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MOLECULAR DETECTION OF BABESIOSIS AND ITS OXIDATIVE STRESS IN BLOOD OF INFECTED CATTLE

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ABSTRACT

This study was conducted on a total of 210 cattle. Eighty cattle were suspected for *Babesia* infection collected from private farms in Giza governorates and 130 from apparently healthy cattle before slaughtering at El Bassatine abattoir Cairo, Egypt. Microscopic examinations revealed that out of 210 cattle, 34 (16.19%) were found to be infected with *Babesia* species. Serological examinations (ELISA) revealed 54 (25.71%) were positive for *babesia* species antibodies. Ten blood samples (5 positive and 5 negative) were conducted for PCR amplification. All samples, that were positive by microscopical and serological examinations were also positive by PCR after using *Babesia* species specific primer (*Babesia* common) and specific primer for *Babesia bovis* at 644pb and 711bp respectively. Whereas, 3 out of 5 negative samples were positive by PCR by using *Babesia* species specific primer (*Babesia* common) and only 2 samples were positive by using specific primer for *Babesia bovis*. After parasitological and serological examinations, 20 samples highly positive for *Babesia* was subjected to hematological and biochemical analysis. In addition, samples from 10 microscopically negative healthy cattle were subjected to the same examination as control. Hematological examination showed a significant ($P<0.05$) decrease in red blood corpuscles count, hemoglobin, packed cell volume accompanied by a significant increase in total leucocytic count, neutrophils, eosinophils, monocytes and lymphocytes numbers. Blood analysis of positive samples for babesiosis revealed a significant ($P<0.05$) increase in malondialdehyde and nitric oxide levels accompanied by a significant ($P<0.05$) decrease of glutathione, superoxide dismutase, glucose-6- phosphate dehydrogenase as well as iron, cupper and vitamin E. Biochemical analysis of positive cases revealed that significant increase in, globulin, aminotranseferases alkaline phosphatase and lactate dehydrogenase activities, T.bilirubin, cholesterol, triglyceride, urea, creatinine whereas a significant($P<0.05$)

decrease in albumin, A/G ratio and glucose were recorded in comparison to the control group.

Key words:

Babesia- cattle- blood film - ELISA -PCR- oxidative stress - hematology - biochemistry.

INTRODUCTION

Babesiosis is caused by intraerythrocytic protozoan parasites of genus *Babesia* that infect a wide range of domestic and wild animals and occasionally man. Babesiosis is a tick transmitted disease of cattle caused by protozoan parasites of species *Babesia bovis* (*B. bovis*), *Babesia bigemina* (*B. bigemina*), *Babesia divergens* (*B.divergens*) and others. Boophilus species of ticks are major vector for the transmission of *B. bovis* and *B. bigemina* . Generally, *B. bovis* is more pathogenic than *B. bigemina* and *B. divergens* **(O.I.E., 2005).** Infections are characterized by high fever, hemoglobinurea, increased respiratory rate, muscle tremors, anemia and jaundice. The fever during infections may cause pregnant cattle to abort and bulls to show reduced fertility lasting six to eight weeks **(Ferreri *et al.,* 2008**). Costs due to babesiosis are incurred not only from mortality, ill-thrift, abortions, and loss of milk/meat production and draft power but also through its impact on international cattle trade **(Abdel Hamid *et al.,* 2014)**. Parasitic infections cause an activation of inflammatory cells which plays an important role in the host defense; and activates various oxidant-generating enzymes. The induction and activation of these enzymes in inflammatory cells are also regulated by many proi-nflammatory cytokines including TNF-α, IL1β, IL-6 and others **(Saleh, 2009)**. In babesiosis caused by *B. bovis*, the infection involves production of IL-1β, IL-12, IFN-γ and TNF-α. The enhanced production of such cytokines might increase cancer risk by inducing or activating enzymes involved in the production of inflammatory cytokines because these enzymes produce large amounts of highly toxic molecules, such as reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide and hydroxyl radicals, and reactive nitrogen species (RNS), including nitric oxide **(Kucukkurt *et al*., 2014)**. The diagnosis of ruminant piroplasmosis is generally based upon microscopic examination of Giemsa stained blood smears and by clinical symptoms in acute cases. After acute infections, recovered animals frequently sustain subclinical infections, which are microscopically undetectable and they can be considered as a source of infection for the potential vector causing natural transmission of the disease **(Schneider *et al.,* 2011)**.Microscopic examination was very

difficult to detect the parasites in cases of low parasitemia. Serological tests, as enzyme-linked immunosorbent assay (ELISA) are capable of detecting the infection in carrier animals and have been broadly used for surveillance and export certification **(Goff *et al.,* 2006** and **Silvia *et al.,* 2014)**. Polymerase Chain Reaction (PCR) technique has been proven to provide reliable results with high sensitivity and specificity in detection of babesial DNA in blood, particularly when the parasitemia is very low or no detectable in microscopic examination **(Figueroa *et al.*,1992**). Consequently, combination of serological and molecular examinations for *Babesia* infection provides powerful tools for accurate diagnosis as well as for epidemiological investigations **(Terkawi *et al.,* 2011)**. The present study aimed to focus light on *B.bovis* infection in cattle by using microscopical, serological and molecular diagnosis. The present investigation also, was designed to find out the effect of babesiosis on antioxidant capacity and hematobiochemical changes in affected cattle.

