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Clinico-Biochemical, Serological and Molecular Study on Tropical Theileriosis in Egyptian Water Buffaloes (*Bubalus Bubalis*)

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Key words:
Biochemical Changes; Clinical, ELISA; PCR, Theileriosis; Water Buffalo

ABSTRACT:
The present study was planned to demonstrate the clinical findings, haematological and biochemical changes and compare the accuracy of different techniques using routine Giemsa-stained film, PCR and ELISA for diagnosis of *Theileria annulata* parasitaemia in Egyptian buffaloes. The study was conducted on 42 buffaloes (3-7 years old), which were divided into three groups: clinical theileriosis (n=10), contact (n=22) and control (n=10). Clinical signs included fever, enlargement of prefemoral lymph nodes, corneal opacity, dark feces, conjunctivitis, dyspnea, and drop in milk yield in lactating buffaloes. Blood films demonstrated the macro and microschizont in lymphocytes and piroplasms in erythrocytes. Haematological examination revealed anemia, neutropenia, eosinopenia, lymphocytopenia and monocytopenia in clinical theileriosis group, and not in contact, compared to control. Biochemical analysis revealed significant decrease (P< 0.01) in the total protein, albumin, globulin, glucose and calcium levels; whereas bilirubin, AST, ALT, ALP, GGT and CK, cholesterol, urea, creatinine, and phosphorous were significantly increased (P< 0.01) in clinical theileriosis, and not in contact, compared to control. The prevalence rate of *T.annulata* based upon blood film examination, ELISA and PCR using specific primers were 23.81%, 33.33% and 42.86%, respectively. Therefore, PCR is more reliable and accurate for detection of theileriosis than other methods. Contact buffaloes should be considered as carriers unless the PCR is tested negative.

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1-INTRODUCTION

Bovine theileriosis, a tick-borne protozoal disease, has adverse effect on the productivity of domestic ruminants. *Theileria parva* and *Theileria annulata* are the most pathogenic and responsible for significant economic and production losses in affected animals. *T. annulata* causes lymphoproliferative disease in the early stage of the disease followed by leukopenia and high mortality and morbidity in cattle, commonly known as Tropical Theileriosis**.** *T. annulata* is endemic in the area around the Mediterranean and the Middle East and reaches the Southern parts of Asia**.** Tropical theileriosis acts as a major constraint on livestock production and improvement in many developing countries. In Egypt, the disease is one of the most destructive factors to livestock production (Al-Gaabary, 1995).

Theileria parasites enter the bovine host as sporozoites during tick infestation, and rapidly invade mononuclear leukocytes. They are

transformed into macroschizonts and induce proliferation of the host cell. Macroschizonts develop further into microschizonts and ultimately into merozoites, which are released from the leukocyte. The merozoites invade erythrocytes and develop into piroplasms (Khan *et al*., 2010). Animals with subclinical infection in endemic regions become carrier of piroplasms and act as a source of infection for the vectors, which are *Hyalomma* ticks**.**

Diagnosis of clinical *T. annulata* infection in cattle is based on the routine detection of macroschizonts in Giemsa-stained lymph node biopsy smears and piroplasms in the red blood cells. However, *Theileria* piroplasms may be difficult to demonstrate in stained blood smears. Furthermore, high experience in piroplasm microscopy is required in subclinical or chronic infections because parasitaemias are often extremely low and may otherwise be missed (Aktas *et al*., 2006)**.**

Since accurate diagnosis is a prerequisite for effective management system, this study was carried out to record the clinical findings, the haematological and biochemical changes in buffaloes infected with tropical theileriosis. Further aim was to detect the subclinical or carrier cases and compare the reliability and accuracy of different diagnostic techniques including the routine microscopic examination, the detection of the specific antibodies by ELISA, and of the parasite DNA detection by polymerase chain reaction (PCR) technique.

