OXIDATIVE STRESS AND HEMATOLOGICAL PROFILE IN *THEILERIA ANNULATA* CLINICALLY INFECTED CATTLE BEFORE AND AFTER TREATMENT

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Received at:17/12/2014

Accepted: 8/1/2015

ABSTRACT

Bovine theileriosis is a destructive disease that affected cattle of all ages, breeds and sex and leads to severe losses in production and reproduction. To assess the antioxidant status and oxidative stress in bovine theileriosis due to *Theileria annulata*, blood samples were collected from 17 clinically infected cattle referred to the Veterinary Teaching Hospital, Assiut University. Complete blood picture, Nitric oxide, Malondialdehyde and total antioxidants capacity were determined and the results were compared with those of 10 healthy controls. The concentration of Nitric oxide (NO), Malondialdehyde (MDA) were significantly higher ($P < 0.0001$) and the total antioxidant capacity (TAC) was significantly lower in the infected cattle than in healthy ones ($P < 0.001$). Conventional and molecular techniques will help in early and accurate diagnosis and enables the effective treatment. The treatment with buparvaquone aims to eliminate the parasite from the blood and lymph node and consequently improvement in the clinical state without any adverse effect on the animal's cells. After treatment noticeable improvement was observed in clinical signs and significant increase in total RBCs count (6.58 ± 0.98 x10^6/mm$^{3)}$ and hemoglobin concentration (9.82 ± 0.98g/dl) compared with (4.37 ± 2.05 X10^6/mm^3 and 6.67 ± 2.76 g/dl) before treatment, respectively. Also, the oxidative stress was significantly altered and a significant increase of the (TAC), significant decrease of (NO) and (MDA) was noticed.

Key words: *Bovine Theileriosis, Oxidative stress, Buparvaquone.*

INTRODUCTION

Bovine theileriosis in cattle is a disease caused by protozoan parasite known as *Theileria annulata*, transmitted by ticks of genus Hyaloma. More than 250 million domestic cattle have been estimated to be at risk (Robinson, 1982). This disease leads to severe losses in production and reproduction of cattle (AL-Gaabary, 1995). *Theileria annulata* infection in cattle resulted in anemia and other adverse effects on the hematological profile, so, supplementation of the diseased animals with supportive treatment is recommended to help those animals to resume their normal productivity early (AbdEllah and AL- Hosary, 2011).

Conventional diagnosis of this disease depends on examination of Giemsa stained thin blood film and lymph smears. This method is limited to the acute stage of the disease where the parasitemia is high enough to be detected microscopically. During chronic and carrier stages the level of parasitemia usually below the microscopical detectable level. The application of genotypic assays for the diagnosis of bovine theileriosis has shown recent advances. Molecular identification provides two primary advantages to phenotypic identification; it is more rapid turnaround time, and improved accuracy of identification (Aktas *et al*., 2005).

Polymerase chain reaction (PCR) offers important advantages such as the higher sensitivity and specificity over conventional techniques in detecting both piroplasm-infected and carrier animals. This has been verified in a number of studies performed on a wide range of animals. The PCR assay has its superiority in separating parasitic infection associated with clinical signs (clinical form) from that without clinical signs (sub-clinical form) (Aktas *et al*., 2005 and AL-Hosary *et al.*, 2009, 2013).

Using 30KDa major merozoite surface antigen of *T. annulata protozoan parasite* (Tams-1 gene) is more appropriate. Tams–1 gene is the most abundant and immune-domoninant antigen on the surface of merozoites and piroplasms of *Theileria annulata*. It is a molecule with a molecular mass of approximately 30 KDa (Altay *et al.*, 2007; Murat *et al.*, 2008 and AL-Hosary *et al.*, 2009, 2013).