MATERIAL AND METHODS

Collection of samples:

A total number of 210 blood samples from cattle were collected for *Babesia* species examination. Eighty cattle from private farms in Giza governorates with suspected symptoms of babesiosis were clinically examined for signs of health and disease with special care to high fever (± 40°C), inappetance, hemoglobinuria, anemia, increased respiratory rate, jaundice, body weight loss and presence of ticks on some animals. In addition, a total of 130 blood samples were collected from apparently healthy cattle at El- Bassatine abattoir. Blood samples were collected as two groups: the first, on silicone coated vacutainer tubes with EDTA (ethylene- diamine tetra acetate) for parasitological, molecular and hematological examination. Plasma was collected by centrifugation of the blood at 3000 rpm for 5 minutes for determination of oxidative stress. The second group on sterile silicone- coated vacutainer tubes centrifuged at 3000 rpm for 5 minutes to collect the serum for serological and biochemical analysis. After parasitological and serological examination, 20 samples highly positive for *Babesia* were subjected to hematological and biochemical analysis. In addition, samples from 10 negative healthy cattle were subjected to the same examination as control.

Parasitological examination:

Thin blood films were prepared from each sample, fixed with methanol, stained with Giemsa stain and microscopically examined for detection of *Babesia* species according to **Coles**

(1986).The percentage of parasitemia was obtained from the middle of the thin blood smear stained with Giemsa stain and examined in a standard way. The number of parasites in twenty microscopic fields was counted. This number was expressed as a percentage of the total erythrocytes in the twenties field **(Thammasirirak *et al.,* 2003)**. **Parasitemia.** The percent of infected RBCs is determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs.

Parasitemia = (No. infected RBCs ÷ Total No RBCs counted) x 100

Serological examination:

A-Antigen preparation:

The ELISA antigen source was obtained from highly positive samples of infected cattle (high parasitemia). Antigen was prepared according to **Madruga *et al.,* (2001**). Briefly, the infected blood was washed three times with phosphate buffer saline (PBS) (83.3mM KH_2PO_4, 66mM Nacl) pH 7.2 by centrifugation at 12,000xg for 30 minutes for buffy coat removal and stored at - 72°C. At the moment of antigen preparation, the blood was thawed at 37°C and the erythrocytes were washed three times with PBS, pH7.2 by centrifugation at 12,000xg, during 30 minutes at 4°C. After the last centrifugation, the intermediate layer of the pellet was collected and lysed with a solution containing 100 mMtris, 10mM EDTA, 0.2 mM N-a-p-tosyl-L-Lysil choromethyl keton, 2 mM phenylmethyl sulfonyl fluoride and 2%Nonidet p40 (v/v), and by ultrasonic disruption (100 W for 10 minutes). The material was centrifuged at 23,700 xg for 60 minutes at 4°C and the supernatant was harvested and used as antigen. The total protein concentration of the antigen, estimated by the Folin's reagent method **(Lowry *et al*., 1951**).

B-Enzyme linked immunosorbent assay (ELISA) :

ELISA was done with antigen concentration of 10 µg/ ml and serum dilution of 1:100 using antibovine IgG alkaline phosphatase conjugate (Sigma). The assay was performed according to **Machado *et al.,* (1997**). Briefly, 100 µl of antigen diluted in 0.05 M carbonate/bicarbonate-buffer, pH 9.6, was added to each well of a micro ELISA plate (Immuion®; Dynatech Laboratories Inc.) and protein concentration was adjusted to 10 µg/m1. The plates were sealed and incubated overnight at 4 °C. After five washes with PBs-tween buffer (phosphate buffered saline, pH 7.2, and tween 20), 100 µl of diluted% 0.05 bovine sera (1:100) in PBS-Tween were added in duplicate to the ELISA plate. Plates were incubated at 37 °C in a humid chamber for 90 minutes and then washed five times with PBS-Tween. 100 µl aliquot of

1:10000 dilution of alkaline phosphatase conjugated anti-bovine IgG (Sigma Chemical Co.) was added to each well and the plates were incubated at 37 °C under the same conditions for 90 minutes. Plates were washed five times with PBS-Tween. The appropriate substrate (p-nitrophenyl phosphate) was added and the plates were sealed and incubated for 15 minutes at room temperature. The plates were then read at 405 nm wave length on a micro-ELISA reader (B.T.-100); Embrabio, São Paulo, Brazil). The tested sera were considered to be positive when the absorbency values were as more than the cut off values (The cut off = double fold of the mean negative sera).

Molecular examination:

Samples for PCR:

After microscopical and serological examinations, 5 positive and 5 negative samples for *Babesia* sp. infection were subjected to PCR using *Babesia* common specific primer and *Babesia bovis* specific primer. DNA positive control for *Babesia* sp. was obtained from cattle with high parasitemia of babesiosis. As well as negative control for *Babesia* sp. was obtained from apparently healthy cattle, negative microscopical and serological examinations. 50 µl of blood from each sample were subjected for DNA extraction according to **Sambrook *et al* (1989).** The samples amplified using the designed and prepared primer according to the published sequence as described in (Table 1).

Table (1): Primers, product size, reference annealing temperature and product size of different primers used in PCR assays.