2-MATERIALS AND METHODS

2.1.Animals and study design:

A total of 42 buffaloes (3-7 years old) were used in this study. Samples were collected from affected animals in a private farm in Qaluobiya governorate and from clinical cases admitted to the veterinary teaching hospital at the Faculty of Veterinary Medicine, Benha University. The affected buffalo showed clinical signs of tropical theileriosis (n=10) including fever, enlarged superficial lymph nodes, corneal opacity, panting and dyspnea. Since carrier animals are always present without clinical manifestation and serve as sources of infection, samples were also collected from the contact animals of the same farm (n=22) to detect the carriers. Contact animals are buffaloes present in the same farm in close proximity to the clinically affected buffaloes but do not express any clinical manifestation, and therefore expected to be carriers. Clinically healthy buffaloes from another farm were used as control (n=10). The control group was examined thoroughly for the presence of any abnormal clinical changes or external parasites, and examined by different laboratory techniques such as direct smear, flotation, sedimentation and blood film to confirm the absence of any internal or haemoparasites

2.2.Blood sampling:

Three blood samples were collected from each animal from jugular vein. Two samples were collected on sodium salt of EDTA for haemogram evaluation (RBCs count, haemoglobin conc., PCV %, total WBCs count and differential leukocytic count) and for PCR test. The third sample was collected without anticoagulant in a clean dry centrifuge tube for serum separation. Samples for serum separation were allowed to coagulate at 37°C for 30 minutes then refrigerated at 4°C for 2 hours for clot retraction then centrifuged at 3000 rpm for 5 minutes. The separated serum samples were aspirated by automatic pipette into clean well dried centrifuge tubes and re-centrifuged at 3000 rpm for 5 minutes. The clear sera were aspirated carefully by automatic pipette and transferred into clean dry labeled Eppendorf tubes and stored at -10°C till examination.

2.3.Preparation and examination of blood film:

Immediately after taking the blood sample from ear vein, three thin blood films were smeared from clinically affected and control buffaloes. To accomplish the comparison study, blood films were prepared from contact animals. The thin blood smears were fixed in methanol for 5 min and stained in Giemsa stain diluted 10% with buffered

water for 30 min. Smears were examined under oil immersion lens for the presence of *Theileria* piroplasms as previously described (Khan *et al*., 2010).

2.4.Haematological examination:

The total erythrocytic count was performed by manual method using improved Neubauer hemocytometer. Haemoglobin (gm/dl) was estimated using cyanomethemoglobin method**.** The PCV% was estimated by microhematocrit method. The red cell indices were calculated from the measured PCV, Hb and RBCs count. The total leucocytic count was performed using improved Neubauer hemocytometer and Turkey's diluting fluid. The blood films were stained by Giemsa stain and differential leucocytic count was conducted by using four-field mender method (Feldman *et al*., 2000).

2.5.Biochemical analysis:

Serum samples were used to determine total protein, albumin, glucose, total and direct bilirubin, ALT, AST, ALP, GGT, CK, urea, creatinine, calcium, phosphorus and cholesterol as previously described (Khan *et al*., 2010).

2.6.Enzyme linked immunosorbent assay (ELISA)

An enzyme linked immunosorbent assay (ELISA) was used to determine antibody levels in buffaloes using antigens prepared from the intra-erythrocytic piroplasm stage of the parasites (whole piroplasm antigen) (Kachani *et al.*, 1996).

2.6.a.Preparation-titration of whole piroplasm antigen

Blood sample were collected from different clinically affected buffaloes, where infection with *T. annulata* was confirmed by PCR using specific primer as described later.The protein concentration was determined by Lowry's method **(**Lowry *et al*., 1951**)**. The protein content of the supernatant was 5.5 mg/ml.

The optimum antigen concentrations was determined by serial doubling dilutions of *T. annulata* piroplasm antigen and known positive and negative control sera, which were obtained from PCR-positive and PCR-negative samples. The optical density was read using ELISA reader at 492 nm. It was found that the optimum antigen concentration was 5 µg/ml. Rabbit anti-bovine immunoglobulin conjugated with horse radish peroxidase enzyme (Sigma, USA) was used as conjugate. The optimum conjugate dilution which gave OD 1 was 1:5000.