Theileria annulata spend part of its life cycle inside the erythrocytes and metabolize hemoglobin then produce free radicals which increase the oxidative stress in the infected animals (Hassanpour *et al.,* 2013). Also, nitric oxide (NO) was assessed as a potential mediator of macrophage anti-Theileria activity. The activated bovine macrophages produce it to prevent *Theileria annulata* trophozoite-infected cells transforming into macroschizont-infected cell (Ignar-ro *et al.,* 1981, Visser *et al.,* 1995). This study aimed to assess the changes in hematological profile and biomarkers of oxidative stress as NO, MDA and TAC in *Theileria annulata* naturally infected cattle and to show the therapeutic impact of treatment on the clinical signs, hematological profile and oxidative stress biomarkers in *Theileria annulata* naturally infected cattle, because there is a lot of information about these points in the diseased animals but little information about the effect of the treatment on the progress of these cases.

MATERIALS and METHODS

1. Animals

A total number of 27 cattle aged from one to five years old were subjected to this study during the period from July to November, 2014; 10 parasitologically free healthy cattle were used as control group and 17 clinically infected cattle with *Theileria annulata*. Infected cattle were selected on the basis of clinical examination and positive blood and/or lymph node smears as well as Tams-1 target based PCR, examination was done before and after treatment.

2. Clinical Examination and Conventional diagnosis

Clinical examination was performed for all animals. The clinical signs of *T. annulata* infection were observed and recorded. Thin blood smears were prepared from the ear veins of all cattle, after preparation stained by 8% Giemsa stain then examined on Olympus Microscope (Olympu, Japan) using oil immersion lens at X1000 magnification (Charles, 2002).

3. Molecular diagnosis:

3. 1. Tams-1 target based PCR assay:

Genetic confirmation of *T. annulata* infection was carried out by using Tams-1 target based PCR.

2.3.2 DNA Extraction:

DNA extraction from whole blood was carried out according to Manufacturer's instructions of the commercial kits QIA amp blood kit, Qiagen, Ltd, UK, Cat No. 51104.

3.3 DNA amplification:

- For the standard PCR, primer Tams1 F (5\ ATG CTG CAA ATG AGG AT) and Tspms1 R (5\GGA CTG ATG AGA AGA CGA TGA G), Amplifying a (785 bp) fragment of the *Theileria annulata* 30 KDa major merozoite surface antigen gene, Tams1, was used (Kirvar *et al.,* 2000 and AL-Hosary *et al.,* 2009, 2013).

3.4. Cycling conditions:

PCR was performed by incubating the samples at three temperatures corresponding to three steps (Denaturation, Annealling, and Extension). 94°C for five minutes, followed by 37 cycles consisting of one minute at 94°C, one minute at 55°C, two minutes at 72°C and final extension step at 72°C for ten minutes longer then the samples were stored at 4 °C until use in the next step. The cycling condition carried out in Biometra thermocycler (Professional basic, Thermocycler, version 11/06 Biometra, An Analytik, Jena Company-Germany).

In addition to the samples positive control sample contain "DNA from *Theileria annulata* infected lymph" and negative control sample that contain no DNA at all were included in the amplification step.

3.5. Gel Electrophoresis:

The electrophoresis chamber was connected to 75 volt power supply for 1:30 hour, 10µl of each PCR product were separated by electrophoresis on 1.8% agarose gel (GX 040.90, Gen agarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno–Train Diagnostic, D–61476, Kronberg/Taunus) Containing Ethidium bromide as 1 µl /ml electrophoresis buffer. Using 100 bp DNA–ladder in (SCi E–PLAS, HU 10, 5636, UK). Then the result obtained through High Performance Ultraviolet Transilluminator, (UV, INC, UK). The image of the PCR products containing the DNA sequence of 785 bp were amplified using DOC–It ®LS, Image acquisition –software, (UVP, INC, UK).