Primer	Primer Design	Reference	Annealing temp.	Product Size
***Babesia* common**	**GTG AAA CTG CGA ATG GCT CA CCA TGC TGA AGT ATT CAA GAC**	***Figueroa et al.* (*1992*)**	**55 °C**	**644 bp**
Babesia bovis	**GGGTTT ATA TAG TCG GTT TTGT ACC ATT CTG GTA CTA TAT GC**	***Fahrimal et al.* (*1992*)**	**65 °C**	**711 bp**

One µg total DNA was subjected for amplification by 50 µl PCR reaction volume with addition of 5 µl of 10X buffer, 2 µl of mixed dNTPs (20 µg) [Roche, Germany], 25 pmol of each primer (primers used for common detection of *Babesia* species) and 2 unit Taq

polymerase enzyme. PCR cycle were composed of denaturing at 93 °C for 1 minutes, annealing 55 °C for 1 minutes and extension at 72 °C for 1 minutes. with initial denaturation at 95 °C for 3 min and final extension for 10 minutes at 72 °C with 40 cycle for a multiplex PCR assay included *Babesia* spp. were tested by common primers. The same conditions were applied on testing samples for *Babesia bovis* with changing the annealing temperature to 65°C. Electrophoresis of 10 µl from PCR product examined on UV and photographed.

Hematological Examination:

The blood collected on EDTA for both groups (infected and control cattle) were subjected for determination of red blood corpuscles count (RBCs), total leucocytic count (WBCs), hemoglobin (Hb %) and packed cell volume (PCV %) according to the methods described by **Schalm** (**1986**). Blood film from each blood sample was stained with Leishman stain and observed microscopically to study WBCs differential count.

Oxidative and biochemical examinations:

The collected plasma was subjected to estimation of malondialdehyde (MDA) according to **Placer *et al.*** (**1966**), nitric oxide (NO) **(Rajaman *et al.*, 1998)**, glucose-6-phosphate dehydrogenase (G6PD) (***Beutler,1984***), reduced glutathione (GSH) **(Beutler *et al.*, 1963**) and superoxide dismutase (SOD) (**Woolliams *et al.*, 1983**). Meanwhile, the obtained sera were also used for assay of copper **(*Mert and Henkin ,1971*)**,iron **(Smith *et al.*, 1981**), vitamin E (**Vasington *et al.*,1960**), total protein **(Hoffamann and Richterrich (1970**),albumin **(Dumas *et al.*, 1971),** aminotranseferases activities **(Reitman and Frankel 1957**), lactate dehydrogenase (LDH) **(Cabaud and Worblewski 1958),** alkaline phosphatase (ALP) **(Kilchling and Freiburg 1951),** total billirubin **(Ducci and Watson 1945**),total cholesterol **(Richmond, 1973**) triglycerides **(Trinder, 1969)** ,urea **(Patton and Crouch, 1977)**, creatinine **Warnick *et al.*,(1983)** and glucose **(Kaplan, 1984).**

Statistical analysis:

The mean values obtained from hemograms and biochemical assays of positive samples were compared with data of negative samples using T- test **(Petrie and Watson, 1999**). Differences were considered to be statistically significant with values of $P<0.0\ 5$.

RESULTS

Parasitological results:

The clinical symptoms were reported as high fever (± 40°C), inappetance, hemoglobinuria, increased respiratory rate, anemia, jaundice, body weight loss and presence of ticks on some

animals. The present study showed that, the microscopical examination of the thin blood films collected from Giza farms (80) and apparently healthy cattle from El -Bassatine abattoir (130) revealed that, the prevalence of *Babesia* infection were 16(20%) and 18(13.85%) respectively (Table 2). Parasitemia was ranged between 0.02 - 0.1%. Piroplams stages of *Babesia* were found small round, pear shape, amoeboid forms and average measurements 2.4x1.5µm. The shape and measurements tend to be *B.bovis* as described by **Soulsbay (1987)** Fig. (1 and 2).

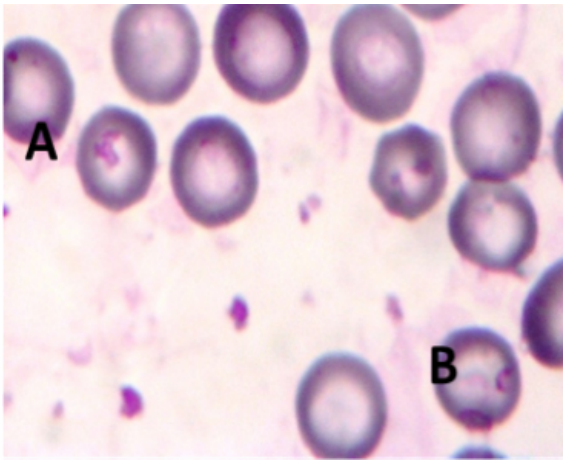

Fig. (1): Thin blood film of naturally infected cattle with *Babesia* spp. stained with **Giemsa x1000:**
(A) Amoeboid shape. **(B)** Pear shape.

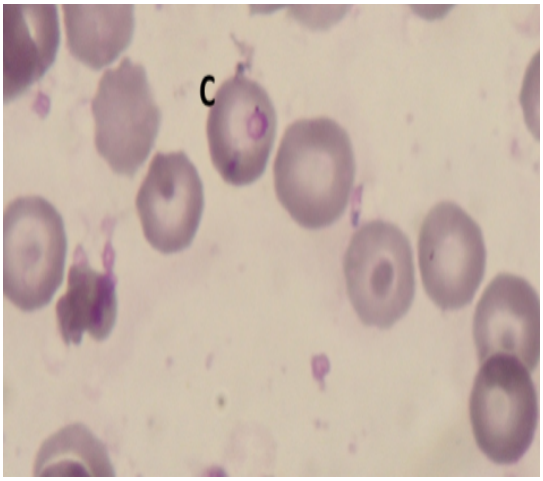

Fig. (2): Thin blood film of naturally infected cattle with *Babesia* spp. stained with Giemsa (round form) x1000.

