudes, it can be seen far beyond the traditional range because of the spread of the midges that are involved in transmission (Afshar 1994, Sperlova and Zendulkova 2011).

BTV-related endothelial injury may cause clinical signs and lesions during replication of the virus (Mahrt and Osburn 1986). Furthermore, endothelial injury may result in oedema, increased vascular permeability, and vascular thrombosis, causing tissue infarction. BT is characterised by fever, lameness due to inflammation of the coronary band, oedema of the lips, catarrhal stomatitis, nasal discharge, torticollis, arthrogryposis hydranencephaly syndrome, decreased milk production, loss of condition and death in sheep (Charanasomboon 1985, Van Aert and others 2008).

The acute phase response is caused by non-specific inflammation. When the acute phase response emerges, acute phase proteins are synthesised in the liver with increased blood concentrations (Murata and others 2004). Ceruloplasmin, an acute phase protein, is an α 2 globulin that has only one polypeptide chain (Coskun and Sen 2005). It plays an important role in lipid peroxidation, oxidation of toxic ferrous ions to non-toxic ferrous ions, and inhibition of free radical production (Murata and others 2004, Coskun and Sen 2005).

Oxidative stress may cause lipid peroxidation and an increase in the malondialdehyde (MDA) concentration, and may cause cytotoxicity (Chaudhary and others 1994). These changes of membrane lipid composition may be induced by free radical initiated lipid peroxidation. This view is supported by increased MDA, one of the aldehydic products of lipid peroxidation, levels in serum, heart, lung, liver and kidney of hypoxic rats (Nakanishi and others 1995).

Antioxidants play an important role in protecting against the deleterious effects of oxidants in organs such as the lungs and kidneys. As well as reduced by oxidants, their serum level and total antioxidant stress decrease gradually (Castillo and others 2003, Aytekin and others 2010, 2011).

Sialic acid is a neuraminic acid derivation and is present in abundance in cell membranes (Taniuchi and others 1981, Schauer 1982, Haq and others 1993). Sialic acid levels increase rapidly in pathologic conditions such as inflammation, tissue degeneration and proliferation (Haq and others 1993).

The diagnosis of BT is often challenging through biochemistry analysis due to the lack of relevant research findings; as such, the aim of the present study was to determine the serum MDA, total antioxidative stress (TAS), total sialic acid (TSA), ceruloplasmin, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), cholesterol, creatinine, albumin, and total protein levels in sheep with and without BT.

MATERIAL AND METHODS
Animals
The study included 13 Sakiz crossbreed sheep, aged one to four years and usually in the last stage of pregnancy, as the BT group and a control group consisting of 10 healthy sheep. All sheep were clinically examined before collecting blood samples. The study was approved by the Food Agriculture and Livestock Ethics Committee (No. 780 and dated 8 September, 2011).

Serum biochemistry analysis
Blood samples were collected from the jugular vein and kept for two hours at room temperature for proper clotting. The samples were then centrifuged at 2500 g at 4°C for 15 minutes and stored at −20°C until analysed. Serum ALT (Archem, Istanbul, Turkey), AST (Archem), cholesterol (Archem), triglyceride (Archem), albumin (Archem), GGT (Archem), total protein (Archem), creatinine (Archem) and TAS (Real Assay Diagnostics, Istanbul, Turkey) levels were measured using commercially available kits as per manufacturer's recommendations using a Biochemistry Auto Analyzer (Sinnowa D280, China).

Serum lipid peroxidation was estimated through a previously described method (Yoshıoka and others 1979) in which MDA reacts with thiobarbituric acid (TBA) to form a coloured complex at a maximum absorbance of 535 nm. The TSA value was measured at 549 nm using the method described by Warren (1959): sialic acid was oxidised to formyl-pyruvic acid, which reacts with TBA to form a pink product (Warren 1959). The ceruloplasmin concentration was measured according to Sunderman and Nomoto (1970): ceruloplasmin and p-phenylenediamine formed a coloured oxidation product that was proportional to the concentration of serum ceruloplasmin.

Statistical analysis was performed using SPSS V.11.5 for Windows (SPSS Inc., Chicago, Illinois, USA). Results were statistically analysed using the independent samples t test.