2.6.c.ELISA procedure

Sera collected from the infected, contact and control animals were tested against *T. annulata* antigen using the ELISA technique as previously described (Kachani *et al.*, 1996).The optical density was measured at 492 nm using microplate reader (Bio-Tek Instruments, Inc.). A cut-off value for the assay was established as the mean OD of the positive sera plus two standard deviations of these results. Serum was considered positive if yielded an optical density equal to or greater than the cut off value (0.370).

2.7.DNA extraction

The DNA was extracted from collected blood samples using Qiagen extraction kit (QIAamp DNA blood mini kit, Qiagen, Germany) according to the manufacturer's manual. The concentration of DNA was determined by estimating the optical density at wave length of 260 nm (Sambrook *et al.,* 2001) using the spectrophotometer (Spectronic, 2000 – USA). The concentration was calculated as follows: 1 OD 260 = 50 µg/ml. The DNA was kept at -20 until assayed

2.8.Polymerase chain reaction for amplification of *T. annulata* gene

Based on conserved regions of the small subunit (SSU rRNA) gene, primers (Metabion Company, Germany) were used to detect common *Theileria* species DNA (D'Oliveira *et al.,* 1995). The primers' sequence were 5'AGTTTCTGACCTATCAG3' and 5' TTGCCTTAAACTTCCTTG3'. The PCR was used to amplify a 30-kDa gene of major merozoite antigen of *T. annulata* (Allsopp *et al.,* 1994) using specific primer (Metabion Company, Germany). The sequence for forward primer was 5'CCTTTCTCCATCGTCCTGAA3' and

for reverse primer was 5'TGGTGTTATCTGCCTGAC-CA3'**.** The PCR reactions were performed in a volume of 50 µl, containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, 4mM $MgCl_2$), 200 µM of each dNTPs, 80 pmol of each primer and 2 U of *Taq* DNA polymerase enzyme (Promega). The PCR mixture was amplified in a programmable thermal cycler (MJ Incorporation, USA) under the following conditions: 95°C for 5 min (initial denaturation) followed by 35 cycles of 94°C, 1 min (denaturation), 55°C, 1 min (annealing), 72°C, 1.5 min (extension) and a final extension of 72°C for 10 min. A negative control PCR reaction with no template was also included in this assay. The amplification products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide and the amplified products were visualized with an UV transilluminator.

2.9.Statistical analysis

The obtained data are presented as means ± SE. The data were analyzed using one-way analysis of variance (ANOVA) with Duncan's post-hoc test to evaluate the significant differences between the 3 groups using Sigma Stat V.3.1 software (SPSS, Inc., Chicago, IL, USA). The differences in means were considered statistically significant at P< 0.01.

3.RESULTS

3.1.Clinical findings

A marked variation in the clinical signs was recorded in the clinically affected buffaloes according to the stage of infection. During the early stage, affected buffaloes showed anorexia, fever (> 40°C), enlarged superficial lymph nodes, nasal and ocular discharge. During the later stages, there were weakness, weight loss and emaciation (Fig. 1), dark tarry feces, corneal opacity (Fig. 2) and coughing, dyspnoea and panting (Fig. 3). Lactating buffaloes showed marked reduction in milk yield.

3.2.Blood film examination:

Examination of Giemsa- stained blood films from buffaloes with clinical theileriosis revealed the presence of macroschizonts (Fig. 4) or microschizonts (Fig. 5) in lymphocytes of Giemsa-stained blood smears. All the clinically affected buffaloes showed intra-erythrocytic piroplasms in the RBCs (Fig. 6). The prevalence using blood film examination for diagnosis of T. *annulata* infection was 23.81% (10/42) (Table 4).

Table 1. Effect of *T. annulata* infection on haematological parameters in Egyptian water buffaloes.