Serological results:

Serological examination revealed that, the incidence of *Babesia* antibodies infection in the Giza farms was 25 (31.25 %) and in the apparently healthy cattle at El Bassatine abattoir was 29 (22.31%) with a total incidence of 54 (25.71 %) (Table 2).

Table (2): Results of microscopical and serological examinations.

Source and No. of examined animals	Microscopical results		Serological results	
	+ve	%	+ve	%
Giza farms (80 samples)	16	20	25	31.25
El Bassatine abattoir (130 samples)	18	13.85	29	22.31
Total number (210 samples)	34	16.19	54	25.71

PCR results:

All samples proved to be positive by microscopical and serological examination were also positive by PCR after using *Babesia* species specific primer (*Babesia* common) at 644pb. Whereas, 3 out of 5 negative samples were positive by PCR technique at 644 pb. All samples positive for *Babesia* species specific primer were also positive by using specific primer for *Babesia bovis* at 711 pb. Whereas, 2 out 5 negative samples were positive by *Babesia bovis* specific primer at 711pb. Only one sample positive by specific primer for *Babesia* (*Babesia* common) but negative by *Babesia bovis* specific primer (Fig 3 and 4).

Table (3): Results of PCR in comparison with microscopical and serological examination.

No. of blood samples	*ME	ELISA	PCR	
			***Babesia* spp. (common)**	***Babesia bovis***
1	**+ve**	**+ve**	**+ve**	**+ve**
2	**+ve**	**+ve**	**+ve**	**+ve**
3	**+ve**	**+ve**	**+ve**	**+ve**
4	**+ve**	**+ve**	**+ve**	**+ve**
5	**+ve**	**+ve**	**+ve**	**+ve**
6	**-ve**	**-ve**	**+ve**	**-ve**
7	**-ve**	**-ve**	**-ve**	**-ve**
8	**-ve**	**-ve**	**+ve**	**+ve**
9	**-ve**	**-ve**	**-ve**	**-ve**
10	**-ve**	**-ve**	**+ve**	**+ve**
Total	**5**	**5**	**8**	**7**

***ME: Microscopical examination**

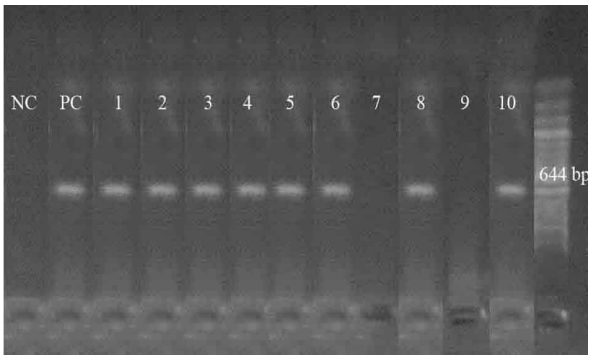

Fig (3): Agarose-gel electrophoresis of amplification products obtained from genomic DNA of *Babesia* species of cattle (644 pb) by using common *Babesia* specific primer.

Lane M**:** Marker, Control positive (PC) and control negative samples (NC).

Lanes 1-6 and 8, 10: Positive for *Babesia* species was isolated from cattle.

Lanes 7, 9**:** Negative *Babesia* species .

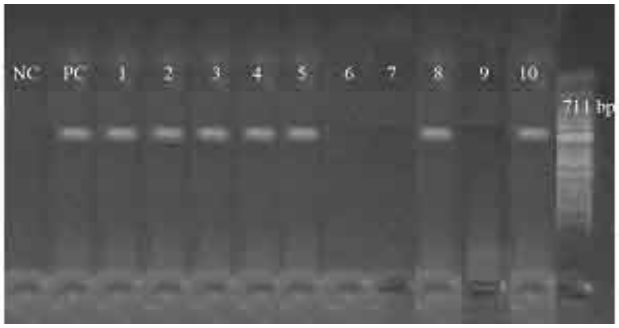

Fig (4): Agarose-gel electrophoresis of amplification products obtained from genomic DNA of *Babesia bovis* of cattle (711 pb) by using specific primer for *Babesia bovis.*

Lane M: Marker, Control positive (**PC)** and control negative samples **(NC).**

Lanes 1-5 and 8, 10: Positive for *Babesia bovis were* isolated from cattle.

Lanes 6, 7, 9: Negative *Babesia bovis*.

Hematological results:

Table (4): Mean value of hematological changes associated with *Babesia* infection in cattle.

Parameter / Groups	T.RBCs (10^6/ µl)×	Hb (g/dl)	PCV %	TLC × (10^3/ µl)	Lympho × (10^3/ µl)	Neutro × (10^3/ µl)	Eosino × (10^3/ µl)	Mono × (10^3/ µl)	Baso × (10^3/ µl)
Control Group	**6.64 ± 0.18**	**11.61 ±0.16**	**32.71 ± 0.39**	**8.18± 0.17**	**4.47 ± 0.09**	**3.28 ±0.057**	**0.12± 0.005**	**0.36 ± 0.003**	**0.05 ± 0.003**
Infected Group	**±4.05 0.095***	**8.23 ± 0.28***	**24.78 ± 0.78***	**10.82± 0.18***	**5.59± 0.04***	**3.98± 0.014***	**0.28 ± 0.004***	**0.51± 0.048***	**0.09 ± 0. 002***

The mean difference is significant at the P<0.05.