4. Blood sampling and routine hematological examination

Whole blood samples were collected directly from jugular vein, in vacutainer tubes contained EDTA for routine hematological examination and into heparinized vacutainer tubes for measurement of total antioxidant capacity (TAC), Nitric oxide (NO) and Malondialdehyde (MDA). Hematological analysis was carried out using automatic blood cells counter (Medonic CA 620, Sweden).

5. Biochemical assays and analysis

Total anti-oxidant capacity (TAC) was determined by using commercial kits supplied from Biodiagnostic Company for diagnostic reagents: Dokki, Giza, Egypt. The determination of the total anti-oxidant capacity was performed by the reaction of anti-oxidants in the sample with a definite amount of exogenously provide hydrogen peroxide (H_2O_2) according to the methods of (Koracevic *et al.*, 2001). The anti-oxidants in the sample eliminate a certain amount of the provided H_2O_2. MDA and NO levels were estimated using commercially available test kits supplied by Biodiagnostic-Egypt, according to the

methods described by (Okawa *et al.,* 1979; Montgomery and Dymock 1961).

6. Treatment protocol

Buparvaquone (Bupaquone) (BVP Ltd .co. Kerry, IRLAND) was used in treatment of the clinically infected animals. Bupaquone was used at dose rate of 2. 5 mg / kg body weight, second dose was required within 48:72 hours from the initial dose. Marbofloxacine (Marbocyl 10%) was used as antibiotic therapy to control the respiratory complications. It used at dose of 2 mg/1kg. (Marbocyl10% 1ml per 50 kg BW, intravenous or intramuscular, Intercova, Animal Health Products-Egypt) according to (AL-Hosary *et al.,* 2010).

7. Statistical analysis

Statistical analysis was conducted using SPSS version 16.0 for windows (SPSS, Chicago, USA). The results were analyzed using one way analysis of variance (ANOVA). Data were expressed as mean ± SD.

RESULTS

1. Clinical and Hematological Examination

The statistics of the measured parameters in healthy and clinically infected cattle before and after treatment are presented in Table 1. Present data showed that, the clinical signs of theileriosis were fever (>40°c), corneal opacity (Fig.1), enlargement of superficial lymph node (Fig.2), lacrimation, respiratory manifestations, nasal discharge (Fig.1), anorexia, paleness of mucous membranes and various degrees of ticks infestation. Treatment with Bupaquone was effective in the improvement in clinical state and in elimination of the protozoan parasites from the blood and lymph nodes.

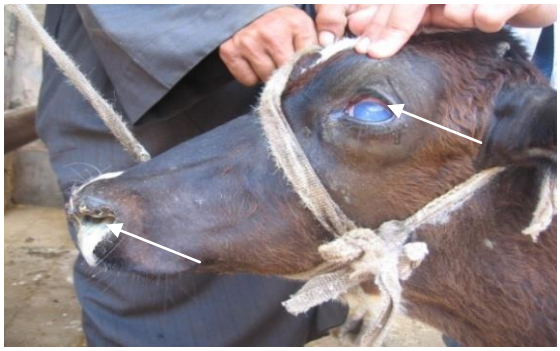

Fig. 1: Cattle showing corneal opacity with nasal discharge.

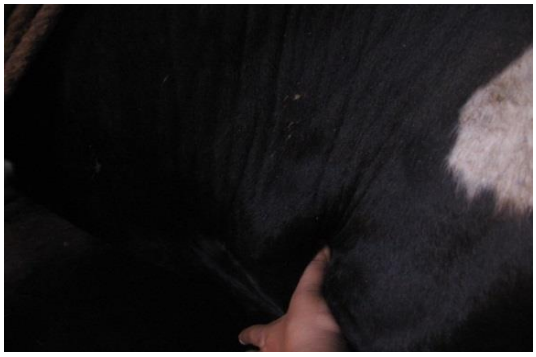

Fig. 2: Cattle showing enlarged prescapular lymph node.