Parameters \ Groups	Control (n=10)	Contact (n=22)	Clinical Theileriosis (n=10)
RBCs (10^6/ µl)	7.8 ± 1.26^a	6.68 ± 1.25^a	4.35 ± 0.88^b
Hb (gm/dl)	12.36 ± 3.33^a	10.02 ± 2.49^b	5.92 ± 0.39^c
PCV %	38.23 ± 2.49^a	35.05 ± 3.68^a	24.08 ± 3.48^b
MCV (fl)*	49.90 ± 2.52^a	52.47 ± 3.72^a	55.36 ± 2.66^c
MCH (Pg)**	15.85 ± 0.20^a	15.00 ± 0.21^a	13.61 ± 0.41^b
MCHC (%)***	32.33 ± 0.33^a	28.59 ± 0.25^a	24.59 ± 0.28^b
TLC (10^3/ µl)****	7.80 ± 0.34^a	7.52 ± 0.15^a	5.03 ± 0.15^b
Neutrophils(10^3/µl)	2.98 ± 0.28^a	2.89 ± 0.26^a	2.46 ± 0.12^b
Lymphocytes(10^3/µl)	4.52 ± 0.37^a	4.33 ± 0.25^a	2.43 ± 0.22^b
Eosinophils (10^3/µl)	0.047 ± 0.007^a	0.041 ± 0.006^a	0.30 ± 0.005^b
Basophils (10^3/µl)	0.030 ±0.006	0.016 ± 0.003	0.014 ± 0.003
Monocytes (10^3/µl)	0.226 ± 0.013^a	0.275 ± 0.039^a	0.126 ± 0.018^b

Values with different superscripts in the same row are significantly different from each other (P < 0.01).
Each value represents as mean ± SE *MCV = mean corpuscular volume
**MCH = mean corpuscular hemoglobin ***MCHC = mean corpuscular hemoglobin concentration
****TLC = total leucocytic count

3.3.Haematological changes

In buffaloes infected with *Theileria*, there was a significant reduction in the total RBCs count, the Hb concentration and the PCV%. MCV was significantly increased ($p<0.01$) while MCH and MCHC were significantly decreased ($p<0.01$) in infected animals compared to control. Contact animals showed non-significant changes. There was significant decrease in total leucocytic count, neutrophils, lymphocytes, monocytes, eosinophils and basophils in infected animals compared to contact animals and control (Table 1).

3.4.Biochemical results

Serum total protein, albumin and globulin were significantly decreased ($p<0.01$) in infected animals compared to control. The total bilirubin, direct bilirubin and indirect bilirubin showed significant increase ($p<0.01$) in infected animals compared to control (Table 2).

On the other hand, the serum activities of ALT, AST, ALP, GGT and CK and urea, creatinine, phosphorous and serum cholesterol revealed significant increase ($p<0.01$). Glucose and calcium levels were significantly decreased ($p<0.01$) compared to control. Contact animals showed non-significant changes in biochemical parameters compared to control (Table 3).

3.5.The results of ELISA

ELISA identified 14 (33.33%) out of 42 buffaloes affected with theileriosis; where 10/10 animals tested as positive from the clinical theileriosis group and 4/22 animals tested as positive from the contact group (Table 4).

3.6.The result of PCR

The PCR using the primers for small subunit (SSU rRNA) gene for common *Theileria* species produced amplicons at the 1098 bp (Fig. 7). The PCR using specific primers for a 30-KDa gene of *T. annulata* showed specific amplicon at 721 bp for positive blood samples. There were no amplicons demonstrated in the negative control where no DNA template was uploaded (Fig. 8).

Table 2. Effect of *T. annulata* infection on different types of proteins in Egyptian water buffaloes.

Groups / Parameters	Control (n=10)	Contact (n=22)	Clinical Theileriosis (n=10)
Total protein(gm/dl)	7.89 ± 0.29^{a}	6.42 ± 0.45^{a}	5.21 ± 0.31^{b}
Albumin (gm/dl)	4.44 ± 0.15^{a}	3.92 ± 0.26^{a}	2.77 ± 0.24^{b}
globulin (gm/dl)	3.45 ± 0.24^{a}	3.50 ± 0.14^{a}	2.54 ± 0.17^{b}
A/G ratio	1.28 ± 0.10^{a}	1.12 ± 0.07^{a}	1.07 ± 0.09^{a}
Total bilirubin (gm/dl)	0.87 ± 0.60^{b}	0.89 ± 0.05^{b}	1.50 ± 0.13^{a}
Direct bilirubin (mg/dl)	0.33 ± 0.07^{b}	0.38 ± 0.07^{b}	1.29 ± 0.02^{a}
Indirect bilirubin (gm/dl)	0.37 ± 0.12^{b}	0.43 ± 0.04^{b}	0.86 ± 0.16^{a}

Values with different superscripts in the same row are significantly different from each other ($P < 0.01$).
Each value represents mean ± SE.