Hematological findings showed a significant decrease (P<0.05) in the RBCs counts, Hb

concentrations and PCV% in the *B. bovis* infected animals as recorded in (Table 4) in comparison to the control group**.** The leukogram revealed a significant increase ($P < 0.05$) in TLC, neutrophils; eosinophil, monocyte, basophil and lymphocytes number as parasitemia rates increased.

Oxidative Stress results:

Table (5): Mean values of some oxidative stress indicators in healthy cattle and diseased ones.

Parameter / Groups	MDA (nmol/ml)	NO (µm/ml)	G_6PD (u/g Hb)	GSH (mmol/L)	SOD (U/ml)	Fe (µg/dl)	Cu (µg/dl)	Vit E (µg/dl)
Control Group	14.18 ± 0.33	11.61 ±0.16	24.96 ± 0.39	44.08± 0.97*	21.47 ± 0.09*	142.18 ±0. 27*	29.16 ± 0.05*	3.11 ± 0.06
Infected Group	77.19* ± 0.95	36.5* ± 0.78	15.18* ± 0.62	25.62*± 0.48	10.59*± 0.66	117.55 *± 0.74	14.28* ±0.48	2.29* ±0.02

The mean difference is significant at the P<0.05.

In our study the obtained results presented in (Table 5) indicated that babesiosis have significant conspicuous oxidant/antioxidant effect on calves by significant increase ($P<0.05$) in oxidant markers (MDA and NO), and significant decrease in antioxidant parameters G6PD, GSH, SOD, Cu, Fe and Vit E.

Biochemical results:

Table (6): Mean values of some biochemical parameters in healthy cattle and diseased ones

Parameter	Control Group	Infected Group
Total Protein (g/dl)	7.08± 0.90	7.41± 0.17
Albumin(g/dl)	3.19± 0.16	2.76*± 0.09
Globulins(g/dl)	3.89± 0.13	4.65*± 0.32
A/G ratio	0.82 ±0.04	0.60* ±0.06
ALT activity level (IU/L)	18.32± 0.87	39.44*± 0.50
AST activity level (IU/L)	24.10± 0.75	57. 62*± 1.18
LDH(IU/L)	426.77±2.77	629.45*±3.23
ALP(IU/L)	44.77±1.05	79.87*±0.99
Total Billirubin(mg/dl)	0. 67± 0.019	1.17*± 0.02
Cholesterol(mg/dl)	115.52±1.21	212.15*±1.76
Triglyceride(mg/dl)	49.72±0.87	83.22*±0.74
Urea(mg/dl)	28.20±0.78	39.88* ±0.20
Creatinine(mg/dl)	0.81±0.07	1.22*±0.12
Glucose(mg/dl)	68.33 ±1.25	47.51*±0.85

The mean difference is significant at the P<0.05.

In the analysis of serum of the infected animals, when compared with controls, as recorded in (Table 6) it was found that there were statistically significant decrease (P<0.05) in serum albumin and A/G ratio and glucose accompanied by significant increase (P<0.05) in globulin levels as well as, in the liver enzymes activity (AST, ALT and ALP and LDH) ,total bilirubin, cholesterol, triglyceride ,urea and creatinine while total protein was insignificantly increased in cattle infected with babesiosis

DISCUSSION

Babesia bovis is the main causative agent of bovine babesiosis and most virulent species in Egypt **(Adham *et al.,* 2009**). The observed clinical findings in cattle affected with babesiosis were high fever, in appetence, hemoglobinurea, increased respiratory rate, jaundice, body weight loss and hemolytic anemia. The reported clinical findings of *Babesia* sp. infection in cattle comes in agreement with what was previously described by **Georgi *et al*.,(1990)** and **Ibrahim *et al.,(*2009)**. The demonstrated high fever could be attributed as response to the effect of unspecific toxic substances produced during the metabolism of *Babesia* on thermoregulatory **(Radostits *et al.,* 2000**). In the present study, the prevalence of *Babesia* sp. infection in cattle was detected at rate of 16/80 (20%) and 18/130 (13.85%) in the stained blood smears collected from Giza farms and El-Bassatine abattoir by microscopic examination respectively. The morphological characters of the detected *Babesia* species using Geimsa stained thin blood smears were small round, pear and amoeboid shape with average measurements of 2.4x1.5µm. Their shape and measurements tend to be *Babesia bovis* **(*Soulsbay 1987*)**. Similar results were obtained in Egypt by many authors namely ***Adham et al.,*** (**2009**), **El-Fayomy *et al*., (2013) and Abdel-Aziz *et al*., (2014**), who reported *Babesia* spp. infection rate of 13% in Giza, 13% in Port Saied and 12% in Cairo governorates respectively.Also,**Abd-El-Gawad (1993) and Mazyad and Khalaf (2002)** reported infection rates 9.9% and 8.1% in Beni-Suef and North Sinai governorates, respectively. On the other hand, high rates of *Babesia bovis* infection (34%) were reported by **Khan *et al.,* (2016)** in Pakistan. Traditionally, microscopic examination has been considered the "Gold standard" for detecting organisms in the blood of infected animals, particularly in acute cases, but not in carriers, where parasitemia is low **(Jacobson,2006)**.In the current research, detection of IgG antibodies in blood samples collected from 80 cattle (Giza farms)and 130 cattle (El- Bassatine