Real-time RT-PCR and conventional RT-PCR
RNA extraction was performed using the blood samples obtained from 13 sheep suspected to have BT through a High Pure Viral Nucleic Acid Kit (catalogue number: 11 858 882 001, Roche, Germany) in accordance with the manufacturer's recommendations. The RNA samples that were obtained were stored at −20°C until analysed. Real-time RT-PCR was performed using BTV SEG1 RSA and BTV SEG1 UNI primers and BTV SEG1 probe (Table 1). Real time RT-PCR and conventional RT-PCR were performed as described by Shaw and others (2007).

Conventional RT-PCR was used for the sequence analysis of positive samples obtained via real-time RT-PCR using ORBI-UNI-F and ORBI-UNI-R sequence primers and a Qiagen OneStep RT-PCR Kit (catalogue number: 210212, Qiagen, Germany), which amplifies a 412 bp section of the orbivirus segment 1 gene section, as described by Shaw and others (2007).

Sequence analysis
The sequencing analysis was performed using purified PCR products. Sequence assembly and editing were performed using BioEdit V.7.0.5.3 (Hall 1999), and the sequence was aligned using the web-based BLAST programme (www.ncbi.nlm.nih.gov/blast). The nucleotide sequence, TR-AFYON2012, was published with the accession number KC924402. The maximum similarity of KC924402 among previously published BTV sequences in GeneBank was 97 per cent. Phylogenetic analysis was carried out using the Mega5 programme, based on matching between the nucleotide sequence, has 412 bp. The construction of a phylogenetic tree was performed via the neighbour-joining (NJ) method using the Kimura 2-parameter model in Mega5 V.5.0 (Tamura and others 2011). The confidence of the NJ tree was assessed by bootstrapping, using 1000 replicates.

RESULTS
Clinical findings
Anorexia, dehydration, oedema of the lips, oral ulceration and fever, and inflammation of the coronary band

TABLE 1: Primer and probe sequences used for RT-PCR, sequencing and real-time RT-PCR assays for the detection of BTV

Purpose		Oligo name	Sequence (5′–3′)	Location*
BTV SEG1 RSA (east)	Forward primer BTVrsa	BTVrsa 291–311F	GCGTTCGAAGTTTACATCAAT	291–311
	Reverse primer BTVrsa	BTVrsa 387–357R	CAGTCATCTCTCTAGACACTCTATAATTACG	387–357
BTV SEG1 UNI (west)	Forward primer BTVuni	BTVuni 291–311F	GCTTTTGAGGTGTACGTGAAC	291–311
	Reverse primer BTVuni	BTVuni 381–357R	TCTCCCTTGAAACTCTATAATTACG	381–357
BTV SEG1 probes	Probe RSA	RSA-BTV 341–320	CGGATCAAGTTCACTCCACGGT	341–320
	Probe 323	BTV 346–323	TCCTCCGGATCAAGTTCACTCCAC	346–323
RT-PCR +sequencing	Forward primer ORBI-UNI	ORBI-UNI-F	YMATCACCGTGCAAGGT	19–35
	Reverse primer ORBI-UNI	ORBI-UNI-R	TGCATYTCGTTTTTMGC	430–414

*Genome location according to GenBank accession number AY154458
BTV, bluetongue virus

were observed in the BT group. All of the 13 infected sheep had oedema of the lips, cyanosis of the tongue and oral mucosa, oral ulceration, fever and lethargy.

Biochemistry findings
Biochemistry analysis of serum showed that in the BT group, TSA (P<0.01), MDA (P<0.001), triglyceride (P<0.001), and ALT and AST (P<0.05) were higher and that ceruloplasmin and TAS (P<0.01) were lower than in the control group (Table 2). Serum albumin, cholesterol, creatinine, total protein and GGT did not differ significantly between the two groups (Table 2).

Real time RT-PCR
Using the real time RT-PCR technique, Afyon 1, Afyon 8, Afyon 9 and Afyon 11 samples, and the negative control sample were negative for any CT. The CT value of positive samples and the positive control sample ranged from 20.26 to 25.91.