The infection was confirmed conventionally by examination of the Giemsa stained thin blood film for detection of the intra-erythrocytic (signet ring) stage of the protozoan parasite *Theileria annulata* (Fig. 3). Also, all samples were subjected to Tams-1 target based PCR for confirmation of T. annulata infection. The results reveled that all examined animals were infected and the positive result was indicated by specific band at 785 bp (Fig. 4).

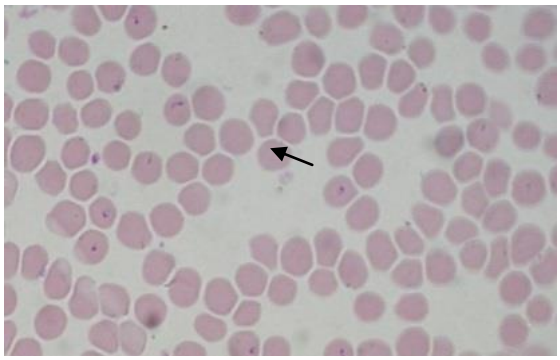

Fig. 3: Blood smears showing intra erythrocytic stage of *Theileria annulata.* (Before treatment).

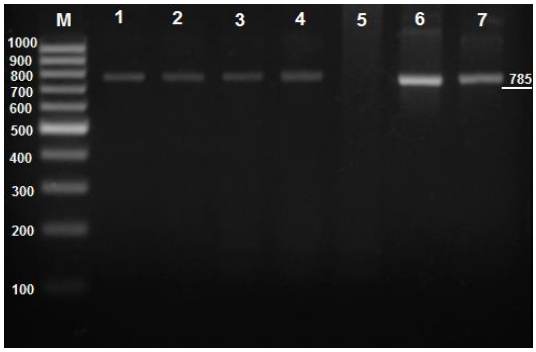

Fig.4: *T. annulata* in blood samples by Tams-1 PCR, M, DNA ladder, (1-4 and 6,7) were positive specific band at 785 bp, (5) was negative sample from the control group.

Hematological examination (Table. 1) revealed significant increase P (<0.01) of MCV, MCH and RDWa values and significant decrease of total erythrocyte count, PCV and hemoglobin in the diseased cattle compared to the control ones which indicated severe anemia. After treatment, the result revealed significant decrease of MCV and RDWa values and significant increase of total erythrocyte count, PCV and hemoglobin.

Table 1: Blood picture in *T. annulata* free and infected cows before and after treatment.

Parameters	Control group n= (10)	Before Treatment n= (17)	After Treatment n= (17)
RBC X10^6/mm3	9.90 ± 0.64	4.37 ± 2.05**	6.58 ± 0.98**
HGB g/dl	10.88 ± 0.62	6.67 ± 2.76**	9.82 ± 0.98**
PCV %	32.92 ± 2.61	16.25 ± 4.69**	31.42 ± 3.40**
MCV fl	33.20 ± 2.59	46.78 ± 3.81**	42.24 ± 5.52**
MCH pg	10.98 ± 0.63	14.11 ± 1.36**	14.77 ± 1.49
MCHC g/dl	33.10 ± 1.24	33.39 ± 1.29	32.74 ± 1.71
RDW%	26.04 ± 1.90	25.83 ± 5.72	23.98 ± 1.84
RDWa um^3	23.90 ± 3.08	41.78 ± 9.36**	32.74 ± 2.81**

RBC: red blood cells; **HGB**: hemoglobin concentration; **PCV**: packed cell volume, **MCV:** mean corpuscular volume, **MCH:** mean hemoglobin concentration, **MCHC:** mean corpuscular hemoglobin concentration, **RDW**: Red cells distribution width. ** P (<0.01) highly significant.