abattoir) by ELISA revealed that, the prevalence of *Babesia* spp. were 25/80 (31.25%) and 29/130 (22.30%) respectively.ELISA showed higher prevalence of infection 54/210 (25.71%) than Giemsa stained blood smears 34/210 (16.19%). All samples positive by microscopical examination were also positive by serological examinations. These results came in agreement with those mentioned by **Abdel-Aziz *et al*., (2014)** (27 .1%) and **Mahmoud *et al.,*(2015)** (26.3%) in Egypt. **Ibrahim *et al.,* (2009)** reported lower percentage (9.3 %) while ***Silvia et al*.,(2009)** reported higher percentage of *Babesia* spp. infection (77 %) in Egypt. The differences between the results may attributed to climate and/or soil, temperature, humidity and spreading the ticks (*Boophilus* species) **(Siliva *et al.,* 2014)**. The results of this study in the examined cattle after using polymerase chain reaction (PCR) for detection of DNA , showing 644bp and 711bp PCR product for the specific detection of *Babesia* common and *Babesia bovis* respectively. All positive samples (5 samples) by microscopical and serological examinations were also positive by PCR amplification for *Babesia* common and *Babesia bovis* specific primers. Three out of 5 negative samples were positive by PCR technique at 644pb by using *Babesia* common specific primer. Whereas, 2 out of 5 negative samples were positive by *Babesia bovis at* 711pb by using *Babesia bovis* specific primer. Only one sample positive by specific primer for *Babesia* common but negative by *Babesia bovis* specific primer. This one sample may be another species of *Babesia*. These results indicated that PCR technique was a highly sensitive tool for detection and confirmation of *Babesia* infection. **Figueroa *et al*. (1992)** used PCR for *Babesia* infection in cattle with primer yielding a 644bp PCR product for the specific detection of *Babesia* common. ***Fahrimal et al***. (***1992***) used PCR for *Babesia* infection in cattle with primer yielding a 711bp PCR product for the specific detection of *Babesia bovis*. **Ibrahim *et al*., (2009), *Zulfiqar et al*., (2012) and Abdel-Aziz *et al.,* (2014)** were successfully detected *Babesia bovis* infection in cattle at 711bp, 541bp, 387bp respectively. **Romero-Salas *et al.,* (2016)** found the specific band in positive cases of *Babesia bovis* infection in cattle was 586bp by using PCR amplification, while in the present study it was 711bp. They added that, the intensity of PCR bands was variable in different test samples depending upon the level of infection in the test samples. Hematological findings showed a significant decrease ($P<0.05$) in the RBCs counts, Hb concentrations and PCV% in the *B. bovis* infected animals as recorded in comparison to the control group. These observations were similar to that reported by **Saleh (2009), Zulfiqar *et al.,* (2012), Swelum *et al*., (2014) and AL-Hosary *et al.,* (2015).**

The cause of anemia during blood parasite infection may be multifactorial. The marked anemia and hemoglobinuria in cattle could be attributed to the severe hemolytic process associated with the presence of *babesia* piroplams inside the erythrocytes and destruction of large numbers of these erythrocytes by the parasite resulting in hemoglobinaemia and consequently hemoglobinuria, the toxic metabolites of parasite which have a suppressing effect on bone marrow interfere with the process of erythropoiesis **(Alkhalil *et al.* 2007). Yukio *et al.*, (2002)** suggested that enhanced oxidation of the erythrocytes may not only be due to increased free radical generation but also could be exacerbated by the inefficient antioxidant capacity that results in erythrocytic destruction and progression of anemia in infected cattle. The leukogram revealed a significant increase ($P < 0.05$) in TLC, neutrophils, eosinophils, monocytes, basophils and lymphocytes number while parasitemia rates increased. The observed leukocytosis in infected animals in this study, are consistent with findings by other researchers [**Esmaeilnejad *et al.*, (2012); Zulfiqar *et al.*, (2012)** and **Swelum *et al., (2014)***]. **Furlanello *et al.* (2005)** reported that leukocytosis occurred due to maturation of neutrophils and lymphocytes. The observed eosinophilia was due to the sensitivity to the foreign protein of a parasite which may be a part of an immune phenomenon. In addition, macrophage activation is known to occur during babesiosis. **Goff *et al.*, (2002)** revealed that hemoparasite-activated macrophages release pro-inflammatory cytokines, including interlukin-1 (IL-1), interlukin-12 (IL-12) and tumor necrosis factor (TNF). Interlukin-1 causes the proliferation of lymphocytes and T helper cells activated by IL-12 produces gamma interferon (IFN-γ). The latter and TNF are also important for activating of blood mononuclear cells (lymphocytosis and monocytosis) and polymorphonuclear cells (neutrophilia). Babesiosis have significant conspicuous oxidant/antioxidant effect on cattle by increasing oxidant markers (MDA and NO), and decreasing antioxidant parameters (G6PD, GSH, SOD, Cu, Fe and vit E). These results are in agreement with that of **Esmaeilnejad *et al.*, (2012), Osama and Gaada (2013) ,Ali *et al.* (2013) and Kucukkurt *et al* .,(2014). *Cimer* (2008)** reported that erythrocytes particularly susceptible to an oxidative damage as a result of high polyunsaturated fatty acids content in their membrane and high concentration of oxygen and hemoglobin. Malondialdehyde (MDA), a product of polyunsaturated fatty acid oxygenation is an indicator to decide the degree of oxidative damage of cell membrane. It reacts with cellular membrane elements and results to increase cellular permeability and