Fig. 1. A buffalo affected with theileriosis showing obvious emaciation

Fig. 2. A buffalo affected with theileriosis showing corneal opacity.

Fig. 3. A buffalo affected with theileriosis showing severe dyspnoea and panting

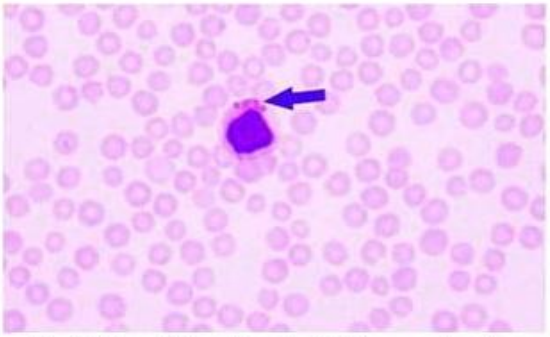

Fig. 4. Blood film stained with Giemsa showing macroschizont of *T. annulata* in a lymphocyte (magnification 1000X).

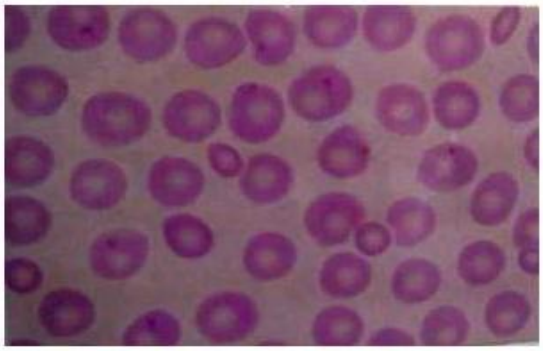

Fig. 5. Blood film stained with Giemsa showing microschizont of *T. annulata* in a lymphocyte (magnification 4000X).

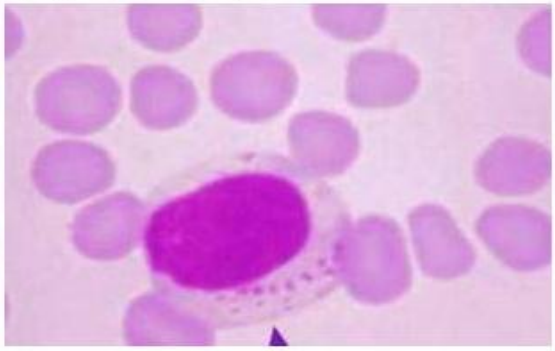

Fig. 6. Blood film stained with Giemsa showing intra-erythrocytic piroplasms of *T. annulata* (magnification 4000X).

4.DISCUSSION

Bovine theileriosis is a tick-borne protozoal disease that imposes a severe constraint on the productivity of domestic ruminants**.** *T. annulata* is recognized as a pathogenic *Theileria* that has been mainly described in temperate areas as the causative agent of Mediterranean theileriosis (Habela *et al*., 1999)**.** In Egypt, the disease is considered to be one of the most destructive obstacles to livestock production (Aktas *et al*., 2006)**.**

Diseased buffaloes (positive blood film), showed anorexia, enlargement of superficial lymph nodes, severe nasal and ocular discharges with congestion of conjunctiva, corneal opacity, salivation (acute form) and respiratory distress in the form of dyspnoea and coughing (subacute form). These signs were suggestive of theileriosis as previously described (El-Deeb and Younis, 2009; Mahmmod *et al*., 2011; Sudan *et al*., 2012).

Table 3. Changes in enzyme activities, metabolites and minerals following *T. annulata* infection in Egyptian water buffaloes.