Conventional RT-PCR
Conventional RT-PCR findings were compatible with those obtained via real-time RT-PCR (Figs 1 and 2).

DISCUSSION
Real-time RT-PCR and conventional RT-PCR were used for the definitive aetiological diagnosis of BT. In recent years, several conventional RT-PCR methods have been used to detect the RNA of BTV serotypes. Conventional RT-PCR can be combined with sequence analysis to study the genetic diversity and molecular epidemiology of BTV isolates. Current RT-PCR methods for BTV detection are sensitive and specific; however, they are not well suited to high-throughput analysis and have

TABLE 2: Oxidant, antioxidant and biochemistry parameters in BT sheep and control group

Parameters	Control group (n=10) $\bar{X} \pm S_{\bar{x}}$	BT group (n=13) $\bar{X} \pm S_{\bar{x}}$	P values
Ceruloplasmin (mg/dl)	41.53±4.39	25.59±1.50	**
Total sialic acid (µg/ml)	547.58±50.32	903.72±70.35	**
MDA (µmol/l)	6.20±0.85	15.61±0.92	***
TAS (mmol Trolox Eq/l)	0.57±0.01	0.48±0.01	**
Albumin (g/dl)	2.29±0.04	2.16±0.06	NS
ALT (U/l)	18.10±0.86	22.70±1.36	*
AST (U/l)	89.80±4.32	114.81±10.53	*
Cholesterol (mg/dl)	63.80±2.40	71.36±5.05	NS
Creatinine (mg/dl)	0.73±0.02	0.64±0.04	NS
GGT (U/l)	50.60±4.28	45.63±3.46	NS
Total protein (g/dl)	6.72±0.23	6.34±0.24	NS
Triglyceride (mg/dl)	28.00±4.48	73.18±8.15	***

*P<0.05; **P<0.01; ***P<0.001
BT, bluetongue; MDA, malondialdehyde; NS, not significant; TAS, antioxidative stress
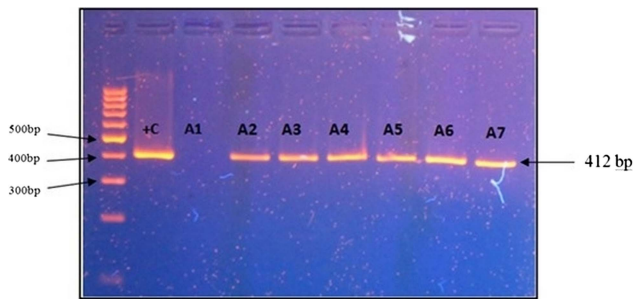
FIG 1: Agarose gel electrophoresis of bluetongue virus (BTV) Seg1 gene based RT-PCR products amplified from field samples. Lane L: 100 bp DNA ladder; lane 1: positive control (+C); lane 2 field sample (Afyon 1): negative; lane 3, 4, 5, 6, 7, 8 field samples (Afyon 2, Afyon 3, Afyon 4, Afyon 5, Afyon 6, Afyon 7): positive

several disadvantages, including the risk of potential cross contamination during sample preparation, and being time consuming (Knowles and Samuel 2003, Jiménez-Clavero and others 2006). As a result, the real-time RT-PCR technique is particularly attractive. When compared with conventional RT-PCR, the real-time method has greater sensitivity and specificity, it is much faster and has less risk of cross contamination because it does not require electrophoresis in agarose gels (Orru and others 2006, Jiménez-Clavero and others 2006, Shaw and others 2007). Because of its advantages over conventional RT-PCR, real-time RT-PCR has been adopted as a useful method for diagnostic purposes (Kimura and others 1999). Although real-time RT-PCR is more sensitive and specific than conventional RT-PCR, the same results were found to be positive by both techniques in this study for nine of the 13 samples. One BTV nucleotide sequence gathered from this study was published in GenBank with accession number KC924402.