enzyme activities **(Sedar *et al.,* 2009).** The significant increase in nitric oxide (NO) may be attributed to that the hemoparasitic diseases stimulates the production of various pro-inflammatory important cytokines from mononuclear cells in animals. These mediators activate the mononuclear phagocytic cells to release oxygen and reactive nitrogen intermediates **(Couret *et al.,* 2001).** The significant decrease in the activity of G6PD in affected cattle suffering of severe anemia is an indicator of a metabolic disturbance of erythrocytes. This enzyme has a key role in the pentose phosphate pathway, which has critical significance in the survival of erythrocytes **(Beutler, 1984**). G6PD enzyme is the principal source of NADPH, which helps in maintaining glutathione in reduced state, thus protecting erythrocytes from oxidative stress. So, G6PD serves as an antioxidant enzyme and the low activity of G6PD has been associated with increased hemolysis in cattle affected by *Babesia* and increased oxidative stress in endothelial cells **(Jonhson *et al.*,2008)** .GSH can protect cells against the damage from ROS and free radicals that arise during conditions of oxidative stress. So, the low G6PD activity can be followed by reduced concentration of GSH, because of the dependence of the activity of glutathione reductase enzyme on NADPH + H levels in the cell **(Saleh, 2009).** Moreover, decrease in cellular GSH content indicates generation of large quantity of ROS (**Tsukahara 2007**). **Ali *et al.,* (2013)** speculated that with increasing parasitemia and oxidative damage in parasitized erythrocytes increased MDA concentration, the activity of SOD enzyme significantly reduced. In this research, serum iron revealed a significant decrease in infected animals when compared with control ones. This agreed with **Lotfallah *et al.,* (2012)** who reported a significant decrease in iron concentration in cattle infected with *Babesia* and this may be due to oxidative damage to hemoglobin cause changes in its structure and function resulting in denaturation and precipitation of hemoglobin and methemoglobin formation inside erythrocyte. It's markedly increased in the onset of anemia in *Babesia* infection **(Saleh 2009)** while **Okubo *et al.,* (2007)** observed that *Babesia* species produce hemolysis of erythrocytes. So, it leads to production of erythrocytes from bone marrow to compensate decrease in erythrocytes level. This lead to decrease serum level of iron which is necessary for erythrocytes production. The decreased serum copper and iron concentrations in *Babesia* infected cattle could be attributed to the inability of the damaged liver to synthesize ceruloplasmin and transferrin, respectively **(Nazifi *et al*., 2011)**. Moreover, **Ali *et al*.,(2013)** reported a decrease in the level of vitamin E activity in sheep infected with *Babesia.* Vitamins E play an important role in keeping stability of erythrocytes and

free-radical scavenger. It protects the cells from damage against free radicals generated as a result of parasites as it reacts with molecular oxygen and free radicals thus preventing autoxidation of polyunsaturated fatty acids in cellular and sub cellular tissues **(EL.Moghazy, 2011)**. Therefore, it could be hypothesized that severity of babesiosis is related to oxidative stress and loss of body's antioxidant reserve. The obtained biochemical analysis reported significant decrease in serum albumin and A/G ratio accompanied by significant increase in globulin levels while total protein was insignificantly increased in cattle infected with babesiosis. These results were in consistence with that illustrated by **Alam and Nasr (2011) and Swelum *et al*., (2014). Kozat *et al* .,(2003)** recorded that babesiosis has harmful effects on the hepatocytes leading to disturbed hepatic functions as decreased synthesis of albumin accompanied by an increase in albumin catabolism urinary loss of albumin associated with renal failure (proteinuria) and anorexia in relation to high rise of body temperature. The observed hyperproteinemia can be attributed to an increase in the globulin concentration which may be due to the stimulation of immune system by the antigens of invaded parasites **(Hussein *et al.,* 2007).** The liver plays a central role in babesiosis. It is the site where the pre-erythrocytic stages of *Babesia* parasites asexually multiply and where host immature mechanisms develop to fight these pre-erythrocytic stages **(Ferreri *et al.,* 2008).** Current study showed a significant increase in the activity of liver enzymes (AST, ALT, ALP and LDH) and total bilirubin in cattle suffering from babesiosis. These results were supported by that obtained before by **Alam and Nasr (2011); Lotfolla *et al.,* (2012) and Swelum *et al.,* (2014). Yeruham *et al.,* (2003)** emphasized that the elevation in liver enzymes in babesiosis may be attributed to sever anemia that lead to hypoxic and toxic liver damages. Also, massive hemolysis occur in conjunction with hypoxia may lead to hepatic cell degeneration and glomerular dysfunction. The increased activity of AST in bovine babesiosis indicates hepatic damage and also hemolytic anemia because this enzyme exists not only in the liver, but also in the heart, kidney, skeletal muscle and erythrocytes **(Kamlie *et al.,* 2005)**. LDH is a cytosolic enzyme, which is essentially present in all tissues involved in glycolysis. Any destructive process of these tissues leads to enzyme leaking into extra cellular fluids and then into body fluids **(Okubo *et al.,* 2007)**. High bilirubin concentrations observed may be related to liver failure during the disease development and to hemolytic anemia as parasites living in erythrocytes may induce massive hemolysis, leading to drop of hematocrit and