2. Biochemical analysis

Free radicals and antioxidants may play integral roles in different aspects of pathogenesis of *Theileria annulata* infection. Present results in (Table 2) revealed a significant increase (P ≤0.01) in the levels of NO and MDA and a significant reduction (P ≤0.05) in the levels of TAC in *T. annulata* clinically infected cattle compared with healthy one (Table 2). After treatment the finding was completely changed. The levels of NO and MDA have been significantly decreased and a significant increase in the TAC was noticed.

Table 2: Level of TAC and oxidants in *T. annulata* free and infected cows before and after treatment.

Parameters	Control group n= (10)	Before Treatment n= (17)	After Treatment n= (17)
TAC mM / L	1.662 ± 0.267	0.628 ± 0.0422**	0.709 ± 0.0553**
NO µmol / L	9.496 ± 0.387	21.981 ± 4.636**	18.054 ± 3.359**
MDA nmol / ml	5.174 ± 0.254	18.054 ± 3.359**	5.01 ± 1.411**

DISCUSSION

Bovine theileriosis is one of the infectious diseases which lead to severe economic losses in cattle, the goal of this study mainly was to investigate the therapeutic impact of treatment on the clinical signs, hematological profile and oxidative stress biomarkers in *Theileria annulata* naturally infected cattle. Our data showed that, the clinical signs of theileriosis were fever (>40°c), corneal opacity, enlargement of superficial lymph node, lacrimation, respiratory manifestations, nasal discharge, anorexia, paleness of mucous membranes and various degrees of ticks infestation. These clinical signs are in agreement with those obtained by (Radostits *et al.,* 2000 and AL-Hosary 2009, 2013). Considerable variation was noticed in the clinical signs of the infected animals after treatment. Recorded results revealed that early treatment with Bupaquone was highly efficient in improvement of clinical signs and also it helps in elimination of both piroplasmic and lymphocytic stages of the protozoan parasites from both blood and lymph nodes within 3–4 days post-treatment. Similar results were reported in cattle by Muraguri *et al.* (2006). All samples were subjected to Tams-1 target based PCR for confirmation of *T. annulata* infection. All examined animals were infected and the positive result was indicated by specific band at 785 bp.

Hematological examination revealed significant increase of MCV, MCH and RDWa values and significant decrease of total erythrocyte count, PCV and hemoglobin concentration in the diseased cattle compared to the control ones which indicated severe anemia. This is in agreement with (Omer *et al.,* 2002). The type of anemia usually classified as normocytic normochromic anemia, it was previously reported by (Sandhu *et al.,* 1998 and Abd- Ellah and

AL-Hosary, 2011). After treatment, the result revealed significant decrease of MCV and RDWa values and significant increase of total erythrocyte count, PCV and hemoglobin. This indicated that the treated animals start the resumption of their normal status.

Free radicals and antioxidants may play integral roles in different aspects of pathogenesis of *Theileria annulata* infection. The results revealed a significant increase P (<0.01) in the levels of NO and MDA and a significant reduction P (<0.01) in the levels of TAC in *T. annulata* clinically infected cattle compared with healthy one. Reduction of TAC level may be attributed to the reduction in antioxidant enzymes as they are consumed by excessive free radicals in the infected animals this was in agreement with (Hassanpour *et al.,* 2013). After treatment the finding were completely changed, the levels of NO and MDA have been significantly decreased and a significant increase in the TAC was noticed.

Treatment of the infected animals for controlling the respiratory complication revealed a significant changes in the hematological profile as well as the oxidative stress and this could be contributed to the elimination of the protozoan parasite because the used drugs was directed to eliminates the parasites and thus prevents the transmission of signals necessary for induction of genes coding for growth factor and for the receptors involved in signal transduction (Mchardy, 1985; Jabbar *et al.,*1992). Marbofloxacine is a new third generation fluoroquinolone and used for control of the respiratory complication. It has broad spectrum bactericidal activity against Gram negative bacteria, including *Mannheimia haemolytica*a and *Haemophillus species*, Gram positive bacteria and Mycoplasma species (Thomas *et al.,* 2001). This allows animals to resume their normal state.