Groups / Variables	Control (n=10)	Contact (n=22)	Clinical Theileriosis (n=10)
ALT (µ/L)	28.66 ± 0.56 [b]	29.77 ± 2.66 [b]	64.48 ± 3.13 [a]
AST (µ/L)	67.64 ± 5.48 [b]	75.91 ± 7.55 [b]	161.56 ± 2.35 [a]
ALP (µ/L)	57.83 ± 1.19 [b]	62.28 ± 3.72 [b]	83.30 ± 2.78 [a]
GGT (µ/L)	32.83 ± 1.22 [b]	35.56 ± 4.79 [b]	47.37 ± 1.71 [a]
CK (µ/L)	146.23 ± 5.02 [b]	155.47 ± 9.5 [b]	408.48 ± 4.78 [a]
Glucose (mg/dl)	63.53 ± 2.66 [a]	55.09 ± 1.35 [a]	41.18 ± 1.53 [b]
Cholesterol (mg/dl)	168.17 ± 5.56 [b]	172.07 ± 4.60 [b]	210.43 ± 8.60 [a]
Urea (mg/dl)	19.37 ± 1.51 [b]	24.53 ± 3.60 [b]	36.31 ± 2.58 [a]
Creatinine (mg/dl)	0.92 ± 0.02 [b]	1.15 ± 0.15 [b]	2.25 ± 0.43 [a]
Calcium (mg/dl)	10.15 ± 0.35 [a]	8.46 ± 0.29 [a]	7.24 ± 1.26 [b]
Phosphorus (mg/dl)	4.37 ± 0.69 [b]	5.05 ± 0.77 [b]	8.47 ± 0.69 [a]

Values with different superscripts in the same row are significantly different from each other ($P < 0.01$). Each value represents mean ± SE.

Table 4. The prevalence rate of *T. annulata* infection in 42 Egyptian water buffaloes.based on blood film, ELISA and PCR examination

Type of examination	No. Positive buffaloes			Total positive	% positive buffaloes from total (42)			*Total %
	Control (n=10)	Contact (n=22)	Clinical theileriosis (n=10)		Control	contact	Clinical theileriosis	
Blood film	0	0	10	10	0	0	23.81	23.81
ELISA	0	4	10	14	0	9.52	23.81	33.33
PCR (common primers)	0	10	10	20	0	23.81	23.81	47.62
PCR (specific T. annulata primers)	0	8	10	18	0	19.05	23.81	42.86

*The total % is calculated by division of the total positive for each technique by the total number of buffaloes examined (42). The result was then multiplied by 100.

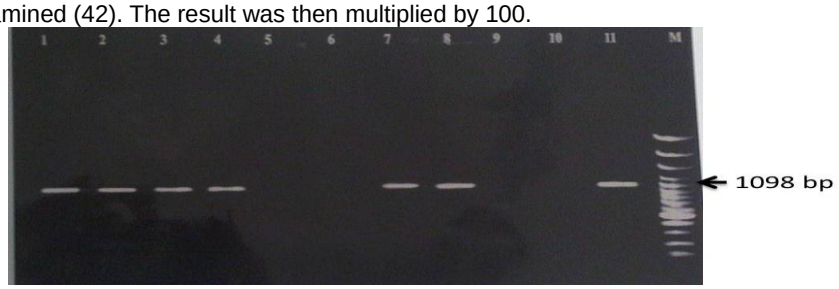

Fig. 7. Agarose gel electrophoresis of amplified SSU rRNA of *Theileria* sp. DNA (1098 bp PCR product) using primers common to *Theileria* sp. Lanes 1, 2, 3, 4, 7, 8, are positive *theileria* blood samples whereas, lanes 5, 6 and 9 are negative blood samples, lane 10 represents negative control sample and lane M represents 1 Kb DNA ladder as a standard size marker.