The common clinical findings in animals with BT include oral ulceration, catarrhal stomatitis, oedema of the lips, arthrogryposis hydranencephaly syndrome, inflammation of the coronary band, fever, lethargy, decreased milk production and death (Charanasomboon
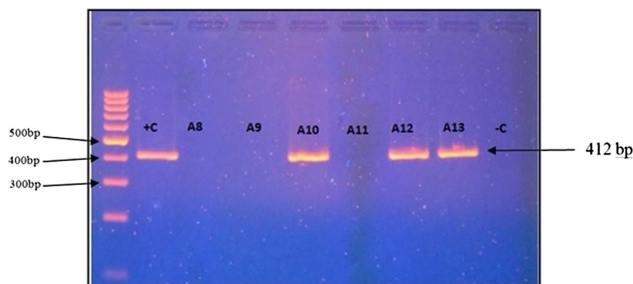

FIG 2: Agarose gel electrophoresis of bluetongue virus (BTV) Seg1 gene based RT-PCR products amplified from field samples. Lane L: 100 bp DNA ladder; lane 1: positive control (+C); lane 2, 3 field samples (Afyon 8, Afyon 9): negative; lane 4, 5, 6, 7 field samples (Afyon 10, Afyon 11, Afyon 12, Afyon 13): positive; lane 8: negative control (–C)

1985, Van Aert and others 2008), which were all observed in the present study.

Plasma concentrations of liver enzymes rapidly and significantly increase in cases of liver damage (Braun and others 2010). Tissue infarction and lipid peroxidation are closely associated with hepatic injury and release of liver enzymes, including ALT and AST, following hepatocyte degeneration (MacLachlan and others 1989, Draper and Hadley 1990). Additionally, inefficient scavenging of reactive oxygen species may cause both oxidative liver damage and increased liver enzyme activity (Sanchez-Compos and others 1999). In the present study, serum AST and ALT concentrations increased in BT ewes compared with healthy controls (Table 2). Atakisi and others (2009) noted an increase in triglyceride levels in ewes in their study on β-hydroxybutyric acid, glucose and triglyceride levels before pregnancy, during pregnancy and after birth. Similarly, Balikci and others (2007) studied certain blood metabolite concentrations, such as glucose, albumin, cholesterol and triglyceride, during pregnancy and postpartum in Sakiz crossbreed ewes, and reported increased triglyceride levels. In the present study, our finding that the sheep in the BT group had elevated serum triglyceride levels agrees with what has been reported by others.

Whilst acute phase proteins do not have sufficient specificity, they are very good indicators of inflammation and tissue injury (Eckersall and Bell 2010). Sanchez-Cordon and others (2013) reported that there was no significant difference in the serum ceruloplasmin level between sheep experimentally infected with BTV-1 and BTV-8. In addition, Aytekin and others (2011) did not observe a significant difference in the ceruloplasmin concentration between sheep with and without naturally contracted ammonium sulphate poisoning. These findings suggest that the serum ceruloplasmin levels were low, however the findings also indicated that other acute phase proteins were elevated. Furthermore, the long storage process of samples may cause this situation as shown by some authors. Meling and others (2012) reported that low serum ceruloplasmin levels were due to holding samples for more than a year in their study on scrapie.

Simsek and others (2006) reported that there was a positive correlation between high MDA levels and liver injury in sheep due to *Dicrocoelium dendriticum*, a trematode that causes hepatic degeneration. Similarly, Kucukkurt and others (2014) found increased concentrations of MDA in naturally infected goats with *Babesia Ovis*, a blood parasite that causes erythrocyte degeneration. Additionally, Crnogaj and others (2010) reported that the increase in the MDA concentration in dogs infected with *Brucella canis* could be associated with increased lipid peroxidation. Furthermore, Aytekin and others (2011) observed a high MDA concentration in lambs due to ammonium sulphate poisoning, which resulted in depletion of adenosine triphosphate and eventually death. This oxidative burden could be

secondary to the heightened state of inflammation which causes the influx and activation of macrophages, neutrophils and eosinophils to the airways. These inflammatory cells are rich sources of reactive oxygen species and exert direct oxidative damage characterised by increased lipid peroxidation, and vascular and tissue permeability (Dworski 2000, Harik-Khan and others 2004). These changes of membrane lipid composition may be induced by free radical initiated lipid peroxidation. This view is also supported by increased MDA levels, one of the aldehydic products of lipid peroxidation, in serum, heart, lung, liver and kidney of hypoxic rats (Nakanishi and others 1995). Lipid peroxidation can contribute to hepatic injury (Draper and Hadley 1990) and this can result in increased serum liver enzymes and MDA levels. In the present study, high levels of MDA and liver enzymes were observed in the BT group, which suggests that tissue infarction and lipid peroxidation were due to BTV.