The same findings had been reported by Shiono *et al.* (2003) who reported that the level of MDA began to increase remarkably in *T. annulata* clinically infected animals. MDA evaluation indicated that lipid peroxidation in erythrocytes of affected cattle was significantly more than those of healthy cattle. In accordance with the findings from other studies (Saluja *et al.,* 1999; Grewal *et al.,* 2005), our results indicated that the lipid peroxidation in erythrocytes of affected cattle increases MDA production. Increased MDA concentration in erythrocytes of affected cattle may be an indication of elevated oxidative stress in theileriosis. Oxidative stress results when the production of the free radicals and reactive metabolites of oxygen exceeds their safe disposal by antioxidant mechanisms. Free oxygen radicals cause lipid peroxidation and the end product of lipidperoxidation is MDA. Determination of MDA allows detection of the degree of lipid peroxidation and level of free oxygen radicals indirectly (Yagi, 1998; Owen, 1996 and Hanan *et al.,* 2013). The erythrocytes membrane is rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals, and is very susceptible to lipid peroxidation (May *et al.,* 1998; Devasena *et al.,* 2001).

CONCLUSION

Bovine theileriosis causes adverse effect on the hematological profile. In addition to oxidative stress which is characterized by significant decrease in the TAC accompanied by increase in both NO and MDA. After treatment of the diseased animals with the specific drugs most of clinical signs were returned to their normal function, blood picture significantly improved and the oxidative stress was significantly declined. Tick eradication programs for animals and surrounding environment must be taken in consideration during treatment of diseased animals.

ACKNOWLEDGEMENT

This work was supported in part by DFG project "Molecular epidemiology network for promotion and support of delivery of life vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa" (DFG AH 41/7-1).

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الاكسده الضاغطه وصوره الدم في الابقار المصابه اكلينيكيا بالثيليريا الحلقيه قبل وبعد العلاج

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يعتبر مرض ثيليريا الماشيه من الامراض المدمره التي تصيب الماشيه من جميع السلالات والاعمار ويؤدي إلى خسائرفادحة. هدفت هذه الدراسه لتقييم صوره الدم الكامله بالاضافه الي اجمالي القدره المضاده للأكسدة والإكسده الضاغطه في حاله الاصابه بثيليريا الماشيه وكذلك دور العلاج في تحسين هذه المعدلات وقد تم تجميع سبع عشر عينه من عينات الدم من الابقار المصابه بالمرض والتي كانت تتردد علي المستشفى البيطري التعليمي بجامعة أسيوط وتم دراسه الاعراض الاكلينيكيه الخاصه بالمرض علي الحالات المحتمل اصابتها بهذا الطفيل. تم قياس كل من المالونديالدهيد واكسيد النيتريك كمؤشرات للاكسده الضاغطه وكذلك اجمالي القدره المضاده للأكسدة وايضا تم اجراء صوره دم كامله لكل حيوان ومن ثم تم مقارنه هذه النتائج بعينات من عشره ابقار سليمه اكلينيكيا. ووفقا للنتائج كانت معدلات المالونديالدهيد واكسيد النيتريك أعلي في الحيوانات المريضه مقارنه بتلك الخاليه من الاصابه. وبمتابعه الحيوانات بعد العلاج لاحظنا الاختفاء الكامل لكل الاعراض التي كان يعاني منها الحيوان من ارتفاع في الحراره واعراض تنفسيه وعتامه في العين وعوده الغدد اللمفاويه لحجمها الطبيعي وكذلك لوحظ ارتفاع في اجمالي القدره المضاده للأكسدة وانخفاض في مؤشرات الإكسده الضاغطه وبالنسبه لصوره الدم فقد ارتفع العدد الاجمالي لكريات الدم الحمراء وكذلك تركيز الهيموجلوبين عن تلك النتائج التي تم قياسها قبل تطبيق العلاج.