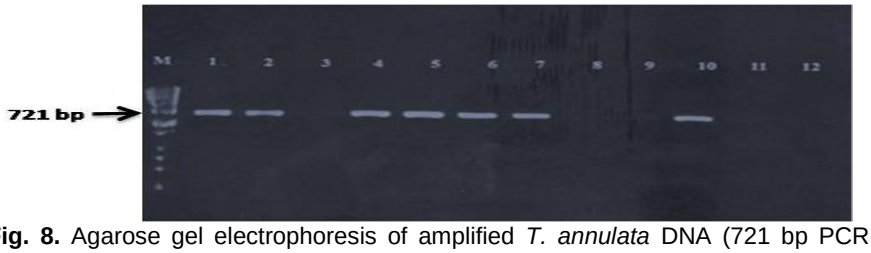

Fig. 8. Agarose gel electrophoresis of amplified *T. annulata* DNA (721 bp PCR product) using specific primers to a 30 KDa gene of *T. annulata*. Lanes 1, 2, 4, 5, 6 and 7, and 10 are positive samples. Lanes 3, 8, 9 and 11 are negative samples while lane 12 represents the negative control. M represents 1 Kb DNA ladder as a standard size marker.

Blood film of buffaloes with clinical theileriosis revealed the presence of piroplasms in the RBCs and macroschizont or microschizont in the lymphocytes of the infected animals. All contact and control animals under investigation were negative with Giemsa-stained blood films. The present results agreed with other studies (Mahmmod *et al*., 2011; Sudan *et al*., 2012). It has been demonstrated that in subclinical or chronic infections, experience in piroplasm microscopy is required because parasitemias are often extremely low and may otherwise be missed. Because of the inaccuracy of blood film results, further examinations had to be carried out.

Haematological examination of *T. annulata* infected buffalo revealed significant decrease in RBCs count, Hb, PCV, MCH and MCHC with significant increase in MCV indicating macrocytic hypochromic anemia. These results are compatible with those previously reported (Osman and Al-Gaabary, 2007; Hasanpour *et al*., 2008; El-Deeb and Iacob, 2012). Anemia could be attributed to destruction of the parasitized erythrocytes by macrophages in the spleen, lymph nodes and other organs of the reticuloendothelial system (Sandhu *et al*., 1998). Moreover, anemia in the some cases of theileriosis such as infection with *T. sergenti* was attributed to the elevation of methemoglobin (MetHB), an oxidized form of Hb (Shiono *et al*., 2001).

The leukopenia observed in infected animals could be attributed to destruction of lymphocytes by schizogony in lymphoid organs and infiltration of these cells into various organs, resulting in a decreased count in the peripheral circulation (Omer *et al*., 2002). Cytokines, particularly tumor necrosis factor (TNF)-α, produced by infected mononuclear cells have been proposed as a cause of the observed panleukopenia in infected animals (Col and Uslu, 2007).

The significant reduction in serum total protein, albumin and globulin was in accordance with previous study (Duncan *et al*., 1994) that attributed these changes to liver disease and severe lymphocytopenia. The increase in bilirubin levels is due to the destruction of parasitized erythrocytes by erythrophagocytosis in the spleen, lymph nodes, and other organs of the reticuloendothelial system (Duncan *et al*., 1994).

ALT and AST are metabolic enzymes mainly occur with high concentrations in liver and muscle. Elevation of these enzymes along with ALP and GGT in blood of infected animals suggests necrosis or disease in these tissues. *T. annulata* infection causes hepatic tissue damage that includes coagulative necrosis and heavy infiltration of lymphocytes in the periportal areas, indicating severe damage to the hepatobiliary system due to hypoxia resulting from anemia and jaundice (Sandhu *et al.,* 1998). In hepatic injury seen in theileriosis, increased serum activities of ALT and AST were closely associated with the hepatic function (El-Deeb and Iacob, 2012). Similarly, the significant rise in CK suggests muscle damage due to the anemic condition of the animals and toxemia resulting in recumbency in the terminal stages of the disease. Increased CK activity is a highly specific marker of muscle damage (Col and Uslu, 2007).

The significant hypercholesterolaemia recorded in infected animals agreed with other authors (Col and Uslu, 2007) who recorded a marked increase in cholesterol level in experimentally *T. annulata* infected animals. Hypercholesterolaemia could be attributed to stimulation of cholesterol synthesis as a compensatory mechanism to reduced osmotic pressure caused by hypoalbuminemia (Col and Uslu, 2007). It could be also a resultant of biliary obstruction which resulted in obstructive jaundice with a reflux of cholesterol into the blood stream from the liver and stimulate hepatic cell membrane for synthesis of cholesterol (Benjamin, 2000).