hemoglobinaemia and to increase in bilirubinemia **(Otsuka, *et al*., 2002).** The present study investigated significant change in cholesterol and triglyceride synthesized in liver as due to toxic effect of *Babesia* on liver when compared with control. These results were agreed with **Rees and Schoeman (2008) and Hamoda *et al.*, (2014)**. The significant decrease ($P<0.05$) in blood glucose concentration could be due to the utilization of glucose by parasites depletion of hepatic glycogen stores and hepatic dysfunction with impaired gluconeogenesis in infected cattle with *B. bovis* **(Durrani and Kamal 2008).** Kidney is susceptible to blood diseases, toxins of parasite and accumulation of immune complex thus impairing the kidney function and structure as manifested by a significant increase of serum urea and creatinine levels. In comparison with the control group these results came in accordance with ***Swelum et al.*, (2014) and Hamoda *et al.*, (2014). Rees and Schoeman (2008**) observed elevation in BUN and creatinine level might have resulted from kidney dysfunction, muscle catabolism, and colonization of *Babesia* in the renal blood circulation.

CONCLUSION

Microscopy and clinical examination are still very helpful for the diagnosis of acute cases of babesiosis,but a combination of serological and molecular diagnosis will detect asymptomatic carriers that are an important reservoir of infection. Generally, parasitic infections cause adverse effects and major changes in the studied blood hematological and biochemical parameters of cattle which will be reflected on health and production.

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الملخص العربى

الفحص الجزيئي للبابيزيا و الإجهاد التأكسدي له في دم الأبقار المصابة

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تهدف هذه الدراسة إلى إلقاء الضوء على الإصابة بطفيل البابيزيا فى الأبقار.لذلك تم تجميع عدد 210 عينة دم من الأبقار (80 عينة من بعض المزارع بمحافظة الجيزة وعدد 130 عينة من مجزر البساتين). تم الفحص الظاهرى لأبقار المزارع وسجلت الأعراض منها نقص فى الوزن و فقر الدم وفقدان الشهيه ويرقان. كما لوحظ وجود قراد على بعض منها فى أماكن مختلفة من الجسم. تم إعداد شرائح دموية من جميع العينات وصبغها بصبغة الجيمسا وفحصها بالميكرسكوب.سجلت نسب الإصابة 16 (31%) و18 (29.21 %) فى أبقار المزارع ألتابعة لمحافظة الجيزة ومجزر البساتين على الترتيب.تم تحضير مولد الضد (الأنتيجين) الخاص بطفيل البابيزيا وقياس نسبة البروتين له وذلك لعمل الفحص السيرولوجى بإختبار الإليزا. سجلت نسب الإصابة 23 (34%) و21 (54 %) على الترتيب.وكانت نسب ألإصابة بالفحص السيرولوجى أعلى من نسب الإصابة بالفحص الميكرسكوبى. كذلك تم أخذ 10 عينات دم الأبقار (5 عينات إيجابيه و5 عينات سلبيه) لإجراء إختبار تفاعل البلمرة المتسلسل بإستخدام نوعين من البوادئ (بادئ للبابيزيا العام وبادئ للبابيزيا بوفيز) وتبين أن جميع العينات الإيجابيه بالفحص الميكرسكوبى والسيرولوجى كانت إيجابيه عند 644 و 711 قاعدة زوجية على الترتيب. إتضح أن جميع العينات الإيجابية بالفحص الميكرسكوبى والسيرولوجى كانت إيجابية بإختبار تفاعل البلمرة المتسلسل أما العينات السلبيه كان منها 3 عينات إيجابية لبادئ البابيزيا العام وعينتان إيجابيتان لبادئ البابيزيا بوفيز.بعد الفحص الطفيلى والسيرولوجى تم إختيار 20 عينة من العينات الأكثر إيجابية للبابيزيا وكذلك 10 عينات سلبية لقياس التغيرات الكيميائية والهيماتولوجية التى حدثت بها. كما أظهر الفحص الخلوي للدم نقصا معنويا فى عدد الكريات الحمراء, نسبة الهيموجلوبين وحجم خلايا الدم المضغوطة مصحوبا بزيادة معنوية في العدد الكلي لكرات الدم البيضاءو قد اظهر العد النوعي لها زيادة معنوية في كل من الخلايا اللمفاوية ,النيتروفيل والأيزونوفيلو المونوسيت. كما اظهرت الفحوصات المعملية بالدم زيادة معنوية في مستوي المالون داي الدهايد وأكسيد النيتريك مصحوبة بنقصا معنويا في مستوي الجلوتاثيون المختزل وانزيم الجلوكوز فوسفات دي هايدروحينيز وسوبر اكسيد والنحاس والحديد ذيزميوتاز فيتامين. اما التحليل البيوكميائي فقد أظهر نقصا معنويا في مستويات الألبيومين مصحوبا بزيادة معنوية في الجلوبيولين كذلك البيلوربين بالأضافة الي الزيادة المعنوية في كل من مستوى نشاط كوليستيرول الدم الكلى AST,ALT,LDH,ALPالكلي، ،ترايجليسريد ,اليوريا والكرياتينين ولوحظ إنخفاضا معنويا فى مستوى الجلكوز .