Wachter and others (1999) observed a decrease in antioxidant activity due to progressive lactation periods in their study on the genetics of antioxidant activity in Holsteins and Jerseys. Castillo and others (2003) reported that the study was carried out in 22 healthy dairy cows divided into two groups: animals with a low production rate and animals with a high milk yield. Results showed that the animals with a high milk yield present higher lipid hydroperoxides levels than the other group. This increase in oxidant compounds is not accompanied by higher levels in protective antioxidant substances. Increased oxidative stress, elevated systemic inflammation and decreased antioxidant defences were common in end-stage disease, particularly in patients with chronic obstructive pulmonary disease and ischaemic heart disease (Stanojkovica and others 2011). Olisekodiak and others (2012) observed decreased TAS levels in rats exposed to intraperitoneal injection of Cd and this could be due to the participation of the body's antioxidant system in combating the increased free radical load and probably the resultant oxidative stress created by the Cd toxicity. The total antioxidant status level decreases due to increased levels of oxidants as lipid hydroperoxides and MDA (Castillo and others 2003). The observed dyslipidaemia and decrease in TAS could be due to increased free radical production causing oxidative stress (Olisekodiak and others 2012). In the present study, TAS levels were lower and MDA levels were higher in the BT group, strongly suggesting that components of TAS are consumed by MDA and reactive oxygen species, which is in agreement with previous reports (Wachter and others 1999, Castillo and others 2003, Stanojkovica and others 2011, Olisekodiak and others 2012).

Evaluation of the TSA concentration may be useful for diagnosing inflammatory diseases due to the increase in sialic acid during the inflammatory process, such as tissue degeneration and proliferation (Singh and others 1980, Haq and others 1993, Citil and others 2004).

Varma and others (1983) studied the protein-bound carbohydrates of seromucoid in normal human serum and reported that many acute phase proteins contain sialic acid. Due to acute phase proteins in the form of glycoprotein, an increase in these proteins in circulation affects the serum TSA level (Taniuchi and others 1981). Wakabayashi and others (1992) studied the relationship between serum sialic acid and lipid concentrations in humans, and reported that there was a positive correlation between serum sialic acid and serum triglyceride levels. Karagenc and others (2005) observed the increased levels of serum sialic acid in acute theileriosis with low percentage of parasitaemia. Increased concentration of sialic acid at the surface of malignant cells in animal and human systems has been related to potential malignancy and changes in immunogenicity (Seyrek and others 2005). The increased TSA levels at the 90th minute continued to increase at the 180th minute and the proceeding reperfusion period in lung injury induced by lower limb ischaemia and reperfusion in rats (Hidıroglu and others 2014). Similarly, in the present study TSA levels were higher in the BT group, as previously reported (Taniuchi and others 1981, Wakabayashi and others 1992, Karagenc and others 2005, Seyrek and others 2005, Hidiroglu and others 2014).

CONCLUSION

The literature includes many studies on the serotype, epidemiology, RT-PCR and virological qualifications of BTV; however, few have investigated biochemical parameters, such as MDA, TAS, ceruloplasmin, TSA, triglyceride, ALT, AST, GGT, cholesterol, creatinine, albumin, and total protein levels, which were investigated in the present study. The biochemical analysis of serum samples showed that TSA, MDA and triglyceride levels, and ALT and AST activity were higher and that ceruloplasmin and TAS concentrations were lower in the BT group compared with the control group. BT can cause serious clinical symptoms and changes in total antioxidant and oxidant levels in sheep. Serum triglyceride, ceruloplasmin, TSA, MDA and TAS concentrations may prove beneficial to the diagnosis, prognosis and biochemical analysis of BT.

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