The hypoglycaemia reported in animals infected with *T. annulata* could be attributed to the utilization of glucose by parasites and damage to the liver associated with with *T. annulata* infection (Duncan *et al*., 1994). It is well documented that the primary source of ruminant blood glucose is hepatic gluconeogenesis from fatty acids that resulted from fermentation of dietary carbohydrates (Col and Uslu, 2007).

The significant elevation of urea and creatinine level in infected animals suggest that theileriosis is associated with kidney damage (Singh *et al.,* 2001)**.** In addition, the hypocalcaemia observed in these animals could be attributed to hypoalbuminemia, decrease dietary intake and kidney damage (Burtis and Ashwood, 1996)**.** Moreover, the hyperphosphataemia detected in infected animals could be attributed to injury of the kidney and decrease its elimination rate (García, 2006).

Although there were significant haematological and biochemical changes in buffaloes with clinical theileriosis compared to control, contact group did not exhibit these significant changes. Therefore, further investigations were necessary to accurately determine the prevalence rate in Egyptian water buffaloes. The percentage of buffaloes infected with theileriosis using blood film examination was 23.81% (10/42). It is now established that examination by blood film has several shortcomings. Blood film could not detect the parasite in contact animals which may suffer from sub-clinical or latent infection. In addition, using conventional microscopic method for diagnosis of tropical theileriosis, although cheap and simple, is limited to the detection of acute cases especially with high level of parasitemia (Mohamed *et al*., 2012). Therefore, microscopic examination is recommend during acute stages when the level of the parasitemia is high enough where parasites can be detected in Giemsa stained thin blood films. However, in later phases of the disease the parasitemia drops to microscopically undetectable levels. ELISA technique was able to detect 14 buffaloes infected with *T. annulata* out of the 42 examined buffaloes (33.33%). However, serological test used for detection of *Theileria* had some shortcomings**.** It is well-known that antibodies against *T. annulata* tend to disappear in long term carriers, whereas piroplasms persist. Therefore, animals with a negative serological test can still be source of infection (Papadopoulos *et al*., 1996)**.** Therefore, the reliability of serological tests could be questionable due to weak specific immune response, lack of determination of antibodies during long term carrier period, immunocompremised animals and possible false negative results (Gubbels *et al*., 2000)**.** Accordingly, the use of PCR was crucial for accurate diagnosis of *T. annulata* infection. The PCR primers based on conserved small subunit (SSU rRNA) gene for common *Theileria* species (D'Oliveira *et al*., 1995) were used to demonstrate the presence of *Theileria* DNA. By using this primer pair, the percentage of buffaloes infected with theileriosis was 47.62% (20/42). However, this pair of primers cannot differentiate between different *Theileria* species. Therefore, specific primers encoding 30-KDa major merozoite antigen was used to specifically amplify *T. annulata* DNA. The PCR using the specific primers for *T. annulata* detected 42.86% (18/21) of buffaloes affected with tropical theileriosis. Consequently, the PCR detected samples that were negative by both microscopic examination and ELISA. In addition, it is noteworthy to mention that the PCR was successful to detect all cases that were positive with ELISA. Consequently, PCR was considered more accurate and reliable method for diagnosis of tropical theileriosis. This conclusion is consistent with many other studies (El-Sebaei *et al*., 2012; Khan *et al*., 2013)**.** According to this result, contact animals should be tested by PCR in order to confirm the infection and accurately determine the infection rate of tropical theileriosis in Egyptian water buffaloes.

In a conclusion, tropical theileriosis in Egyptian water buffaloes causes a significant clinical, haematological and biochemical changes with disturbance in hepatic and renal function. The PCR using specific primers to determine the subclinical, chronic or carrier cases is more accurate and sensitive than the microscopic examination and the ELISA technique. Therefore, PCR results should be highly considered during the epidemiological studies of tropical theileriosis in buffaloes.

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